MONASH University

The evolution and ecology of heterospory

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BSc. (Hons.)

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0.0 – Thesis Abstract

Heterospory was one of the most innovative adaptations in land plants, independently evolving in multiple lineages. However, very little is known about the adaptive significance of heterospory and why it was so successful. There are scant attempts to explain the origin in the literature, and where there is it usually is based around inbreeding avoidance. However, chapter two describes in detail how inbreeding arguments stand up poorly to scrutiny. Haig and Westoby (1988) proposed a theory, albeit the only complete theory, for the origin heterospory. They not only described the process by which heterospory arose, but suggested the conditions under which it would arise. They proposed that heterospory arose in early Devonian vegetation that was starting to become complex and competitive. This thesis not only tested their theory and assumptions, but investigates a consequence of heterospory – sex allocation. To test the hypothesis that heterospory is favoured in complex environments, associations between spore size and habitat variables were studied in two lycophyte genera: *Selaginella*, a terrestrial free-sporing plant genus, and *Isoetes*, a mostly aquatic free-sporing plant genus. The free-sporing heterosporous lycophytes are particularly appropriate models for heterospory as they closely resemble ancient fossil heterosporous plants from the Devonian. For the terrestrial genus *Selaginella*, leaf area index (inferred as level of shade) and net primary productivity were selected as appropriate measures of habitat type. For *Isoetes* a different approach was required as they are predominantly aquatic in habit. Habitat groupings for *Isoetes* were based on the typical duration of inundation. For both of these groups, megaspore and microspore size were measured from herbarium data, or for unavailable *Isoetes* species, from the literature. The findings of these two studies suggested that heterospory is favoured in complex habitats. Habitats that were highly shaded, or in water (light is restricted by the water column) had species that produced much larger megaspores. In fact, the response in *Selaginella* was so distinct they never produced very large spores in open environments. The main conclusion from these two portions of the thesis is that heterospory is selectively advantageous in environments where establishment is difficult due to nutrient competition. This conclusion is particularly relevant to the conditions in which heterospory has appeared. The last chapter uses *Selaginella* as a novel empirical model for sex allocation in free-sporing heterosporous plants. Angiosperms have clearly been shown to usually be female biased in their resource allocation, but this thesis shows *Selaginella* is extremely male biased in 13 of 14 species studied. The vast difference in *Selaginella* sex allocation bias calls for potential re-evaluation of sex allocation in heterosporous plants.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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Date: 30/11/2017

Publications during enrolment

Thesis Chapter 2

Petersen KB, Burd M. 2017. Why did heterospory evolve? *Biological Reviews*. ***92(3)*:** 1739-1754.

Thesis Chapter 5

Petersen KB, Burd M. 2018. The enigma of sex allocation in *Selaginella*. *Annals of Botany*. *in press*.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes (*2)* original papers published in peer reviewed journals and (*2)* submitted publications. The core theme of the thesis is "The adaptive ecology of heterospory". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Martin Burd.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of 2, 3, 4 and 5 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status *(published, in press, accepted or returned for revision, submitted)*	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	Why did heterospory evolve?	Accepted	70%. Concept and writing first draft	1) Martin Burd, input into manuscript drafts and concept 30%	No
3	Spore size and the adaptive nature of heterospory: evidence from *Selaginella*	Submitted	70%. Concept, data collection and writing first draft.	1) Martin Burd, concept, data collection and input into manuscript 30%	No
4	Aquatic environments select for more extreme heterospory in the free sporing lycopod genus *Isoetes*	Submitted	70%. Concept, data collection and writing first draft.	1) Martin Burd, concept, data collection and input into manuscript 30%	No
5	The enigma of sex allocation in *Selaginella*	Accepted	60%. Concept and data collection and writing first draft	1) Martin Burd, concept, data collection and input into manuscript 40%	No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 30/11/2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date: 30/11/2017**

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Contents

1.0 – Thesis Introduction

Heterospory appeared very early in land plant history but early predecessor land plants were homosporous and not always vascular. The earliest land plants appeared between the mid-Ordovician (~476 Myr) to the early Silurian (~432 Myr), and by the late Silurian and early Devonian fossils resembled that not only of liverwort-like plants, but hornworts and mosses (Kenrick and Crane, 1997). These plants were all gametophyte (haploid) dominant as an adult plant, and the diploid sporophyte generation only functioned to produce and release spores (non-vegetative). The period that followed, the mid-Silurian to early Devonian, was a particularly important and interesting part of land plant evolutionary history; there was an increase in plant diversity, vegetation complexity, and it marked the undeniable appearance of vascular plants (Kenrick and Crane, 1997). The vascular plants included many lineages that are now extinct, but they also included the homosporous clubmoss ancestors of the extant lycophyte lineage and other important vascular plant groups (Bateman and DiMichele, 1994; Kenrick and Crane, 1997). The early vascular plants were homosporous and the vegetative stage was dominated by the diploid sporophyte generation. Early Devonian (~398 Myr) plant evolution marked the appearance of increasing spore diversity through the fossil record and the appearance of heterosporous plants (Chaloner, 1967; Kenrick and Crane, 1997). The heterosporous plants were associated with a time of fast increasing environmental diversity and complexity. Furthermore, as indicated in the spore fossil record (Chaloner, 1967), the appearance of heterospory marked the beginning of the dominance of heterosporous plants. But what made heterospory so advantageous in early land plant evolution? Chapter 2 discusses in detail the shortcomings in the literature on the evolution and ecological advantage of heterospory. The later evolutionary consequences of heterospory (*e.g.* seeds) are well understood in the angiosperms and gymnosperms. However, nobody fully understands the ecological advantage that heterospory itself gave to early free-sporing plants. Additionally, the evolution of heterospory had many more ecological implications. For example,

heterospory allowed sex allocation to be controlled by the sporophyte where previously it was controlled by the gametophyte. Sex allocation (Chapter 5) has been well studied in seed plants, but vastly unexplored in free-sporing plants.

This thesis aims to fill the gaps in knowledge on the adaptive origin of heterospory in the land plants and some of its ecological consequences. The first chapter reviews in detail the history and what is currently known about the adaptive origin and gives a short background on the fossil record of heterospory. The published fossil record on heterospory is particularly detailed but no empirical study exists on the adaptive origin of heterospory. The chapters thereafter present results of studies using habitat and spore size to infer selection of heterospory in two free-sporing heterosporous lineages, the Selaginellaceae and the Isoetaceae. These two lycophytes lineages are related, but inhabit vastly different habitats. These free-sporing heterosporous plants are very appropriate models for heterospory evolution as they differ very little from their ancient heterosporous ancestors. The final chapter investigates the sex allocation in *Selaginella*. As most sex allocation evidence is based around what has been learnt from the seed plant lineages, the free-sporing *Selaginella* offers an alternative perspective on the current understanding of sex allocation in plants.

1.1 – References

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2.0 – Why did heterospory evolve?

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2.1 – Abstract

The primitive land plant life cycle featured the production of spores of unimodal size, a condition called homospory. The evolution of bimodal size distributions with small male spores and large female spores, known as heterospory, was an innovation that occurred repeatedly in the history of land plants. The importance of desiccation-resistant spores for colonization of the land is well known, but the adaptive value of heterospory has never been well established. It was an addition to a sexual life cycle that already involved male and female gametes. Its role as a precursor to the evolution of seeds has received much attention, but this is an evolutionary consequence of heterospory that cannot explain the transition from homospory to heterospory (and the lack of evolutionary reversal from heterospory to homospory). Enforced outcrossing of gametophytes has often been mentioned in connection to heterospory, but we review the shortcomings of this argument as an explanation of the selective advantage of heterospory. Few alternative arguments concerning the selective forces favouring heterospory have been proposed, a paucity of attention that is surprising given the importance of this innovation in land plant evolution. In this review we highlight two ideas that may lead us to a better understanding of why heterospory evolved. First, models of optimal resource allocation – an approach that has been used for decades in evolutionary ecology to help understand parental investment and other life-history patterns – suggest that an evolutionary increase in spore size could reach a threshold at which small spores yielding small, sperm-producing gametophytes would return greater fitness per unit of resource investment than

would large spores and bisexual gametophytes. With the advent of such microspores, megaspores would evolve under frequency-dependent selection. This argument can account for the appearance of heterospory in the Devonian, when increasingly tall and complex vegetative communities presented competitive conditions that made large spore size advantageous. Second, heterospory is analogous in many ways to anisogamy. Indeed, heterospory is a kind of re-invention of anisogamy within the context of a sporophyte-dominant land plant life cycle. The evolution of anisogamy has been the subject of important theoretical and empirical investigation. Recent work in this area suggests that mate-encounter dynamics set up selective forces that can drive the evolution of anisogamy. We suggest that similar dispersal and mating dynamics could have underlain spore size differentiation. The two approaches offer predictions that are consistent with currently available data but could be tested far more thoroughly. We hope to re-establish attention on this neglected aspect of plant evolutionary biology and suggest some paths for empirical investigation.

Key words: anisogamy, endospory, evolution, gametophyte, homospory, land plants, lycophytes, seed habit, sporophyte, water ferns.

2.2 – Introduction

Heterospory was a pivotal innovation in the history of land plants. The divergence that defines heterospory – small, male microspores and large, female megaspores – resembles in many ways the evolution of anisogamy, but heterospory emerged within the distinctive context of the land plant alternation of generations (see Table 1 for a glossary of terms used in this article). This novel life cycle arose among the earliest colonists of the land as an adaptation to the terrestrial environment (Niklas & Kutschera, 2009; Shaw, Szövényi & Shaw, 2011). Like their charophycean algal ancestors, the first land plants released sperm into free environmental water. On land, this limited sperm mainly to dispersal by rain splash (Graham, 1993; Niklas & Kutschera, 2009). But

very early in their history, land plants evolved greater reliance on wind dispersal of spores, which had acquired desiccation resistance at least by 470 Ma in the Ordovician (Strother, Al Hajri & Traverse, 1996; Wellman, 2010) and so provided an advantage over gametes as units of dispersal and propagation (Graham, 1993). Land plants did not abandon gametes, but a new generation in the life cycle, the multicellular diploid sporophyte, evolved as an extension of the formerly haploid-dominant life cycle of charophycean algae (Shaw *et al*., 2011). Multicellular sporophytes amplified the spore production ultimately derived from each zygote, enhancing the ability of land plants to colonize terrestrial habitats (Niklas & Kutschera, 2009).

Charophycean algae lack an equivalent of the sporophyte generation (Graham, 1993). The photosynthetic adults, called gametophytes, are haploid, and when their sperm and eggs undergo syngamy to produce a diploid zygote, it is the only diploid cell in the life cycle. The zygote directly undergoes meiotic reduction division, possibly after dormancy but without intervening mitotic growth (Fig. 1A). [This is the standard interpretation of charophyte life cycles. Haig (2010) has pointed to the often meagre and unreplicated cytological evidence of zygotic meiosis in charophycean algae. Whatever the details of zygotic cell division, however, charophycean algae clearly lack the equivalent of a distinct sporophyte generation (Graham, 1993)]. Land plants inherited this gametophyte-dominant life cycle, but interpolated mitotic growth in the zygote before meiosis (Shaw *et al.*, 2011). The developing diploid embryo (whence the name ‘embryophytes’ for the land plants is derived) draws nutrition from its parent gametophyte and eventually grows into the sporophyte (Fig. 1B).

The first sporophytes produced spores that were small and unimodal in size, a condition known as isospory or homospory. With the appearance of vascular plants in the Silurian, sporophytes evolved larger size, increased longevity, and greater structural complexity than their gametophytes (Kenrick, 1994; Kenrick & Crane, 1997). The first known heterosporous plants – the fossil taxa *Cyclostigma* (Chaloner, 1968) and *Bisporangiostrobus* (Chitaley & McGregor, 1988; Kenrick & Crane, 1997) – appeared in the Late Devonian following the evolution of sporophyte

dominance. Heterospory eventually arose independently in as many as 11 separate vascular plant lineages, including zosterophyllopsids, lycophytes, equisetophytes, the water ferns, and several progymnosperm lineages, among them the ancestor of the extant seed plants (Bateman & DiMichele, 1994). Heterospory encompasses a variety of details that sometimes differ among the various lineages in which it evolved, but it is characterized in extant plants by three important features: size differentiation of small microspores and large megaspores, development of gametophytes within the confines of the spore wall (endospory), and unisexuality in which male gametophytes develop from microspores and female gametophytes from megaspores (Bateman & DiMichele, 1994) (Fig. 1C).

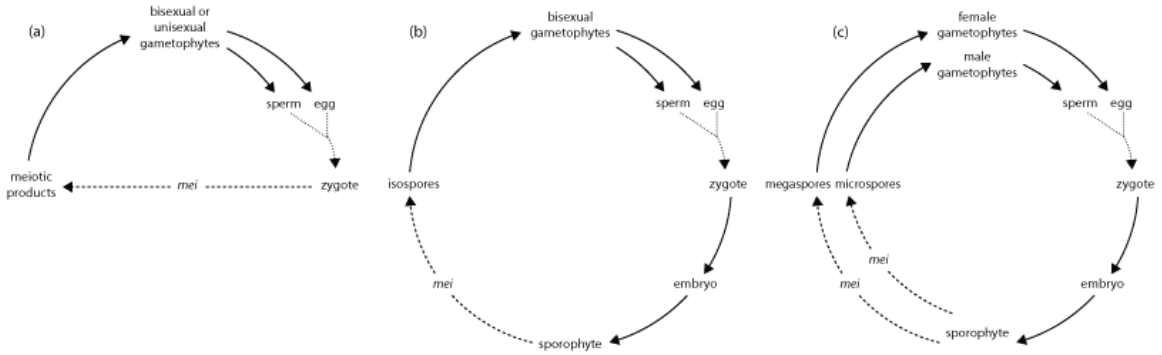

Fig. 1. Simplified life cycle diagrams of (A) a charophycean alga, (B) a typical homosporous vascular plant such as *Lycopodium*, and (C) a representative heterosporous free-sporing plant such as *Selaginella*. Solid arrows represent mitotic growth or gametogenesis; dashed arrows with '*mei*' represent meiotic reduction division; dotted arrows represent syngamy. The algal life cycle in A lacks a sporophyte phase; the haploid gametophytes are bisexual in some species and unisexual in others. In B, mitotic growth and differentiation starting from the zygote yield a diploid sporophyte, an important innovation characteristic of all land plants. Isopores produced by the sporophyte develop into potentially bisexual gametophytes (although only one sex may be expressed in particular cases). Heterospory in C entails two spore types that develop into unisexual

gametophytes. Male and female identities are therefore functionally segregated at the spore stage of the heterosporous life cycle.

The oldest extant heterosporous lineage is the *Selaginella*/*Isoetes* clade within the lycophytes. This lineage originated in the Late Devonian, judging from the *Isoetes*-like fossil *Lepidosigillaria* (Grierson & Banks, 1963). *Selaginella* and *Isoetes* together account for about 900 extant species, while their homosporous sister lineage, the Lycopodiaceae, contains about 400 species (Judd *et al.*, 2008). The water ferns, families Marsileaceae and Salvineaceae, are a second group of living heterosporous plants (Bateman & DiMichele, 1994). They appeared in the fossil record around the Cenomanian in the Late Cretaceous and have a current diversity of about 80 species in six genera, greatly outnumbered by the 12,000 species of homosporous leptosporangiate ferns (Judd *et al.*, 2008; Taylor, Taylor & Krings, 2009). The seed plants form a third and largest group of heterosporous plants. Heterospory appeared in the Late Devonian and Early Cretaceous among the progymnosperm grade leading to the seed plants (Taylor *et al.*, 2009), of which there are about 300,000 extant species (Judd *et al.*, 2008). Although the universally heterosporous angiosperms are now the dominant lineage of land plants, they possess other features that contribute to their evolutionary success (Bodribb, Feild & Sack, 2010). A fourth instance of heterospory may be seen in the weakly developed heterospory of the leptosporangiate fern *Platyzoma microphyllum* – weak because size differentiation is not pronounced and unisexuality is inconsistent ('megagametophytes' that initially produce eggs can later produce sperm), and because gametophytes are free-living, photosynthetic plants, unlike the endosporic gametophytes of the three major heterosporous groups (Tryon, 1964). *Platyzoma*, thought to be of recent origin, is monotypic (Tryon, 1964). Although patterns of diversity in the geological past have differed greatly from current diversity, of course, it is clear from the species richness in these groups that heterospory has not, by itself, been an automatic key to evolutionary success (Duckett & Pang, 1984).

Why did such a profound transformation occur, and occur repeatedly, among the land plants? What selective forces were in play? Here we will review current understanding of the origin of heterospory and highlight where that understanding falls short, particularly on the fundamental question of adaptation. We will see that some longstanding perspectives on heterospory have overlooked the issue of its adaptive significance, and we will propose new avenues for investigation of this issue.

It is surprising how little research addresses the selective factors underlying the evolution of heterospory. A solid foundation of palaeontological evidence on the origin of heterospory has been uncovered and the morphology and development of heterospory has been well described (Bateman & DiMichele, 1994; Taylor *et al*., 2009), but the adaptive significance of heterospory remains little explored. We hope the present review helps to redress this gap.

2.3 – Conventional views on heterospory

2.3.1 – Progression to the seed

As early as the 18th century the existence of two spore types in certain plants had been documented, albeit very imperfectly understood. de Jussieu (1741, 1742) described the large and small spores of the water ferns *Pilularia* and *Lemma* (*Marsilea*), but in the absence of a general understanding of the alternation of generations and uncertainty at the time over the universality of sexual reproduction in plants, he did not correctly identify their role in the life cycle. We now know these two spore types to be the megaspores and microspores of the heterosporous Marsileaceae.

By the mid-19th century, accumulated evidence pointed to the likelihood of sexual reproduction among all land plants. Hofmeister's (1851) detailed observations closed the debate by providing convincing evidence of gametogenesis and syngamy in the life cycles of all major plant groups. Moreover, in locating when and where these events occurred in each group, Hofmeister was

able to draw the links between life stages that established the universality of the alternation of generations among land plants, later recognized as an alternation of diploid and haploid phases (Strasburger, 1894). A central element of Hofmeister's argument was the similarity between the heterospores of plants like *Selaginella* and their homologous (as we now understand it) counterparts in the ovules and pollen of conifers. He summarized the evidence of this correspondence in the final chapter of his book, quoted here in the 1862 English translation:

"In more than one respect the formation of the embryo of the Coniferæ is intermediate between the higher cryptogams [such as *Selaginella*] and the phænogams [flowering plants]...The filling of the embryo-sac by the endosperm may be compared with the production of the prothallium [gametophyte] of the Rhizocarpeae [water ferns] and Selaginellæ... The embryo-sac of the Coniferæ may be looked upon as a spore remaining enclosed in its sporangium... Moreover, the development of the pollen of the Coniferæ...exhibits vital phenomena similar to those met with in the microspores of *Pilularia*, *Salvinia*, and *Isoetes*" (Hofmeister, 1862, p. 438).

The significance of the universal alternation of generations was grasped immediately. Henfrey (1852) quickly presented Hofmeister's ideas to the English-speaking world with a summary, in very nearly modern terms, of reproductive cycles in vascular plants. Repeating Hofmeister's theme, he identified the heterosporous 'higher cryptogams', such as *Selaginella* and *Isoetes*, as intermediate between homosporous vascular plants and seed plants. This emphasis on free-sporing heterospory as a step in the progression of land plants to seed production became a common theme in botanical literature and textbooks (Bateman & DiMichele, 1994), one that persists to the present (Table 2).

Table 2. Examples of literature emphasizing the role of heterospory as a precursor to the evolution of seeds.

Sachs (1887, p. 225)	"In the Selaginelleæ the prothallium is formed within the spore, and this class therefore establishes a transition to Phanerogams, where the prothallium is altogether rudimentary and is found in a spore-like structure, the embryo sac, within the ovule..."
Coulter (1898, p. 478)	"...the appearance of heterospory among the pteridophytes is one of the most important contributions to plant progress made by the group, but it is impossible to escape the conclusion that heterospory was attained independently by several lines... If heterospory appeared independently in several lines the same conclusion must be reached in reference to its natural outcome, the seed..."
Pettitt (1970, p. 403)	"Since current hypotheses aim to find the phylogenetic origin of the seed habit in the free-sporing heterosporous, haploid dioecious condition, the importance of studies that determine the similarities and distinctions, both in structure and behaviour, of sexually differentiated sporangia at all levels, needs no further emphasis."
Bell (1979, p. 58)	"An explanation of the true nature of the two kinds of spores of the heterosporous ferns and lycopods, little understood before Hofmeister, soon followed. The way was now clear to recognise the essential homologies between the archegoniates and seed plants."
Steeves (1983, p. 3551)	"It is generally accepted today that the seed habit is a derivative of free-sporing heterospory, that is, a condition such as is found in modem *Selaginella*... In the epochs preceding the first appearance of

	seeds in the fossil record there was a steady increase in the occurrence of heterospory and in the differentiation between the two spore types...”
Chaloner & Pettitt (1987, pp. 41, 43)	“The progression from homospory to seed was accomplished in some 40 million years (within the Devonian period)... we outline the changes in the sequence of developmental events that have been involved in the transition from heterospory, exemplified by *Selaginella*, to the seed, exemplified by *Pinus*.”
Linkies *et al.* (2010, p. 824)	“Three major evolutionary trends were important for the transition from the progymnosperms to the seed plants... the evolution from homospory to heterospory, ... the evolution of the integuments; and the evolution of pollen-receiving structures.”
Willis & McElwain (2014, pp. 105–106)	“The transition from plants that were homosporous (one spore size) to heterosporous (two spore sizes) is considered one of the most important evolutionary trends in the development of seed-bearing plants...”

The seed habit is clearly linked to heterospory. All extant and fossil lineages that evolved seeds did so from heterosporous origins, and heterospory was an essential and not merely a coincidental step in this evolutionary transition: retention of megaspores on the parent sporophyte and fertilization of eggs by dispersed microspores are core elements of the seed habit that require heterospory as an antecedent. But heterospory could not have arisen in order to allow the later evolution of seeds. The authors cited in Table 2 were not making this teleological error, but the longstanding emphasis given to the role of heterospory in the evolutionary transition to the seed habit seems to have diverted attention from the adaptive significance of heterospory in its own right.

Statements like those in Table 2 about the progression from heterospory to seed are seldom preceded by a substantial consideration of why homospory progressed to heterospory. Ingrouille & Eddie (2006, p. 145), for example, offered the following:

"Perhaps it [heterospory] has adaptive value where the environment is very heterogeneous and two different reproductive strategies, dispersal (small spores) and establishment (large spores) are favoured."

This conjecture offers few directions for investigation. Any environment would favour both dispersal and establishment. Why would the establishment of new sporophytes benefit more from large spores than from large gametophytes that grew from smaller spores? Such advantages may well exist, but they need explicit consideration and investigation. Moreover, attention to the adaptive foundations of the homospory-to-heterospory transition also provokes the question of evolutionary reversions from heterospory to homospory. We know of no such instances (which might be very difficult to detect in exclusively fossil lineages of heterosporous plants), but the possibility of such reversions highlights the need to account for heterospory as an adaptation in its own right independently of its association with the seed habit.

2.3.2 – Avoidance of inbreeding

Prevention of inbreeding and promotion of outcrossing form a second important theme in the literature on heterospory. Sporne (1962, p. 15) provided a typical argument: "[M]onoecious gametophytes [those that produce both egg and sperm]...are much more likely to be self-fertilized than cross-fertilized, unless they are actually submerged in water. Yet, dioecious prothalli [unisexual gametophytes] in a terrestrial environment would be at an even greater disadvantage, for they might never achieve fertilization at all, so long as the antherozoid [sperm] has to bear the whole responsibility of finding the archegonium [egg-bearing organ]. This is where heterospory may operate to the advantage of plants with dioecious prothalli." Steeves (1983) and Kar & Dilcher

(2002) also noted that the separation of sexes in heterospores may be advantageous because it promotes outbreeding, and Qiu, Taylor & McManus (2012) repeatedly invoked the outcrossing advantages of heterospory in their review of the origins of the land plant life cycle.

As with the focus on transition to the seed habit, the argument that heterospory is a solution to inbreeding contains a truth but is nonetheless an unsatisfactory explanation of the selective factors underlying the repeated origins of heterospory. To start, we can note that gametophytic self-fertilization could be precluded by unisexuality of spores and gametophytes without size differentiation between microspores and megaspores (Steeves, 1983). Exactly this combination of reproductive traits – unisexuality within isospory – has evolved repeatedly among the three basal haploid-dominant lineages of land plants, the liverworts, mosses, and hornworts, with the result that about 40–60% of species in these groups have unisexual gametophytes (Wyatt & Anderson, 1984; McDaniel, Atwood & Burleigh, 2012; Villarreal & Renner, 2013). Other mechanisms exist to impede gametophytic self-fertilization. Homosporous ferns with potentially bisexual gametophytes circumvent the potential for self-fertilization through temporal separation of their egg and sperm production and *via* chemical signals (antheridiogens) that trigger sperm production in neighbouring gametophytes (Lloyd, 1974). Empirical studies of homosporous lycophytes and ferns indicate that gametophytic self-fertilization is often rare or absent (Haufler & Soltis, 1984; Holsinger, 1987; Soltis & Soltis, 1987, 1990, 1992; Pryor *et al.*, 2001). Heterospory, then, may be sufficient but is not necessary to block gametophytic selfing.

Furthermore, heterospory in itself prevents only self-fertilization within a single gametophyte, but not mating between sibling gametophytes from the same sporophyte parent. That is, sperm from a microgametophyte could conceivably fertilize an egg on a sibling megagametophyte derived from the same sporophyte. When exactly such mating occurs in angiosperms (an ovule is fertilized by pollen from the same flower or from another flower on the same plant), it is described as self-fertilization and produces inbred zygotes. This source of inbreeding, which we will term sporophytic selfing, occurs frequently among angiosperms

(Goodwillie, Kalisz & Eckert, 2005), universal heterospory in the angiosperms notwithstanding. Thus, heterospory prevents an extreme type of inbreeding, but does not itself preclude the kind of inbreeding thought to have important evolutionary consequences in flowering plants. Indeed, the evolutionary dynamics of sporophytic selfing in angiosperms have been one of the most intensively studied topics in plant evolutionary ecology. This body of work, particularly the role of inbreeding in the evolution of unisexual sporophytes (a condition called *dioecy*), offers a perspective on the potential role that inbreeding might play in the evolution of heterospory and unisexual gametophytes.

Selection against sporophytic selfing is driven by inbreeding depression, the reduction in fitness caused by homozygosity of recessive deleterious mutations (Charlesworth & Willis, 2009). The mere existence of this genetic load in a population does not, however, lead inevitably to the evolution of mechanisms that enforce outcrossing. On the contrary, genetic models suggest that it is possible for selection to drive a population to complete sporophytic selfing under the dual effects of a genetic transmission advantage for an allele promoting self-fertilization and the purging of genetic load as a result of exposure to selective scrutiny through selfing (Lande & Schemske, 1985). Elaborations of this basic model that account for ecological factors like pollen limitation (shortfalls in pollinator service that limit ovule fertilization) indicate that mating systems with intermediate levels of self-fertilization between complete selfing and complete outcrossing can evolve and persist in populations (Johnston, 1998; Porcher & Lande, 2005). Empirical evidence shows that many angiosperm species are highly or completely self-fertilizing (Schemske & Lande, 1985; Goodwillie *et al.*, 2005) and patterns of inbreeding depression in relation to selfing rate suggest that partial self-fertilization ('mixed mating') can be an evolutionarily stable breeding system (Winn *et al.*, 2011). The evidence from angiosperms, then, shows that selection does not always oppose self-fertilization by sporophytes.

Moreover, it is not evident that outcrossing entails such a strong selective advantage that it can account for the repeated evolution of unisexual sporophytes (dioecy) in angiosperms. If this

were the case, dioecy should evolve less frequently in lineages that already possess genetic incompatibility mechanisms that prevent sporophytic selfing. The evidence available to test this hypothesis is limited (Winn *et al.*, 2011), but does not strongly support the idea that dioecy evolves as an escape from self-compatibility. A compilation of angiosperm families by Charlesworth (1985, Table 6) showed that dioecy occurred in 58 families and was absent from 55 families in which no self-incompatibility mechanisms were known, while dioecy occurred in 38 families and was absent from 24 families in which the occurrence of self-incompatibility was well established. That is, separate sexes have evolved in about 48% of families in which self-fertilization can occur, and in about 60% of families in which some other mechanism that interferes with selfing also occurs. These proportions are little altered if families in which self-incompatibility is suspected but not firmly established are included. This family-level analysis, conducted before reliable estimates of the angiosperm phylogeny were available and before phylogenetic comparative techniques were developed, can provide only heuristic insight into the role of inbreeding in the evolution of dioecy. Nonetheless, the evidence does not unequivocally point to inbreeding as the lynchpin in the evolution of separate sexes among angiosperm sporophytes (see also Givnish, 1982).

In comparing gametophytic unisexuality to sporophytic unisexuality, we must recognize that inbreeding depression is potentially far more severe in the former than in the latter, because gametophytic self-fertilization involves syngamy of genetically identical, mitotically derived gametes, thus creating homozygosity at every locus in the new sporophytic genome. Yet the potential severity of inbreeding depression from gametophytic selfing may, paradoxically, ameliorate the evolutionary consequences of inbreeding on the breeding system. Purging of genetic load in sporophytically expressed genes would occur very rapidly and effectively under gametophytic selfing, so that inbreeding depression at a mutation-selection equilibrium would be low under even modest rates of selfing in a population (Hedrick, 1987). Some evidence that this occurs comes from a comparison of two moss species, one with unisexual gametophytes and therefore incapable of gametophytic selfing, the other with combined sexes in the gametophyte.

Sibling mating (sporophytic selfing) in the former species produced inbreeding depression of approximately 0.6 for seta and capsule length in the sporophytes, while complete homozygosity from gametophytic selfing in the species with bisexual gametophytes produced inbreeding depression of only 0.04 in the sporophytes (Taylor, Eppley & Jesson, 2007).

Whatever the severity of inbreeding depression, however, heterospory is an unlikely evolutionary solution to rare occurrence of gametophytic selfing. Indeed, if complete homozygosity is effectively lethal in the zygote or early embryo before it draws much on gametophytic resources, eggs may be lost through self-fertilization with little or no diminution of the gametophyte's opportunity to produce outcrossed progeny through another egg, provided the gametophyte can produce multiple archegonia and outcrossing sperm are available in the environment. That is, a degree of self-fertilization would be absorbed by bisexual gametophytes through overproduction of eggs. Rapid death of self-fertilized progeny would effectively hide selfing from selection. Such blunting of selection against self-fertilization at the gametophytic stage has some parallels with the 'selective interference' that lethal recessives impose on the purging of genetic load through sporophytic selfing (Lande, Schemske & Schultz, 1994). Again the result is that inbreeding depression, however severe, need not lead inevitably to the evolution of unisexuality as a solution.

Furthermore, gametophytic selfing would allow colonization of new habitats by a single spore. This advantage seems to characterize many pioneer fern species, which often have high levels of homozygosity with little genetic load (Sessa, Testo & Watkins, 2016). Indeed, experimental tests indicated that 61 of 96 species in a sample from throughout the monilophyte phylogeny were capable of gametophytic selfing (Sessa *et al*., 2016). Such widespread retention of the capacity for selfing in the homosporous ferns suggests that selection to eliminate gametophytic selfing is an unlikely explanation for the repeated evolution of heterospory in other lineages.

2.4 – The model of Haig and Westoby

The most complete theory for the adaptive origins of heterospory – indeed, the only argument of which we are aware that is sufficiently comprehensive to be called a theory – is the model of Haig & Westoby (1988). Their argument rests on patterns of accrual of male and female fitness in relation to resource investment, a style of modelling common in the literature of evolutionary ecology. They drew on an earlier model of optimal offspring size by Smith & Fretwell (1974), adapting it for the case of spore size and the role of spores in the plant life cycle. The Smith–Fretwell model, now a cornerstone of evolutionary ecology, involves a straightforward maximization of parental fitness given two size-related effects: (1) a nonlinear effect of an offspring's size on its fitness, and (2) a trade-off between the size of individual offspring and the number of offspring a parent can produce. Offspring fitness is assumed to increase with size but with diminishing marginal returns (a negative second derivative), starting from a minimum size required for viability. The trade-off is assumed to entail a single division of a fixed pool of resources, so that offspring size is inversely proportional to the number of offspring produced. Under these assumptions, the optimal offspring size occurs at a point that maximizes fitness gain per unit of parental investment. This optimum can be represented graphically as the point of tangency between a line passing through the origin and the curve of offspring fitness in relation to size (Fig. 2A). The slope of this line has the units of fitness gain per unit of resource investment, and the maximum slope of a line that still touches a point on the curve of possible fitness is the point of tangency. The optimum maximizes the parent's but not necessarily the offspring's fitness. In animals, this discrepancy produces parent–offspring conflict over the amount of parental care (Clutton-Brock, 1991).

In the Haig–Westoby model, a gametophytes's expected fitness depends on the size of its antecedent spore. This would necessarily be true for endosporic growth of gametophytes, but could also characterize exosporic, photosynthetic gametophytes in seasonal or competitive environments

in which spore reserves determine the rapidity of gametophyte establishment and expansion. The model begins with a consideration of bisexual gametophytes, and so patterns of fitness accrual are considered separately for male and female function. Fitness through each sexual function follows a curve of diminishing marginal returns as a function of spore size. Additionally, the minimum spore size that allows sperm production and male fitness is assumed to be smaller than the minimum size that brings non-zero female fitness (Fig. 2A). The total expected fitness for such a spore is the sum of the male and female contributions, forming a complicated nonlinear function with respect to size (Fig. 2A). The optimal spore size, s^*, given the fitness function in Fig. 2A is sufficient to allow both sperm and egg production by the gametophyte.

The Haig–Westoby model then postulates that the minimum spore size needed for successful female reproduction increases while the minimum size for male success remains unchanged (Fig. 2B). This change might have arisen initially as land plants evolved greater height and more complex canopies and as vegetative communities became denser and more diverse. A young sporophyte in such an environment needs greater size before it achieves positive photosynthetic balance and nutritional independence. Greater adult size in turn entails a longer period of juvenile growth. These changes in the sporophyte life history would require an egg-producing gametophyte to supply greater resource reserves to subsidize a longer period of early development of its new sporophyte. Under these conditions, the optimum spore size increases, much as shady regeneration niches and large plant habits favour large seeds among spermatophytes (Salisbury, 1974; Rees, 1996; Moles *et al.*, 2005; Quero *et al*., 2007). The model then predicts that potentially bisexual isospores may reach a size at which a parental sporophyte could achieve a higher rate of fitness return per unit investment by investing in small spores adequate for sperm production but below the minimum size required for female fitness (s_0^* in Fig. 2B). The fitness advantage of small spores would, of course, be frequency dependent, diminishing as small spores spread in a population. From this initial step, selection for canalized sex expression and other

functional specializations in each spore type could stabilize heterospory at an equilibrium in which both spore types obtained equal rates of fitness return per unit investment (Haig & Westoby, 1988).

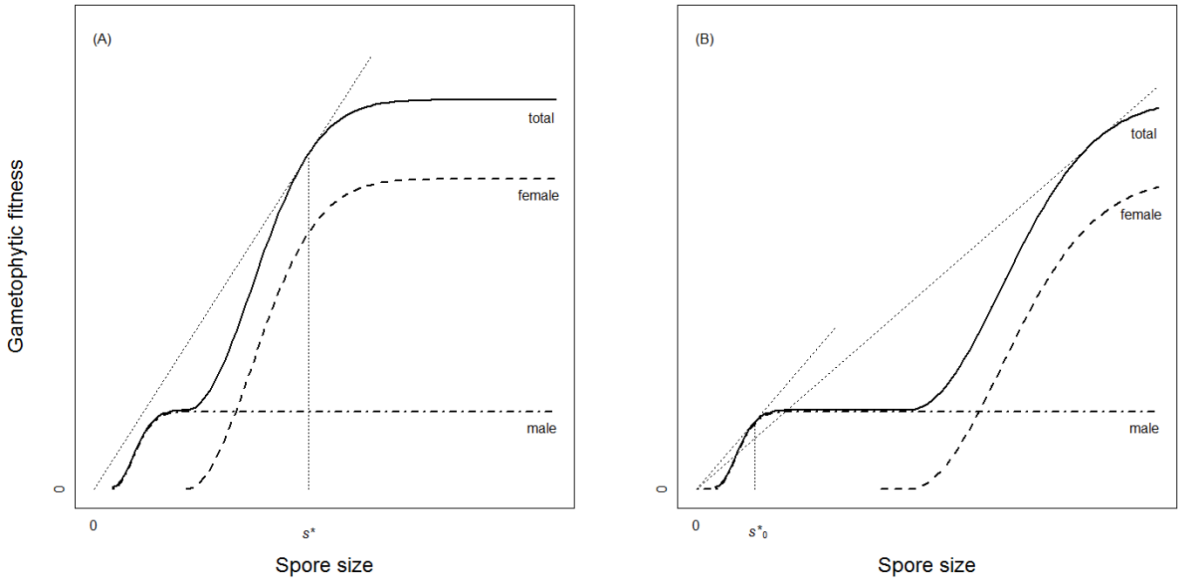

Fig. 2. Graphic depiction of optimal spore size in the model of Haig & Westoby (1988). (A) A gametophytes emerging from a small spore can gain some fitness through sperm production but only a larger spore can produce a gametophyte capable of gaining fitness through female as well as male function. The maximum fitness gain per unit of resource investment in a spore developing into a bisexual gametophyte (indicated by the slope of a line through the origin and tangential to the curve of total fitness) occurs at a spore size s^*. (B) The male fitness curve is the same as in A but spores must be larger before their gametophytes can attain female fitness (e.g. if establishment conditions for new sporophytes are competitive). Production of microspores of size s^*_0 now yields greater fitness per unit investment than would production of larger spores giving rise to bisexual gametophytes. As microspores establish in the population, frequency-dependent selection would favour megaspore production until equal average fitness returns are attained through each sex function.

This model makes several distinctive predictions:

(1) The initial step leading to heterospory would involve an evolutionary increase in the size of isospores and their bisexual gametophytes (rather than, say, the evolution of unisexual isospores followed by enlargement of the female spores).

(2) This increase in spore size would be associated with the evolution of increasing vegetative density and complexity that required young sporophytes to draw on greater nutrient reserves for their successful establishment.

(3) Above a certain size threshold, microspores would be favoured by selection. Although there is no requirement that the threshold be the same among all species and environments, any similarity in the threshold sizes should result in little or no size overlap between the largest isospores and the smallest heterosporous megaspores.

(4) Further evolutionary increases in megaspore size above the threshold at which heterospory is favoured should not be accompanied by correlated increases in microspore size.

The limited available evidence tends to support these predictions:

(1) Palynological data suggest that spore sizes increased steadily from the Silurian through the Devonian to the Carboniferous (Chaloner, 1967; Traverse, 2007). Homospory likely prevailed through the initial size increase. Spores of extant free-sporing homosporous plants rarely exceed 100 µm diameter (fewer than 3% of species in Table 5.2 of Traverse, 2007), while spore diameters reached 50 µm only in the Silurian, did not pass the 100 µm threshold until the Pragian stage of the Early Devonian, and reached the 200 µm mark only in the Emsian, just over 400 million years ago (Traverse, 2007; Chaloner, 1967). By the Givetian stage of the Middle Devonian, megaspores were abundant and phylogenetically diverse and extreme heterospory had evolved, including the occurrence of so-called seed-megaspores with diameters in excess of 1000 µm (Traverse, 2007; Steemans *et al.*, 2011). A diameter of 200 µm is the conventional threshold for assigning megaspore status to dispersed fossil spores (Bateman & DiMichele, 1994), although the size at which weak heterospory initially emerged may have been somewhat less. The first firm evidence of incipient

heterospory in the fossil record, from spores in sporangia still attached to the parent plant in the fossil progymnosperm *Chaleuria cirrosa* (late Emsian or early Eifelian, *c.* 393 Ma), involved microspores 30–48 μm in diameter and megaspores 60–156 μm in diameter (Andrews, Gensel & Forbes, 1974). This degree of heterospory is comparable to that in the extant fern *Platyzoma microphylla*. It is clear, in any case, that heterospory emerged after an extended period of evolutionary increase in isospore size.

(2) The Mid-Devonian appearance of pronounced heterospory was preceded by an Early Devonian rise in the diversity and size of vascular plants (Knoll *et al.*, 1984; Kenrick & Crane, 1997). The number of fossil spore genera and sporophyte macrofossil genera increased about fourfold between the Early and Middle Devonian (Knoll *et al.*, 1984). Silurian vegetation generally had been only a few centimetres tall, but innovations in vascular stem support and branching habit in the Early Devonian led to increasing height and canopy complexity of sporophytes (Edwards & Selden, 1992; Kenrick & Crane, 1997). The advent of lycophytes with their microphylls would have increased light interception by the canopies of Devonian vegetation (Taylor *et al.*, 2009), and in the Emsian stage of the Early Devonian there is evidence of height-stratified communities with zosterophyllophytes forming a low, dense understorey beneath a taller canopy of trimerophytes (Edwards & Selden, 1992). Cambial meristems and secondary stem thickening evolved in the Middle Devonian, signalling the evolution of shrubby and arborescent forms that would have further increased the height of vegetation and the spread of crowns (Edwards & Selden, 1992; Kenrick & Crane, 1997; Niklas, 1997). The Devonian increase in spore size was coincident, therefore, with increasingly complex plant communities that must have created shady and competitive conditions in the ground layer where regeneration occurred.

(3) Comparative data on spore sizes among extant species suggest that an approximate ‘heterospory threshold’ may occur at a spore diameter near 200 μm. The largest isospores, in the fern genus *Ceratopteris* (Pteridaceae), have diameters of about 160 μm, just below the size range of 163–190 μm for megaspores of the weakly heterosporous fern genus *Platyzoma* (Tryon &

Lugardon, 1991). Megaspores in the heterosporous lycopod *Selaginella* range from about 200 μm diameter upwards (to over 1000 μm), and the minimum megaspore size in other free-sporing heterosporous groups is somewhat larger: about 300 μm diameter in the Marsileaceae, and 400 μm in the Salvineaceae and in *Isoetes* (Tryon & Lugardon, 1991). Thus, the border between homospory and heterospory among extant species seems to occur at spore diameters of 160–200 μm.

Heterospory in fossil species involved megaspores of comparable or somewhat smaller size. A progymnosperm from the Late Devonian with documented heterospory, *Archaeopteris roemeriana*, had megaspores 110–403 μm in diameter, with a mean of 214 μm (Fairon-Demaret, Leponce & Streel, 2001). The early Carboniferous equisetophyte *Protocalamostachys farringtonii* had megaspores with diameters ranging from about 100 to 250 μm (Bateman & DiMichele, 1994). Bimodal size distributions of spores in other Carboniferous equisitophytes and progymnosperms also suggest that megaspore diameters occurred somewhere in the range of 100–200 μm (Bateman & DiMichele, 1994). But the size gap between isospores and the smallest megaspores may have increased over time. Dispersed spore species with size variation that spanned 200 μm diameter steadily increased in frequency from the Early to the Middle Devonian, but then later declined in the Late Devonian and nearly disappeared in the Carboniferous (Chaloner, 1967).

(4) While maximum megaspore sizes increased during the Devonian and Carboniferous, microspores appear to have remained small throughout the history of heterosporous lineages, as would be expected if the two spore types were subject to different selective pressures on size: nutrient reserves in megaspores for establishment of juvenile sporophytes, and minimum size requirement for effective dispersal and function of microspores, yielding a high return per unit investment. Where the preservation allows the two spore types to be associated in a single species, heterospory in Devonian and Carboniferous fossils invariably involves microspore diameters less than 100 μm – often less than 50 μm – even as megaspores evolved to diameters of several hundreds or thousands of micrometers (Brauer, 1980; Chitaley & McGregor, 1988; Marshall & Hemsley, 2003; Taylor *et al.*, 2009). Among extant free-sporing heterosporous plants, microspores

are less than 80 μm diameter (Tryon & Lugardon, 1991), and among seed plants, pollen grains rarely exceed 100 μm (Traverse, 2007).

The model of Haig & Westoby (1988) seems, then, to offer insights into the evolution of heterospory that have some support in the fossil record and among extant free-sporing heterosporous taxa. But the overview of evidence that we have provided here reveals the promise of further exploration of the assumptions and predictions of the model rather than the definitive success of the model.

2.5 – Anisogamy as an analogy for heterospory

A clue to the adaptive significance of heterospory may lie in a comparison with anisogamy, a much earlier innovation involving size differentiation of reproductive cells. Heterospory involves endospory and unisexuality of gametophytes, elements unique to the role of spores in the land plant life cycle, but the common element of size differentiation in both anisogamy and heterospory makes the comparison useful. Indeed, heterospory can be considered a reinvention of the male–female distinction, but a reinvention for spores rather than gametes. It allowed sporophytes to adjust reproductive investment between male and female function independently (Fig. 1C), with the (presumed) added feature of a trade-off between the production of many small spores or fewer larger ones.

The evolution of anisogamy has received considerable attention going back to Kalmus (1932), Kalmus & Smith (1960), and Scudo (1967), and the topic has been especially well scrutinized theoretically (Parker, Baker & Smith, 1972; Charlesworth, 1978; Maynard Smith, 1978; Dusenberry, 2006; Iyer & Roughgarden, 2008; Lehtonen & Kokko, 2011) along with some empirical work (e.g. Randerson & Hurst, 2001; Togashi & Bartelt, 2011). But the similarity between anisogamy and heterospory mostly has gone unrecognised. Haig & Westoby (1988) provide an exception, although they emphasized differences rather than commonalities. Here we

focus on a recent model of anisogamy that we believe offers new insights relevant to the origin of heterospory, and draw attention to some empirical work on anisogamy in green algae that supports its predictions.

2.5.1 – The model of Lehtonen & Kokko

Much as isospory preceded heterospory, isogamy is thought to be the ancestral state from which anisogamy evolved. Parker *et al.* (1972) offered a highly influential hypothesis concerning the selective advantages favouring differentiation of gamete size. Because more gametes can be produced from a given investment of resources if each one is smaller, selection could favour individuals producing proto-sperm in scramble competition for mates. At the same time, zygote fitness would be a function of the cytoplasmic endowment from the two contributing gametes, and so selection would also favour a provisioning strategy, leading to proto-eggs. Since matings between small gametes would offer little prospect for zygote success, proto-sperm would be under strong selective pressure to target the larger proto-eggs, while selection on proto-eggs to resist syngamy with proto-sperm would likely be less intense, as a zygote from such a union would still be sufficiently provisioned for initial growth. The Parker *et al.* (1972) model showed that anisogamy could evolve under these selective pressures, but zygote fitness had to be an accelerating function of size. Subsequent models explored modified assumptions that expanded the conditions favouring anisogamy (Charlesworth, 1978; Maynard Smith, 1978; Dusenberry, 2006; Iyer & Roughgarden, 2008).

An important shortcoming in the Parker *et al.* (1972) model and its modifications was highlighted by Lehtonen & Kokko (2011). The earlier arguments had modelled resource investment in gametes and their subsequent mating interactions as a single, time-independent event. Lehtonen & Kokko (2011) pointed out that the true dynamics would be based on the flux of gametes into and out of the mating environment. Proto-sperm and proto-eggs would enter the environment at rates

that depended on their production by parental organisms, and would exit *via* mortality if they failed to find a partner or *via* syngamy if they did, each of which were rate-dependent processes. When they included these elements, their model confirmed the prediction of Parker *et al.* (1972) that numerical advantage in scramble competition could favour anisogamy, but it also revealed a new set of conditions under which anisogamy can evolve. Even in the complete absence of scramble competition, they showed that selection for strongly divergent gamete sizes occurs if there are low rates of gamete production, gamete survival, or gamete encounters. In these circumstances, proto-eggs risk going unfertilized, yet the resource investment they represent has already been made by the parent organism and cannot be recovered. Anisogamy eases the risk of proto-egg death before contact with a mate, in that size differentiation allows smaller (and better dispersed) proto-sperm to enter the mating environment at a faster rate. Thus, in a world where mating is highly uncertain, the evolutionary interests of proto-sperm producers and proto-egg producers are concordant, and selection can drive anisogamy to extremes of size differentiation (Lehtonen & Kokko, 2011).

The extension of this model to heterospory is straightforward. Given that spores took over the principal dispersal role from gametes in the course of land plant evolution (Niklas & Kutschera, 2009), ultimate mating success would have depended strongly on the flux of spores and their resulting gametophytes in the environment. As we have already noted, selection favouring large spore size likely intensified while survival rates for spores and gametophytes may have declined with the evolution of increasingly complex vegetation in the Devonian. Increased spore size would, all else equal, reduce the rate of spore production. Thus, the flux of spores and gametophytes into the mating environment would be reduced just when monospecific stands that might saturate a local site with their spores were disappearing in the increasing diversity of Devonian vegetative communities. The conditions of low entry rate, high mortality, and infrequent mating encounters may well have characterized the spores and gametophytes of many Devonian species. The selective dynamics envisioned for gametes in the Lehtonen & Kokko (2011) model could equally drive size

differentiation among spores, in which numerous microspores that specialize in dispersal relieve the mating deficit experienced by megaspores/megagametophytes.

2.5.2 – Evidence from green algae

The Lehtonen–Kokko model, as we have applied it to heterospory, suggests that spore size divergence will be adaptive in environments where gametophytes occur at low density or where mate encounters are rare. As spores play a dispersal role on land similar to that of gametes in aquatic environments, we can ask whether divergence in gamete size has been favoured in aquatic environments of low gamete density and difficult mate location. For this evidence we turn to the green algae.

Anisogamy among marine green macroalgae tends to be associated with habitat depth (Togashi & Bartelt, 2011) (Fig. 3). In shallow intertidal habitats, species of *Ulva*, *Monostroma*, and *Enteromorpha*, among others, tend to produce isogamous or nearly isogamous gametes. Several *Bryopsis* species characteristic of the lower intertidal zone have distinct anisogamy in which female gametes are about 4–55 times larger than male gametes. In even deeper waters, species in other genera of the Bryopsidales like *Penicillus* and *Udotea* have even more extreme anisogamy, with female gametes up to thousands of times larger than their male counterparts (Togashi, Cox & Bartelt, 2007). Thus, isogamy is dominant in shallow, bright environments, but gamete size differentiation tends to increase in deeper, darker waters.

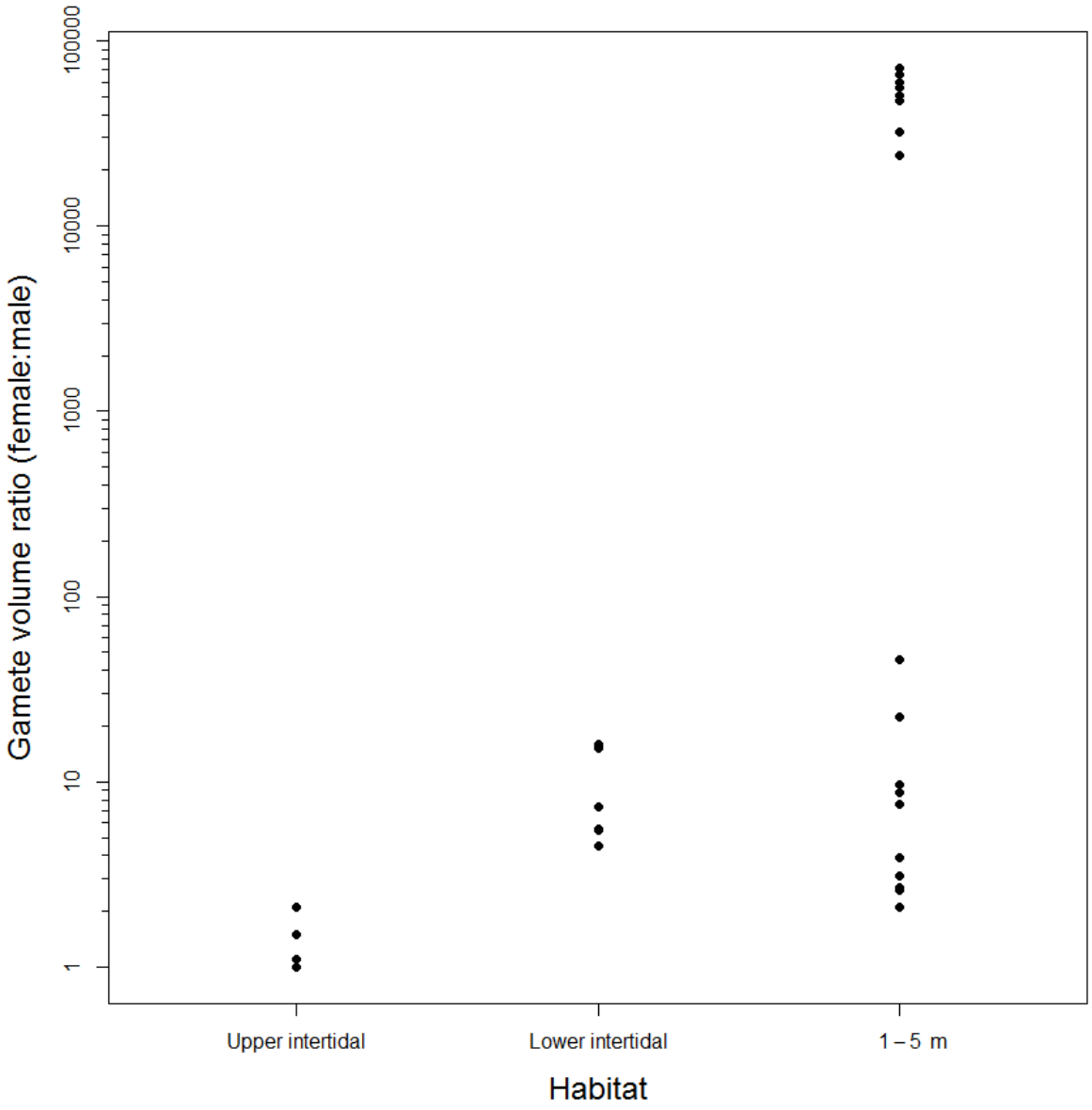

Fig. 3. Anisogamy in marine green algae in relation to depth of habitat. Data from 37 species in the orders Ulotricales, Ulvales, and Bryopsidales (Togashi *et al*., 2007, Appendix A). Gametes are taken to be spheres and their volumes are calculated from their diameters.

Depth is also associated with mating kinetics among these green algae (Togashi & Bartelt, 2011; Togashi *et al.*, 2012). The biflagellated isogametes of shallow-water taxa possess phototactic eyespot systems that direct them to the surface where they mate. Thus, gamete encounters occur in

an essentially two-dimensional arena at the surface, resulting in a higher rate of gamete encounter and fertilization than occurs in the three-dimensional water column (Togashi *et al.*, 1999). Following syngamy at the surface, the motile zygotes of these shallow-water species immediately display negative phototaxis, returning to the substratum for settlement (Togashi & Bartelt, 2011).

For species in deeper waters, the time needed for gametes to reach the surface and for zygotes to descend to the substrate may obviate any advantage they could obtain from the reduced dimensionality of the search space at the surface, given potential loss to currents or predators during the ascent and descent. Male gametes of macroalgae in these habitats lack an eyespot, and the most strongly anisogamous species in the deepest habitats lack eyespots in the gametes of either sex (Togashi *et al.*, 2007). Fertilization in these species takes place at depth, in a three-dimensional search space, thereby lowering the effective density of gametes relative to the same gamete output by a surface-mating species. Models of mating kinetics at low gamete density suggest that fertilization success will be greater under anisogamy than under equivalent isogamy (equivalent in the sense of yielding the same zygotic volume), in part because the much superior motility of very small gametes gives anisogametes a higher encounter rate than that of isogametes (Togashi *et al.*, 2007). The Lehtonen & Kokko (2011) model predicts the same effect: low encounter rates favour anisogamy. Furthermore, the need to establish on substrates under low illumination would be a separate source of selection favouring large zygotes among deep-water specialists. As zygote size increases, the fertilization advantage of anisogamy persists over a wider range of gamete densities (Togashi *et al.*, 2007).

Green algae, then, provide empirical evidence that anisogamy is favoured in environments that make gamete contacts rarer. High effective gamete density has favoured isogamy among surface-mating species, while lower gamete encounter rates and the need to establish under low illumination at depth among bryopsidalean algae has favoured marked anisogamy. These environmental features seem analogous to those facing land plants at the origin of heterospory: greater community diversity and vegetative density could have lowered spore densities on the

ground or made competition for light more important during establishment of sporophytes. We suggest that theories and evidence relating to the evolution of anisogamy can usefully be employed to motivate future work on heterospory.

2.6 – Heterospory in aquatic and amphibious environments

Fossil evidence tends to support the idea of a wet lowland origin of heterospory among Devonian lycophytes, Carboniferous progymnosperms, and water ferns (Kenrick & Crane, 1997; Kar & Dilcher, 2002). This fossil history and the fact that *Isoetes* and the heterosporous water ferns are aquatic plants led Bateman & DiMichele (1994) to suggest that heterospory is favoured in 'aquatic–amphibious habitats'. More supporting evidence comes from *Platyzoma microphyllum* which appears in ephemeral water-edge habitats in the wet season of northern Queensland (DiMichele, Davis & Olmstead, 1989; Tryon, 1964).

Establishment in permanently or seasonally inundated sites poses challenges for young sporophytes of amphibious species because they must grow through an aquatic layer that restricts light and carbon dioxide diffusion before they emerge and attain photosynthetic competence (Keeley, 1998). Juvenile sporophytes could overcome these challenges more readily with large nutrient subsidies from their parental gametophytes and megaspores. Indeed, the selection imposed on spore size in aquatic sites would have parallels with the selection imposed by habitat depth on gametes and zygotes of marine algae. As set out in the Haig & Westoby model, evolution of larger spore size to cope with establishment in low light, low carbon dioxide amphibious environments would eventually initiate the selective dynamics leading to heterospory. The introduction of sperm-producing microspores would increase the flux of sperm into the environment, relieving mate limitation of larger spores and further driving spore size differentiation, analogous to the process predicted by the Lehtonen & Kokko (2011) model for anisogamy. We have noted above that the increasing stature of sporophytes and increasing diversity of plant communities in the Devonian

would give rise to competitive establishment conditions that could favour heterospory. Amphibious habitats would reinforce the selection for heterospory.

Just as there are parallels in the selective forces, there are intriguing parallels between the mating dynamics of aquatic heterospores and algal gametes. Kar & Dilcher (2002) noted that the spore walls of Devonian and Carboniferous megaspores have an alveolate ultrastructure that resembles the spore walls of extant water ferns and *Isoetes*. The spongy spore wall created by this ultrastructure enhances the buoyancy of megaspores, thereby promoting mating on the water surface. Exine processes on fossil megaspores may have functioned to trap microspores floating on the water surface. Kar & Dilcher (2002) attributed the advantage of surface mating to outcrossing, but this seems unlikely to us, both because outcrossing is an inadequate explanation for the origin of heterospory (Section II.2) and because it is not apparent that the outcrossing rate would be any higher under surface mating than by mating in the water column. Buoyancy and surface mating would, however, provide search and encounter advantages similar to the fertilization advantage of phototactic isogametes of marine green algae (Section IV.2).

The morphology and behaviour of megaspores and microspores of extant water ferns in the Marsileaceae reinforce this interpretation of the selective advantage of heterospory for amphibious plants. Both megaspores and microspores of *Marsilea*, *Pilularia*, and *Regnellidium* are enveloped in a gelatinous layer of the outer spore wall that expands upon hydration and allows the spores to float at the water surface for about 12 h. Megaspores have a funnel-shaped invagination in the gelatinous layer, the ‘sperm lake’, where sperm accumulate and eventually have access to a single archegonium and egg. After about 12 h, the gelatinous layer begins to disintegrate and the megaspore sinks toward the substrate with its sperm lake or zygote or embryo (Schneider & Pryer, 2002). Heterospory in the Marsileaceae thus captures the advantageous fertilization dynamics of surface mating with the establishment advantages of large megaspores.

These arguments throw attention onto the ecology and mating dynamics of released spores and gametophytes of *Isoetes*, the water ferns, and *Platyzoma*. Our search of the literature suggests that extremely little is known on this topic at present.

2.7 – Nutrients and spore size

Gametophyte structure in extant heterosporous species provides a clue to the adaptive function of spore size. Within microspores, differentiation of sperm from central cells is accompanied by disintegration of a layer of jacket cells, so that essentially the entirety of the microgametophyte is devoted to sperm production (Gifford & Foster, 1989). Megagametophytes, by contrast, develop as a relatively thin layer of differentiated cells overlying a reserve of nutrients that they do not exploit. In some *Selaginella* species, there is even a diaphragm between the megagametophyte and the reserve (Robert, 1971). In *Isoetes* and water ferns, the reserve becomes cellularized only after the new embryonic sporophyte has begun growth (Gifford & Foster, 1989). Large megaspores evidently did not evolve to produce large gametophytes.

Spore size variation at the origin of heterospory appears to be related to nutrient partitioning or competition within and among sporangia. Evidence of this was noted by several workers more than a century ago. Williamson & Scott (1894) observed that sporangia of the Carboniferous equisetophyte *Calamostachys* containing aborted spores also contained mature spores of larger than normal size. Although the variation was modest (a threefold difference in diameter between putative megaspores and microspores in *C. casheana*), they suggested that abortion and nutrient competition formed the developmental basis of incipient heterospory. A similar phenomenon occurred in another fossil equisetophyte, *Sphenophyllum* (*Bowmanites*) *dawsoni*, although the size differentiation was even less pronounced (Thoday, 1906). Megasporangia of the undoubtedly heterosporous progymnosperm *Archaeopteris latifolia* contained 2–5 tetrads, hence 8–20 megaspores, and those megasporangia with fewer tetrads tended to have larger megaspores

(Chaloner & Pettitt, 1987). A parallel occurs between such fossil evidence and the differentiation of megaspores and microspores of living *Marsilea quadrifolia*: microspores develop from all 16 sporocytes in a sporangium, yielding 64 microspores, while megaspore formation involves abortion of all sporocytes but one (Shattuck, 1910).

The pace of cell division in sporogenous tissue may be a mechanism of spore size determination complementary to that of abortion. Exogenous application of an ethylene-releasing compound caused megasporangia to develop in normally microsporangiate positions along strobili of two *Selaginella* species (Brooks, 1973). Ethylene retards cell division in plants, so that fewer sporocytes differentiate where ethylene concentrations are higher. In the normal megasporangium, only a single functional sporocyte develops, producing a single tetrad of spores.

Spore size differentiation within a species could, then, have arisen easily in Devonian plants by simple mechanisms of spore abortion or control of cell division in sporogenous tissue, sporocytes or spores. Megaspore sizes in early heterosporous plants were often quite variable, even within single sporangia (Taylor *et al.*, 2009). Spore size would likely have interacted with sexual expression in the gametophyte stage. Even today, the mechanisms by which gametophytes produce only antheridia or archegonia are not well understood (Banks, 2009), but experimental evidence going back to the 19th century has long indicated that environmental conditions affecting gametophytic growth and longevity can affect sexual expression in normally bisexual or unisexual gametophytes (Shattuck, 1910; Tryon, 1964). This finding accords with the Haig & Westoby (1988) model, which starts from the assumption that only larger gametophytes would have the resources to sustain egg production and eventual provisioning of an embryo, while smaller gametophytes would produce only sperm.

Habitats where a newly emerged sporophyte needs a substantial nutrient reserve for successful establishment would also be highly competitive for diminutive, thallose, autotrophic megagametophytes. Thus, the environmental factors favouring large megaspore reserves would also have favoured endosporic gametophytes, if they did not already exist. We can therefore see in the

Devonian increase in spore size, both among isospores and heterospores, a reflection of the same ecological function that seeds would eventually provide. There are, of course, important differences between megaspores and seeds, particularly the elimination of dependence on external water for fertilization and the ability of parental sporophytes to make post-fertilization investment in seeds. But the functional similarity of nutrient reserves in megaspores and seeds is apparent, and this analogy provides a path for empirical investigation of the ecology of heterospory.

2.8 – Prospects for testing the adaptive significance of heterospory

Free-sporing plants can serve as model organisms for observational or experimental approaches to address the ecological advantages of heterospory. The evidence and ideas reviewed thus far suggest that heterospory arose following an amplified provisioning role for spore reserves during sporophyte establishment, that is, when megaspores became ecologically similar to seeds. This core idea could be tested by examining the regeneration ecology of species that vary in megaspore size. Reproductive success should become more dependent on megaspore size as establishment conditions become more severe. The inspiration for this endeavour can come directly from studies of seed ecology (e.g. Salisbury, 1974; Rees, 1996; Quero *et al*., 2007).

The greatest variation in spore sizes outside the seed plants occurs among *Selaginella* species: megaspores vary approximately a thousandfold in volume and microspores a hundredfold (Tryon & Lugardon, 1991; K.B. Petersen & M. Burd, unpublished data). The establishment of new sporophytes under low light should be more successful the larger the megaspore. While this prediction is unremarkable, we can find no evidence that it has been tested with free-sporing plants. The effect of shading and competition on photosynthetic performance of young sporophytes and of megaspore size on establishment success could be examined directly in the field among coexisting species or among a wider range of taxa under controlled laboratory environments. In many *Selaginella* species, large and small megaspores are produced in a single sporangium, offering the

potential to detect spore size effects without confounding effects of other species-specific differences.

Species ranges provide another means to relate spore size variation to environmental conditions. Habitats characterized by high leaf area index (LAI, the total leaf upper surface area, possibly in multiple layers, above a unit of ground area) would tend to have shaded regeneration sites (although open microhabitats from, say, disturbance could also exist in regions with otherwise high LAI). We have analysed geographic occurrence data for 115 *Selaginella* species and found that species with large megaspores tend to occur in habitats with high LAI (K.B. Petersen & M. Burd, unpublished data). This pattern is broadly consistent with the Haig & Westoby (1988) hypothesis, but a more detailed measurement of species-specific regeneration niches across the genus would test the provisioning function of meagspore size further. Similar analyses for homosporous lycophytes would provide a useful comparison to this pattern.

Isoetes and the water ferns are mostly amphibious or aquatic, making them particularly good models for testing the issues raised in Section V. The high diffusional resistance of water makes carbon acquisition a challenge for submerged plants (Keeley, 1998). Species that establish under a water layer may benefit from subsidies to early sporophyte growth in the same way as terrestrial species in shaded sites. Most but not all species of *Isoetes* grow as permanently submerged plants, while many species in the Marsileaceae occupy seasonally wet habitats (Tryon & Tryon, 1982). Associations between megaspore size and the duration of submergence during early growth in these taxa would be worth investigation, although megaspore sizes within *Isoetes* and the Marsileaceae are much less variable than in *Selaginella* (Tryon & Lugardon, 1991). Species in the Salviniaceae float on the water surface rather than having a submerged phase, and they have substantially smaller megaspores than *Isoetes* or the Marsileaceae (Tryon & Lugardon, 1991), perhaps indicative of reduced dependence on early nutrient provisioning, although the regeneration ecology of these species, like all the free-sporing heterosporous taxa, is imprecisely known.

We have also suggested, drawing on the work of Lehtonen & Kokko (2011), that low rates of mate encounter may have promoted the evolution of heterospory. This idea has a counterpart in the concept of pollen limitation of seed set in flowering plants, a topic that has received much attention in primary field work, comparative analyses, and theoretical development (Burd, 1994; Ashman *et al*., 2004). It would be a challenge to infer from modern ecology how mate limitation in the Devonian might have led to the advent of heterospory, but there are elements of the argument that could be pursued. In particular, spore production and fertilization rates of megagametophytes could be measured in the field in free-sporing heterosporous taxa to determine if the degree of spore size difference is associated with 'microspore limitation' of reproductive success. Further theoretical development along the lines of the Lehtonen & Kokko (2011) argument would be welcome. A model tailored to the plant life cycle and parameterized for (probably different) production rates and mortality rates of microspores and megaspores may allow field measurements of reproductive success to be interpreted in terms of the potential for selection favouring heterospory.

2.9 – Conclusions

(1) The adaptive nature of heterospory has been poorly understood and insufficiently investigated. There exists a large body of information on fossil heterospores and on the developmental biology of the heterosporous life cycle, and now on the genomics of a free-sporing heterosporous species (e.g. Kirkbride, Fischer & Harada, 2013). But adaptation is an issue of ecological function and selective advantage, and on these matters we know little. The undoubted role of heterospory in the evolution of the seed habit seems to have absorbed attention to the point that the adaptive nature of the homospory-to-heterospory transition is rarely addressed.

(2) The theoretical model for the evolution of heterospory by Haig & Westoby (1988) points to optimal resource deployment as a central issue in the adaptive significance of heterospory.

According to their argument, evolutionary increase in spore size was the trigger that made microspores advantageous. Thus, unisexual microspores were the primary evolutionary innovation, followed by frequency-dependent selection for megaspores, thus establishing heterospory. Spore size variation among extant free-sporing plants might be used as a model system to identify the ecological conditions that favour large spores. Experiments can determine if spore size correlates with sporophyte growth, the time needed to reach positive photosynthetic balance, and establishment success under low light or other environmental stress. Multispecies comparative approaches could also be profitably employed to examine environmental and geographic factors that correlate with spore size. More generally, the ideas and techniques that have been applied to field and greenhouse studies of the reproductive ecology of spermatophytes could be applied to free-sporing plants far more than has been done.

(3) The theoretical model of Lehtonen & Kokko (2011) and the interesting work of Togashi *et al*. (1999, 2007) and Togashi & Bartelt (2011) on anisogamy provides a useful analogy for the study of heterospory. Their work throws attention on the dynamics of mate encounter as a critical factor shaping selection for mating propagules of different size. Differences among extant species in the degree of heterospory might provide appropriate model systems for empirical investigation of fertilization dynamics, difficult as that might be to achieve in the field or even in controlled laboratory environments. The water ferns are a relevant starting model for comparison to the mating dynamics already known from green algae (Togashi & Bartelt, 2011).

(4) Heterospory may have originated in wet or amphibious environments. The reproductive ecology of *Platyzoma microphyllum*, which grows in ephemerally wet habitats and represents a very recent appearance of heterospory, deserves careful study. *Isoetes* and the water ferns are also potentially useful models of the ecology of heterospory in aquatic and semi-aquatic environments.

(5) The seemingly settled story of heterospory contains a wealth of unanswered and even unasked questions about selective factors and adaptive function. The abundance of existing fossil and morphological evidence on heterospory needs to be augmented with ecological experiment and

phylogenetic comparative analysis to advance our understanding of why this pivotal innovation appeared.

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2.11 – Glossary of terms

Table 1. Glossary of terms appearing in this article.

Alternation of generations	The land plant life cycle of sexual reproduction. This life cycle progresses through a multicellular diploid phase that produces spores, which then germinate and grow into a multicellular haploid phase that produces gametes. The two phases are conventionally called 'generations', although each phase is only part of a single complete life cycle. See Fig. 1.
Anisogamy	The production of gametes of different size. Among some green algae, the larger, female gamete may be flagellated, and because of its motility is not considered an egg.
Archegonium	The egg-bearing organ of gametophytes (the archegonium is evolutionarily lost in the megagametophytes of flowering plants).
Charophycean algae	A group of freshwater green algae that includes the stoneworts. The charophytes are the algal sister lineage to the land plants.
Cryptogam	A term for free-sporing plants, referring to the inconspicuousness of fertilization in their life cycles.
Dioecy	Unisexuality of individual plants, used especially to describe the sporophytes of some seed plants, which will individually produce either megaspores (ovules) or microspores (pollen) but not both. *Cf. monoecy*.
Egg	The female gamete of land plants. Note, however, that eggs are produced by haploid gametophytes and are products of mitotic, not meiotic, cell division.
Embryo	As in other organisms, the land plant embryo is the immature multicellular structure that develops from a zygote. The embryo gives

	rise to the mature sporophyte.
Embryo-sac	The megagametophyte of seed plants, contained within the ovule.
Endosperm	The nutrient reserve of angiosperm seeds, derived partly from cells of the megagametophyte.
Endospory	Development of a gametophyte inside, or mostly inside, the wall of the spore from which it derives. Endosporic gametophytes thus depend for their growth on nutrients contained in the spore when it was released.
Equisetophytes	One lineage of the fern radiation, commonly called horsetails or scouring rushes. Although a minor group today, arborescent equisetophytes were an important component of Carboniferous forests.
Exospory	Development of a nutritionally independent gametophyte outside the wall of the spore from which it derives.
Free-sporing	Referring to plants that disperse spores rather retaining them as part of seed production. All land plants other than the seed plants (gymnosperms and angiosperms) are free-sporing.
Gametophyte	The haploid, gamete-producing phase of the alternation of generations. See Fig. 1.
Homospory	The production of spores of a unimodal size; equivalent to isospory.
Isogamy	The production of gametes of a unimodal size, common among green algae. The lack of size differentiation means isogametes are not distinguished as male or female, although they play the same role in the life cycle as sperm and eggs.
Isospory	The production of spores of a unimodal size; equivalent to homospory.
Leptosporangiate ferns	The largest lineage of ferns. The term refers to a narrow stalk supporting each sporangium.
Lycophytes	A lineage of vascular plants, also called lycopods, represented today by

	about 1300 species of herbaceous plants. The extant lycophytes are divided into a homosporous lineage and a heterosporous lineage. Arborescent lycophytes were important components of Devonian and Carboniferous forests.
Monoecy	Bisexuality of individual plants. When used to refer to gametophytes, monoecy implies production of both eggs and sperm; when used to refer to sporophytes, monoecy implies production of both megaspores and microspores.
Ovule	The structure of seed plants that develops into a seed. It is largely equivalent to a megasporangium (but contains additional tissue layers).
Phænogam	An older term for seed plants, especially flowering plants, meant to contrast to *cryptogam*.
Pollen	The microspore and endosporic microgametophyte of seed plants.
Progymnosperm	A grade of several lineages preceding the appearance of the extant seed plants. The earliest diverging progymnosperms had not evolved the seed habit, but seeds characterized all later progymnosperm lineages.
Prothallium	A term for the gametophyte of ferns.
Seed, seed habit	Seeds have three important characteristics: (1) they develop from megaspores that are retained on the parent sporophyte (rather than being dispersed as in free-sporing plants); (2) the megaspores grow and develop into egg-bearing megagametophytes inside enclosing tissue layers of the parent sporophyte; (3) microspores (pollen) must be transported to the megaspore-bearing plants in order to effect fertilization. *Seed habit* refers to reproduction *via* seeds rather than *via* freely dispersed spores.
Seed plants	Seeds evolved multiple times among land plants. Extant seed plants –

	the gymnosperms (conifers and related plants) and angiosperms (flowering plants) – derive from one origin of the seed habit.
Sperm	Male gametes of land plants, products of mitotic cell division within haploid gametophytes. Among free-sporing plants, sperm must be transported by external water in the environment to reach eggs. Among seed plants (except Cycads and Ginkgo), sperm are delivered *via* pollen, which frees seed plants from dependence on external water for reproduction.
Spore	Single-celled meiotic product; a propagule in the land plant life cycle. In contrast to animal life cycles in which the meiotic products are gametes, spores do not undergo fusion with other spores; rather, they germinate and grow into gametophytes, which then produce gametes.
Sporangium	A multicellular structure containing initially sporocytes and eventually spores. A sporangium producing megaspores is a megasporangium; one producing microspores is a microsporangium.
Sporocyte	A cell that undergoes meiosis to give rise to spores.
Sporophyte	The diploid, spore-producing phase of the alternation of generations. See Fig. 1.
Thallose	Referring to the flat, prostrate tissue forming the photosynthetic gametophytes of some free-sporing plants.
Water ferns	A monophyletic lineage of two families of leptosporangiate ferns, Marsileaceae and Salviniaceae, that are heterosporous.

3.0 – Spore size and the adaptive nature of heterospory: evidence from *Selaginella*

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3.1 – Abstract

Heterospory was a pivotal evolutionary innovation for land plants, but the selective advantages underlying the size differentiation of male and female spores are very poorly understood. Here we use the geographic distributions of 114 species of the heterosporous lycophyte genus *Selaginella* to infer the functional ecology of microspore and megaspore size, traits that would be correlated with many aspects of a species' regeneration niche. We characterized habitats at a global scale using leaf area index (LAI), a measure of foliage density and thus shading, and net primary productivity (NPP), a measure of growth potential. Microspores are smaller among *Selaginella* species in habitats with higher LAI and NPP. Megaspores are larger among species that inhabit regions of high LAI, while megaspore size declines as habitat productivity rises, but only in relatively open habitats with low LAI. Filtering of wind-borne microspores by leaf surfaces would give species in leafy environments with smaller microspores an advantage derived from size-number trade-offs in spore production. Megaspore volume in *Selaginella* reflects nutrient storage for establishment of the next sporophyte generation, a functional role similar to that of seed size among spermatophytes. The geographical distribution of *Selaginella* suggests that larger megaspores provide an establishment advantage in shaded habitats. These results support previous theoretical arguments that heterospory was originally an adaptation to the increasing height and

density of Devonian vegetative canopies that accompanied the diversification of vascular plants. However, habitat productivity can generate selection on spore size independently of foliage density.

3.2 – Introduction

Although the fossil record tells us when, how, and in which lineages heterospory arose (1), we have remarkably few insights into why it evolved (2). Heterospory was an essential precursor to the seed habit and thus to modern terrestrial vegetation, but the eventual evolution of seeds cannot explain the ecological advantage provided by heterospory at the time it arose (2). Prevention of gametophytic self-fertilization, sometimes posited as the selective advantage underlying heterospory (3, 4), also has many shortcomings as an adaptive explanation (5). While heterospory precludes self-fertilization by gametophytes, it does not prevent sporophytic inbreeding (e.g., self-pollination in angiosperms), nor is it obvious from angiosperm evolution that sporophytic inbreeding is strongly or universally disfavored by selection (5, 6). Despite the pivotal role of heterospory in the evolution of land plant life, the ecological factors that might have created selection favoring large, female megaspores and small, male microspores have received only meagre attention and empirical investigation.

There is a substantial fossil record of early heterospory (1), but fossils allow only limited inferences about the functional ecology of heterospory. Extant plants that are heterosporous and also free-sporing (i.e., releasing both megaspores and microspores into the environment, in contrast to seed plants that retain megaspores within ovules on the parent sporophyte) could serve as models for investigating the adaptive nature of heterospory. However, few such lineages remain (1, 7), and direct field study of mating interactions and regeneration ecology in the extant lineages is difficult. Thus, what is known about the ecology of heterospory is poor in comparison to the accumulated knowledge of pollination, seed dispersal, seed bank dynamics, seed germination, and seedling

establishment that is available for gymnosperms and angiosperms. Nonetheless, the difficulty of direct field study of reproduction in extant heterosporous lineages can be circumvented by examining broad patterns of biogeography in relation to environmental conditions. We apply this strategy to the ancient lineage *Selaginella*.

Selaginella is a genus of approximately 700 extant species in the early diverging lycophyte lineage of vascular plants (7, 7b). The lycophytes contributed important arborescent elements to early forests (8), but *Selaginella* itself is a herbaceous linage that had appeared by 310 Ma (9). Most *Selaginella* species are tropical and occur in wet forest, but they are found worldwide in habitats as varied as tundra (e.g., *S. sibirica*), desert (e.g., *S. lepidophylla*), and temperate forest (e.g., *S. uliginosa*), and vary in morphology from prostrate, moss-like plants to scrambling climbers several meters in length. Megaspores of *Selaginella* range from about 200–1200 μm in diameter, a modest size compared to seeds (10), but much larger than the spores of homosporous plants (11). Microspores are about 20–60 μm in diameter (11), a size range comparable to pollen grain sizes among seed plants (12).

How might Late Devonian environments have created selection that led to this degree of size difference between male and female spores? Haig and Westoby (13) proposed that the first evolutionary step toward heterospory was an increase in the size of bisexual isospores, a trend known from the fossil record (14). This increase would, according to the argument, have been driven primarily by the advantage of having greater resource reserves to support establishment of the next sporophyte generation, a female function of bisexual gametophytes. Male fitness, in contrast, is assumed to be governed primarily by a minimum size for spore viability; above that minimum, male fitness is largely insensitive to spore size. Thus, an increase in the size of bisexual isospores would provide more fitness benefits to the female function than to the male function of bisexual gametophytes. Drawing on a model of optimal offspring size (15), Haig and Westoby argued that bisexual isospores could reach a size at which smaller but more numerous unisexual male spores would provide greater average male fitness per unit of resource investment by the

sporophyte (13). Under this resource allocation advantage, production of microspores would spread. Indeed, the Haig-Westoby hypothesis emphasized that the evolution of microspores was the central evolutionary novelty in the origin of heterospory (13). Finally, frequency-dependent selection created by the spread of microspore production would simultaneously favor canalized expression of unisexual female development in the large isospores, leading to megaspores and the establishment of heterospory. Some available evidence is consistent with this argument (2), but the hypothesis has not been well investigated.

Here we test two key elements of the Haig-Westoby hypothesis: microspore size should be insensitive to habitat, and shaded and competitive habitats should favor larger megaspore size (13, 16). Increasing height and complexity of vegetative canopies (43, 44) and the evolution of microphylls that increased light interception (8) accompanied the Early Devonian rise in the diversity and size of vascular plants approximately 400 million years ago (45, 46). The advent of these novel terrestrial environments preceded the first appearance of heterospory in the Emsian stage of the Early Devonian (12, 46). Thus, we quantified the modern regeneration environment faced by *Selaginella* spores using worldwide patterns of leaf area index (LAI), the number of leaf layers in the vegetative canopy above a given point on the earth's surface (Fig. 1a), and of net primary productivity (NPP), the photosynthetic fixation of carbon by the vegetation cover on a unit area of the earth's surface per unit time (Fig. 1b). LAI measures foliage density, thus understory shade, an important limitation to photosynthetic assimilation rates; NPP measures plant growth, thus the leniency of the abiotic environment but also the potential competition faced by young sporophytes during their establishment. Worldwide, these two metrics are only moderately correlated ($r = 0.57$) (17). Among habitats where the 114 species of *Selaginella* in the present study occur (Fig. 1c), the association of foliage density and primary productivity is complex. LAI and NPP are strongly correlated ($r = 0.78$) for those species in relatively open habitats (LAI < 4) but much weaker ($r = 0.36$) for those species that occur in denser vegetation (LAI > 4). That is, LAI and NPP are largely redundant measures at low foliage density but not at high density. This

complexity proves important for the interpretation of *Selaginella* reproductive ecology because LAI and NPP may exert opposite selective effects on spore size, particularly on megaspore size. Increasing shade under higher LAI canopies should favor larger megaspores (13, 16), much as shady habitats favor large seeds among spermatophytes (18–22). But increasing survival and growth in higher NPP habitats could create selection for smaller and more plentiful megaspores, much as permissive juvenile environments can favor smaller eggs in animals (23, 24).

3.3 – Methods

Spore size. Spore size data for the 114 species in our sample were obtained from specimens held in the United States National Herbarium (US) at the Smithsonian Institution and the National Herbarium of Victoria (MEL). Megaspores and microspores were extracted from strobili, hydrated in distilled water, and digitally photomicrographed with a length calibration bar in every frame. We then measured spore size from the photomicrographs by fitting an ellipse to a spore profile and extracting the lengths of the major and minor axes. We treated the spore shape as an ellipsoid of rotation and calculated its volume as $V = 4\pi r_1 r_2^2/3$, in which r_1 and r_2 are the major and minor semi-axis lengths.

Phylogeny of *Selaginella*. In order to conduct a phylogenetically informed analysis, we inferred the phylogeny of *Selaginella* species based on *rbcL* sequence data. Sequences (including duplicate taxa sequences) were downloaded from GenBank (retrieved January 17, 2017). A preliminary analysis led to identification and removal of unreliable, duplicated and misidentified gene sequences in conjunction with BLAST (GenBank search). The final alignment represented 203 species including an outgroup of eight species of *Isoetes*, the sister lineage to *Selaginella*. The sequences were aligned by ClustalX2 (53), and the aligned sequences were checked for obvious mistakes and modified manually in MEGA version 7.0 (54). The best-fit model of evolution was identified as GTR with

gamma, invariant sites and equal frequencies in jModelTest 2.1.10 (55, 56). However, better mixing of the MCMC and better ESS values were obtained with estimated frequencies in runs under a relaxed lognormal clock. The hypothesis of a strict molecular clock was tested using PAUP (57). However, the null hypothesis was rejected under a log likelihood ratio test (chi-test: $p < 0.05$). A relaxed (uncorrelated) lognormal clock was preferred due to the long evolutionary time (~370 Myr) of the dataset. The tree model used was a birth-death model with incomplete sampling (58). Two basal fossils were applied as calibrations for divergence date estimation. One of the fossils constrained the divergence of Isoetaceae from Selaginellaceae at 370 Myr (prior: exponential, $\bar{x} = 5$) using the fossil *Lepidosigillaria whitei*, considered to be isoetalean due to similarities in rooting system architecture with *Isoetes* (59, 60). The second fossil constrained the crown group of *Selaginella* at 310 Myr (prior: exponential, $\bar{x} = 5$) based on the early anisophyllous, dichotomously branching heterospore fossil of *Selaginella suissei* (9, 61). The MCMC analysis was prepared in BEAUti for use in the BEAST software package version 1.8.4 (62). Four separate Markov chain Monte Carlo runs with random starting topology were conducted. Each run lasted 20 million generations with sampling every 5,000 generations. Twenty million burn-in states in each run were removed; however the fourth run converged later, reaching the same plateau as other MCMC at a much later than the others. The burn-in was taken at that point of plateau. All runs were combined to create a larger sample set and enhance the effective sample size (ESS). The maximum clade credibility tree was generated using TreeAnnotator (BEAST package) and then pruned to include only the 114 *Selaginella* species for which we have spore size data.

The resulting tree is shown in Fig. S1. largely retains the structure of the phylogenetic analysis of Korall and Kenrick (63) and Weststrand and Korall (70), in particular the division of most of the genus into major clades A and B and the named subgenera within them. Our phylogeny differs substantially in only a single clade composed of *S. australiensis* and *S. sinensis*, which diverges early in our tree (after subgen. *Selaginella* containing *S. selaginoides* and *S. deflexa*: Fig. S1), thus sitting outside subgen. Stachygynandrum to which Weststrand and Korall (70) assigned

the clade. The positioning of *S. australiensis* and *S. sinensis* had been considered unreliable by Korall and Kenrick (63). We removed these two species and repeated all the analyses described below, without any substantial change to the results. We therefore report results from the full tree with *S. australiensis* and *S. sinensis*.

Biogeography of *Selaginella* species. Occurrence records for the *Selaginella* species in our sample were obtained from the Global Biodiversity Information Facility (GBIF, www.gbif.org) and Tropicos (tropicos.org) databases on 26 May 2016. Records without latitudinal and longitudinal coordinates of the collection site as well as duplicate collection records from identical geographic coordinates were removed. The remaining records were individually checked to remove locations outside the native range of a species (64) and locations within urban areas, farmlands, plantations, clear felling and water bodies. After cleaning the data, we had from 1 to 327 occurrence records for individual species (97.6% of species had over 3 occurrence records).

Environmental data. We obtained data on global patterns of leaf area index (LAI), and net primary productivity (NPP) from the NASA Earth Observations (NEO) database (http://neo.sci.gsfc.nasa.gov). Leaf area index represents the number of leaf layers in the vegetation above a point on the earth's surface. Global patterns of LAI were estimated from satellite observations of surface reflectance spectra in conjunction with a model of expected radiances from canopy structures over the range of natural conditions (65). Net primary productivity is the net amount of carbon fixed per unit area of the earth's surface per unit time, i.e., gross photosynthetic assimilation less respiratory loss. NPP can be estimated from remote measurement of the fractional absorption of phytosynthetically active radiation by vegetative cover (66). We obtained monthly summary data for LAI and NPP in GeoTIFF maps at 0.1 degree spatial resolution for each month from 2001 through 2016 (2015 for NPP). We extracted the maximum LAI observed for a given pixel in the maps over the course of each year, and then averaged these yearly maxima over the 16

years of data to obtain a single representation of average maximum LAI over the terrestrial surface (Fig. 1a). Higher values of LAI represent more shaded conditions at the ground surface. We summed monthly NPP over the twelve months of a year and then averaged the yearly sums over the 15 years of data to obtain an average measure of total yearly plant growth (Fig. 1b).

We characterized the environment in which each *Selaginella* species occurs by using the *extract* function in the R package *raster* (67) to obtain the LAI and NPP values at the collection location for each geo-referenced occurrence in our cleaned data set. The average LAI and NPP variable across all the records for a given species were taken as the typical habitat for that species.

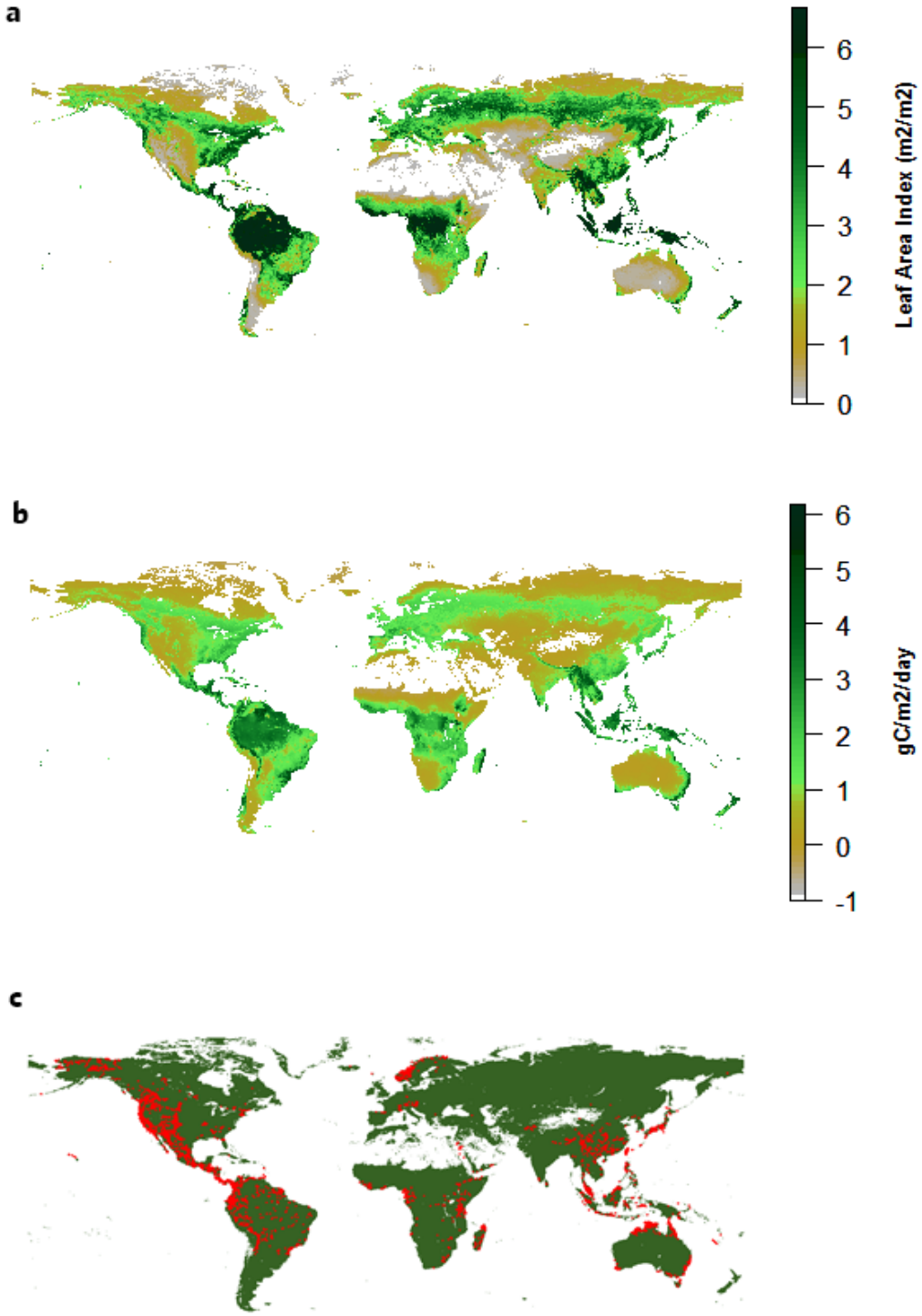

Fig. 1. Global distribution of (a) average maximum annual leaf area index (LAI) and (b) average annual net primary productivity (NPP); and (c) geographic occurrences used to estimate characteristic environments of the Selaginella species in the sample.

Statistical analysis. We compared the relationship between megaspore or microspore volume (transformed to common logarithms for all analyses) and the habitat variables of LAI and NPP using phylogenetic generalized least-squares (pGLS) regression. The analyses were carried out in the R package *caper* (68). Unlike ordinary least-squares regression, which assumes complete independence of all cases in the analysis, pGLS accounts for differing degrees of relatedness among species through a matrix of expected covariances between species in their regression residuals; the expectations are based on branch lengths in the phylogenetic tree structure (69). We used the untransformed tree (i.e., $\lambda = 1$) in Fig. S1 for the analyses. In the initial analysis of megaspore size, we calculate four pGLS models: one each using LAI or NPP as the sole independent variable, one with both variables together, and one with both variables and their interaction effect. The best supported model judging by the Akaike Information Criterion (AIC) included the interaction. To visualize the interaction, we plotted phylogenetically adjusted values of the logarithm of megaspore volume (the value for each species predicted by pGLS regression plus the phylogenetic residual) against either LAI or NPP (Fig. 2a, b).

Data availability. Spore size data and habitat LAI and NPP mean values for individual *Selaginella* species, and the *Selaginella* phylogenetic tree are available in Figure S1.

3.4 – Results

From occurrence data for 114 *Selaginella* species (Fig. 1c), we could determine the average environment that each species inhabits (Table S1). Phylogenetically informed regression techniques then allowed us to examine the association of environmental characteristics with the spore sizes of species in those environments.

The best models for microspores showed that their average volume decreases as LAI or NPP increases (Fig. 2). The slope of the LAI effect (Table 1a) indicates that average microspore volume

changes by a factor of $10^{-0.066} = 0.86$ with every unit increase in habitat LAI, i.e., a loss of 14% in size. A unit increase of 1 g C m^{-2} day^{-1} in NPP changes average microspore volume by a factor of $10^{-0.116} = 0.77$ (Table 1b).

Table 1. Phylogenetic regressions of $\log_{10}$(microspore volume in μm^3) in relation to (a) LAI (dimensionless) and (b) NPP (C assimilation in g m^{-2} day^{-1}). (a) Model $F_{1, 112} = 18.62$; $R^2 = 0.14$. (b) Model $F_{1, 112} = 19.79$; $R^2 = 0.15$.

	Model Term	Estimate	Std. Error	*t*	*P*
(a)	Intercept	4.495	0.624	7.20	< 0.0001
	LAI	–0.066	0.015	–4.32	< 0.0001
(b)	Intercept	4.544	0.622	7.30	< 0.0001
	NPP	–0.116	0.026	–4.45	< 0.0001

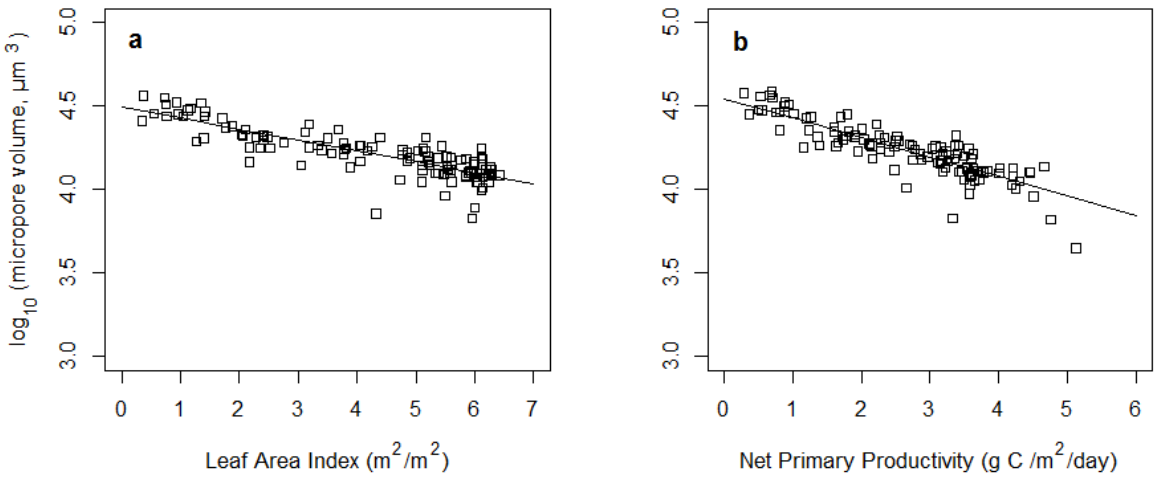

Fig. 2. Microspore size in relation to (a) Leaf Area Index and (b) Net Primary Productivity.

Each panel shows results from separate regression models with either LAI or NPP as a sole independent variable. Both panels show the phylogenetically adjusted values of microspore size, i.e., the prediction of the regression equation plus the phylogenetic residual for a given species.

The best model for megaspore size indicated significant effects of NPP and an interaction of LAI and NPP (Table 2). Visualization of the interaction shows that megaspore size follows an approximate U-shaped pattern with respect to foliage density, tending to become smaller as LAI increases up to a value of approximately 4 leaf layers per unit ground area, but then becoming larger as LAI increases further (Fig. 3a). With respect to NPP, megaspore size follows an even more complex, non-monotonic pattern (Fig. 3b).

Table 2. Phylogenetic generalized least-squares regression of the common logarithm of megaspore volume (μm^3) in relation to LAI (dimensionless), NPP (C assimilation in g m^{-2} day^{-1}), and their interaction effect. Model $F_{3, 110} = 4.54$; $R^2 = 0.11$.

Model Term	Estimate	Std. Error	*t*	*P*
Intercept	8.305	0.645	12.88	< 0.0001
LAI	–0.005	0.058	–0.09	0.927
NPP	–0.501	0.149	–3.37	0.001
LAI × NPP	0.062	0.024	2.51	0.014

We dissected this complexity by splitting the data into two sets of species: those inhabiting environments with LAI < 4 (“low LAI”) and with LAI > 4 (“high LAI”). Megaspore sizes among *Selaginella* species in low LAI habitats are unaffected by the LAI variation from 0 to 4 (open symbols in Fig. 3c), but in high LAI habitats, greater foliage density is associate with significant increases in megaspore size (filled symbols in Fig. 3c). The slope of the LAI effect for the high LAI

species (Table 3b) indicates that every unit increase in LAI above 4 brings an average $10^{0.241}$ = 1.74-fold increase in the megaspore volume of the *Selaginella* species present.

Higher levels of primary productivity correlates with a decline in megaspore size in the open, low LAI habitats (open symbols in Fig. 3d) but not in dense, high LAI habitats (filled symbols in Fig. 3d). The contrast suggests that a lenient environment with higher survival rates of megagametophytes and higher growth rates for young sporophytes favors greater megaspore numbers at the expense of megaspore size, but only in habitats with low density of foliage. The slope of the NPP effect for the low LAI species (Table 3a) indicates that every increase in habitat productivity by 1 g C m^{-2} day^{-1} is associated with an average decline in megaspore volume to $10^{-0.459}$ = 0.35 of the reference value.

The contrasting effects of LAI on megaspores and microspores are not due to an interspecific negative relationship (i.e., evolutionary trade-off) between megaspore and microspore size. To the contrary, megaspore and microspore volumes are positively, albeit very weakly, related across the species in our data set (phylogenetic GLS regression using log-transformed spore volumes: $F_{1,112} = 15.08$, $p < 0.001$, $r^2 = 0.11$).

Table 3. Phylogenetic regressions of $\log_{10}$(megaspore volume in μm^3) in relation to LAI (dimensionless) and NPP (C assimilation in g m^{-2} day^{-1}) for data separated into species inhabiting (a) open environments (LAI < 4), and (b) closed environments (LAI > 4). (a) Model $F_{2, 43} = 4.54$; $R^2 = 0.17$. (b) Model $F_{2, 65} = 8.08$; $R^2 = 0.20$.

	Model Term	Estimate	Std. Error	*t*	*P*
(a)	Intercept	7.832	0.610	12.85	< 0.0001
	LAI	0.188	0.095	1.97	0.056
	NPP	–0.459	0.155	–2.96	0.005
(a)	Intercept	6.629	0.628	10.55	< 0.0001
	LAI	0.241	0.068	3.52	< 0.001
	NPP	–0.045	0.080	–0.56	0.576

3.5 – Discussion

Microspore size. Microspores of *Selaginella* tend to be larger in open and low productivity habitats, and smaller in dense or productive vegetation (Fig. 3). Unlike the functional similarity between megaspores and seeds, it is more difficult to draw an analogy between the ecology of microspore size and pollen size, even though angiosperm pollen are approximately the same size as *Selaginella* microspores (12). What we can infer about *Selaginella* microspores is limited by what we know in the spermatophytes, *e.g.* pollen-stigma interactions (26, 27), the special roles of animal pollination vectors (25), and pollen tube growth and competition (28). Nonetheless, two effects of habitat on angiosperm pollen size may be relevant. The first concerns desiccation and pollen longevity. The reduction in surface area:volume ratio of larger pollen grains will tend to promote

desiccation resistance (29, 30). Accordingly, species that release pollen under conditions that are more arid tend to have larger pollen grains (30), an effect noted also for spore size in basidiomycete fungi (31). Equivalently, pollen tends to be larger in more arid environments. The decline in *Selaginella* microspore volume as habitat NPP increases (Fig. 3b) is consistent with the angiosperm pattern. Higher humidity associated with high NPP would promote microspore viability, allowing a reduction in size and concomitant increase in microspore production under a size-number trade-off. This effect is similar to the effect of artificially elevated productivity on egg size documented in chinook salmon (24).

A second habitat effect relevant to both pollen and microspore dispersal is filtration by vegetation surfaces. Wind-borne pollen is filtered, sometimes strongly, from the air by leaves (32) and bark (33). Similar filtration of *Selaginella* microspores is likely to be correlated with foliage density in the habitat. Species with smaller microspores and greater microspore production per unit investment could therefore be favored in high LAI habitats, leading to the geographic pattern we found (Fig. 3a).

Megaspore size. On a global scale, *Selaginella* megaspores tend to be larger where leaf density is higher and the habitat relatively closed (Fig. 2c) and smaller where net primary productivity is greater, provided the habitat is relatively open (Fig. 2d). The effect of foliage density is consistent with a functional advantage for large nutrients reserves to support early growth of the new sporophyte generation (Nutrients are not used by the megagametophyte that develops directly from the spore) (2, 34). The reserve function is even morphologically reinforced in some *Selaginella* species by a septum separating the megagametophyte from the bulk of the megaspore contents (35). Similar nutrient stores are observed in the megaspores of other lineages of free-sporing heterosporous plants (36–38). While it remains to demonstrate experimentally that these reserves provide an advantage for establishment of new sporophytes under natural conditions, the mechanisms that would create an advantage for *Selaginella*—greater survival rate and better

etiolation response under litter, shade or competitive pressure (19)—are no different to those acting in seeds, where such effects have been abundantly demonstrated (18–22, 39, 40).

The functional similarity between megaspore size and seed size is reflected in similar biogeographic patterns. Seeds also tend to be larger in habitats with greater LAI (41), and mean seed mass within communities shows a pronounced rise at the latitudinal transition from relatively open sub-tropical vegetation to tree-dominated tropical vegetation (10). These geographic patterns point to the advantage of a large nutrient reserve for establishment of seedlings in regeneration sites subject to shade and heavy litter layers. Exceptions to this generalization, such as small seeded species in tropical wet forest environments, often specialize at establishing on steep, clear microslopes, while larger seeds establish elsewhere (42).

Implications for the evolution of heterospory. Our analysis of *Selaginella* supports a central element of the Haig-Westoby theory: that the first step toward heterospory occurred when shaded, competitive environments created selection for larger spores that could better assist the early growth and establishment of young sporophytes (13). High-LAI environments supporting larger megaspores in *Selaginella* may provide the best extant model for the environmental conditions in which the spore size differentiation of heterospory first evolved. Because homospory is the ancestral condition in vascular plants, an initial increase in spore size would have involved isospores that gave rise to bisexual gametophytes. Comparison of the largest bisexual isospores of extant homosporous plants, the smallest megaspores of extant heterosporous plants, and the megaspores sizes of fossil plants suggests that the differentiation of spore sizes and sexual functions is favored when isospore size approaches an approximate threshold of 200 μm diameter (2, 13).

In the model of Haig and Westoby (13), microspores become adaptive when bisexual isospores become “too big,” that is, due to advantages arising from options for resource allocation. The decline we found in microspore volume among species in environments of higher LAI and NPP (Fig. 3) suggests that additional direct selective pressures on male spore size arising from the

environment may also have occurred. The filtering effect of foliage, inimical to spore dispersal, would have accompanied the evolution of leaves and would have been well established in the plant communities of the Devonian (43). Selection for smaller spores from this effect could have acted in concert with the resource allocation advantage posted by Haig and Westoby (13).

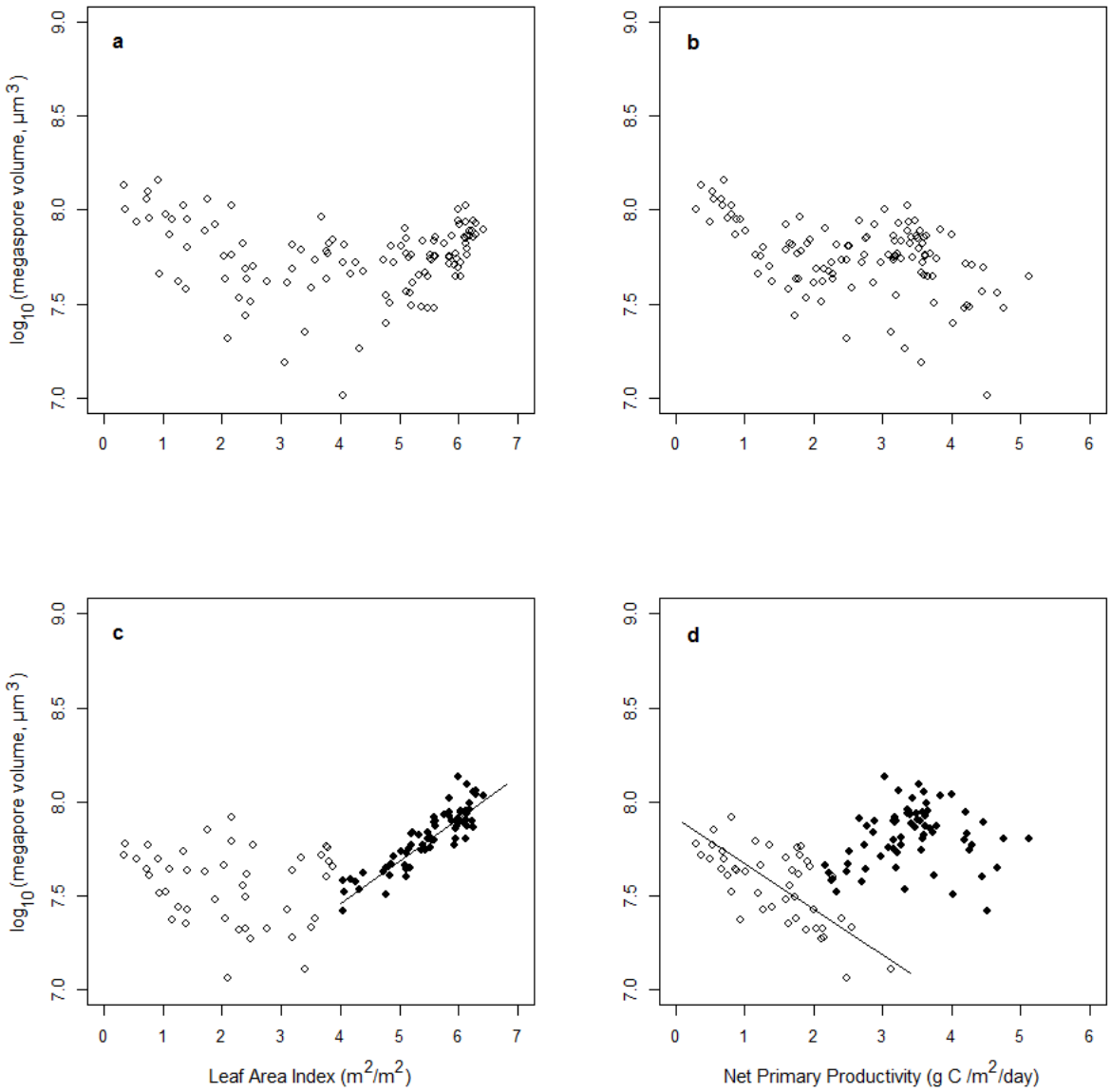

Figure 3. Megaspore size in relation to (a, c) Leaf Area Index and (b, d) Net Primary Productivity. (a, b) Results of a single model with LAI, NPP, and the LAI×NPP interaction as independent variables, plotted against LAI in panel (a) and against NPP in panel (b). (c, d) Results of models with LAI and NPP (without the interaction) as independent variables, applied separately to *Selaginella* species in habitats with LAI < 4 (open symbols) and with LAI > 4 (filled symbols). Results from the two separate analyses are superimposed in panels (c) and (d). All panels show the phylogenetically adjusted values of megaspore size, i.e., the prediction of the regression equation plus the phylogenetic residual for a given species.

Heterospory in other lineages. The genus *Isoetes*, the heterosporous sister lineage to *Selaginella*, prefers aquatic habitats (some terrestrial species exist), and the heterosporous water ferns (Marsileaceae and Salviniaceae) are fully aquatic (47). Fossil evidence and this distribution of heterospory among extant lineages has prompted suggestions that heterospory may have originally (or additionally) evolved in aquatic environments (1, 3, 45). Aquatic habitats, like canopy foliage, restrict light availability because of filtering in the water column (48–50) and restrict photosynthetic productivity through limitation of CO_2 diffusion (51). Thus, regeneration under water and regeneration in shaded terrestrial environments should present comparable challenges that favor the evolution of large nutrient reserves in spores. If so, the evolution of heterospory in aquatic lineages may have occurred following selection pressures similar to those suggested by our analysis of *Selaginella*. *Isoetes* and the water ferns therefore offer additional opportunities to investigate empirically the adaptive nature of heterospory.

Perspectives on the history of land plants. The role of heterospory in the evolutionary history of land plants is ambiguous. On one hand, multiple, independent origins of heterospory (1) point to the existence of strong selective advantages in the early forest habitats in which it arose, and the seed plants, all of which are heterosporous, dominate the earth's current terrestrial plant diversity (7). On the other, extinctions of most heterosporous lineages that have existed in the past and the meagre species diversity of heterosporous water ferns in comparison to homosporous ferns (2) suggest that the advantages of heterospory do not always provide long-run evolutionary success (52).

Our results here suggests that megaspores were early functional equivalents of seeds, that is, a means of conveying nutrient reserves between sporophyte generations. Botanists traditionally draw a distinction between freely dispersed megaspores and seeds because of the presence of integuments enclosing first the megasporangium and later the mature seed. Integuments do present a number of advantages such as protection and regulation of dormancy, and these advantages no doubt contributed to the dominance of seed plants in the earth's vegetation. But megaspores of free-

sporing heterosporous plants appear to have been selected originally because they filled an ecological role now predominantly occupied by seeds (16). Repeated independent origins of heterospory seem to follow from a ubiquitous advantage for a seed-like establishment strategy once tall and dense vegetation evolved. Heterospory, then, evolved because megaspores were the functional seeds that appeared before true morphological seeds evolved.

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3.7 - Supplementary Information

Table S1. Species (n = 114) used in the analysis, their mean megaspore and microspore volumes, and mean leaf area index (LAI) and net primary productivity (NPP) of their habitats. All species collected from (US), except *S. gracillima* which was collected from (MEL).

Species	Megaspore volume (μm^3)	Micropore volume (μm^3)	LAI (m^2/m^2)	NPP (g C/m^2/day)
Selaginella alopecuroides Bak.	17616668	7021	6.42	1909.6
Selaginella amblyphylla Alston	6588927	16459	5.98	1886.9
Selaginella apoda (L.) Spring	21836192	11782	5.04	1390.8
Selaginella arenicola Underw.	17814288	25181	4.25	1463.6
Selaginella arizonica Maxon	10534738	27176	0.76	605.4
Selaginella arsenei Weath.	29051392	32120	3.51	1400.2
Selaginella articulate Kze.	162401844	5653	6.24	1794.4
Selaginella asprella Maxon	32031972	38610	1.17	768.2
Selaginella australiensis Baker	95966407	8227	5.17	2239.0
Selaginella biformis A. Braun ex Kuhn	67410421	5941	5.60	1653.8
Selaginella bigelovii Underw.	13994520	29033	1.27	944.0
Selaginella bodinieri Hieron.	10664943	20624	3.77	1111.7
Selaginella bombycina Spring	13381167	9916	6.01	1530.8
Selaginella boninensis Bak.	12500505	12098	5.93	2094.3
Selaginella brooksii Hieron.	14191874	4236	6.04	1835.3
*Selaginella caffrorum (*Milde) Hieron.	6203715	48115	1.39	1037.6
Selaginella chrysocaulos (Hook. & Grev.) Spring	41726676	56669	3.57	1343.3
Selaginella ciliaris Retz.	11630187	5003	2.42	1101.5
Selaginella cinerascens A.A.	41521369	66034	0.95	867.0

Eaton				
Selaginella davidii Franch.	10633149	12700	4.04	1285.6
Selaginella deflexa Brack.	78320844	29911	5.38	2074.6
Selaginella delicatula Alston	31454295	24621	5.84	1749.4
Selaginella densa Rydb.	44151442	23015	2.04	882.5
Selaginella denticulate (L.) Spring	37724215	23200	1.41	745.2
Selaginella diffusa (K. Presl)				
Spring	34560912	18239	6.12	1728.1
Selaginella doederleinii Hieron.	24673819	8380	5.89	1639.4
Selaginella douglasii (Hook. &				
Grev.) Spring	39583129	15444	4.17	1293.1
Selaginella dregei (Presl) Hieron.	24710306	41392	2.48	1231.6
Selaginella echinata Bak.	54405270	73951	3.40	1627.9
Selaginella effusa Alston	28305960	29498	3.78	1092.4
Selaginella eremophila Maxon	8211327	50629	0.35	537.6
Selaginella erythropus (Mart.)				
Spring	4399176	6148	4.86	1384.4
Selaginella exaltata (Kze.)	626606189	5249	6.29	1672.3
Selaginella extensa Underw.	24802049	252301	4.85	1874.1
Selaginella firmuloides Warb.	10989133	4042	5.12	2152.8
Selaginella flabellata (L.) Spring	6785357	6559	5.94	1851.7
Selaginella flagellata Liebm.	5521794	5346	6.12	1811.6
Selaginella fragilis A. Br.	209447893	9335	6.24	1765.8
Selaginella frondosa Warb.	8103259	3970	6.26	1819.5
Selaginella gracillima (Kuntze)				
Alston	28289656	19145	3.06	1805.8
Selaginella haematodes (Kze.)				
Spring	6862064	4761	6.18	1830.8
Selaginella hansenii Hieron.	16778181	24689	2.41	1078.5
Selaginella helferi Warb.	12848102	9388	4.90	1567.4
Selaginella helicoclada Alston	27430988	31561	2.29	1145.0

Selaginella helvetica (L.) Spring	7100520	3405	3.68	1104.1
Selaginella heterostachys Bak.	17865736	19173	5.55	1641.6
Selaginella imbricata (Forsk.) Spring ex Decaisne	16854721	69882	0.77	693.6
Selaginella indica (Milde) Tryon	17956620	18855	3.88	1163.2
Selaginella intermedia (Bl.) Spring	14847418	5186	6.20	1721.5
Selaginella involvens (Sw.) Spring	71614008	11299	5.60	1653.2
Selaginella kraussiana (Kunze) A. Braun	10650719	16724	4.32	1710.7
Selaginella labordei Hieron.	11314930	30448	5.15	1640.9
Selaginella landii Greenm. & N. Pfeiff.	14167962	36046	3.78	1295.8
Selaginella lepidophylla (Hook. & Grev.) Spring	15352265	166469	1.88	1027.8
Selaginella leucobryoides Maxon	24677486	30323	0.37	508.6
Selaginella limbata Alston	102496152	14204	3.81	1148.0
Selaginella lingulata Spring	103238974	15972	6.00	1594.1
Selaginella longiaristata Hieron.	13430518	4331	5.39	1688.8
Selaginella longipinna Warb.	15129282	4091	5.49	2275.1
Selaginella lyallii (Hook. & Grev.) Spring	301626122	5501	5.96	2421.3
Selaginella martensii Spring	14131804	12914	5.99	2157.1
Selaginella mayeri Hieron.	5632323	17124	6.11	1744.0
Selaginella moellendorffii Hieron.	13846891	10928	5.52	1611.4
Selaginella mollis A. Br.	7338031	4289	5.44	1801.1
Selaginella mutica D.C.Eat. Ex Underw.	12292378	17777	0.73	657.0
Selaginella myosurus (Sw.) Alston	227362363	14869	6.00	1445.2
Selaginella nipponica Franch. & Sav.	19353918	20039	5.11	1481.2
Selaginella nivea Alston	35416537	23609	2.10	1373.6

Selaginella novae-hollandiae (Sw.) Spring	13896137	12028	5.47	1864.4
Selaginella oregana D.C. Eaton	23866645	61549	5.11	1667.1
Selaginella ornata (Hook & Grev.) Spring	6040047	10220	6.14	1791.3
Selaginella pallescens (C. Presl) Spring	19956195	31930	4.77	1654.7
Selaginella peruviana Hieron.	32058577	29715	2.36	1048.8
Selaginella phillipsiana (Hieron.) Alston	17707902	50369	1.05	714.1
Selaginella picta (Griff.) A.Br. ex Bak.	20056013	5647	4.06	1319.3
Selaginella pilifera A.Br.	18282594	12874	2.39	1200.3
Selaginella plana (Desv. Ex Poir.) Hieron.	24581968	25519	6.14	1827.6
Selaginella pulvinata (Hook. & Grev.) Maxim.	36910470	44161	2.17	850.7
Selaginella pygmaea (Kaulf.) Alston	29759771	37915	2.06	1081.6
Selaginella radiata Baker	6154347	3709	5.77	1735.8
Selaginella remotifolia Spring	207831924	46206	5.59	1649.2
Selaginella repanda (Desv. & Poir.) Spring	7333543	49855	5.34	1819.2
Selaginella roxburghii (Hook. & Grev.) Spring	7682479	3075	6.13	1721.1
Selaginella rupestris (L.) Spring	14455120	143883	3.33	1027.4
Selaginella rupincola Underw.	12947460	31389	1.71	794.6
Selaginella sanguinolenta (L.) Spring	21517912	23488	1.35	667.3
Selaginella sartorii Hieron.	33332633	43315	3.19	1064.9
Selaginella selaginoides (L.)	88973693	15157	2.17	715.1

Schrank & C.F.P.Mart.				
Selaginella sellowii Hieron.	11773877	16255	3.11	1187.4
Selaginella sericea A.Br.	310812847	9117	6.14	1774.5
Selaginella shakotanensis (Franch.				
Ex Takeda) Miyabe & Kudo	5593101	37829	5.10	1250.1
Selaginella siamensis Hieron.	22825545	17262	5.53	1685.4
Selaginella sibirica (Milde)				
Hieron.	23182163	30678	1.77	612.3
Selaginella simplex Baker	5941260	22099	5.58	2049.9
Selaginella sinensis (Desv.) Spring	111373415	13429	2.52	932.2
Selaginella stauntoniana Spring	13174424	6365	5.84	1821.9
Selaginella steyermarkii Alston	13279191	51891	4.77	1982.2
Selaginella suavis Klotzsch	281681143	6115	5.21	2060.1
Selaginella sulcata (Desv. Ex				
Poir.) Spring	77435202	6658	5.85	2059.5
Selaginella tamariscina (Beauv.)				
Spring	26820552	47799	5.23	1525.0
Selaginella tortipila A.Braun	30974902	57188	5.61	1491.5
Selaginella uliginosa (Labill.)				
Spring	83192297	23140	4.05	2175.9
Selaginella umbrosa Lem. Ex				
Hieron.	3908500	4134	6.30	1977.3
Selaginella uncinata (Desv. Ex				
Poir.) Spring	12509905	6538	4.73	1373.3
Selaginella underwoodii Hieron.	18081025	18209	1.13	739.6
Selaginella utahensis Flowers	36024248	61983	0.55	593.7
Selaginella vogelii Spring	47078429	10090	5.20	1478.3
Selaginella wallacei Hieron.	5432165	56014	3.19	1245.4
Selaginella watsonii Underw.	39775288	30202	0.93	670.9
Selaginella weatherbiana R.M.				
Tryon	18753100	26386	1.41	893.1

Selaginella wightii Hieron.	13835292	16406	6.05	1811.7
Selaginella willdenowii (Desv.) Bak.	63092426	10845	6.13	1777.2
Selaginella wrightii Hieron.	26822607	27578	2.76	1235.6
Selaginella xipholepis Bak.	13132215	56044	4.39	1272.3

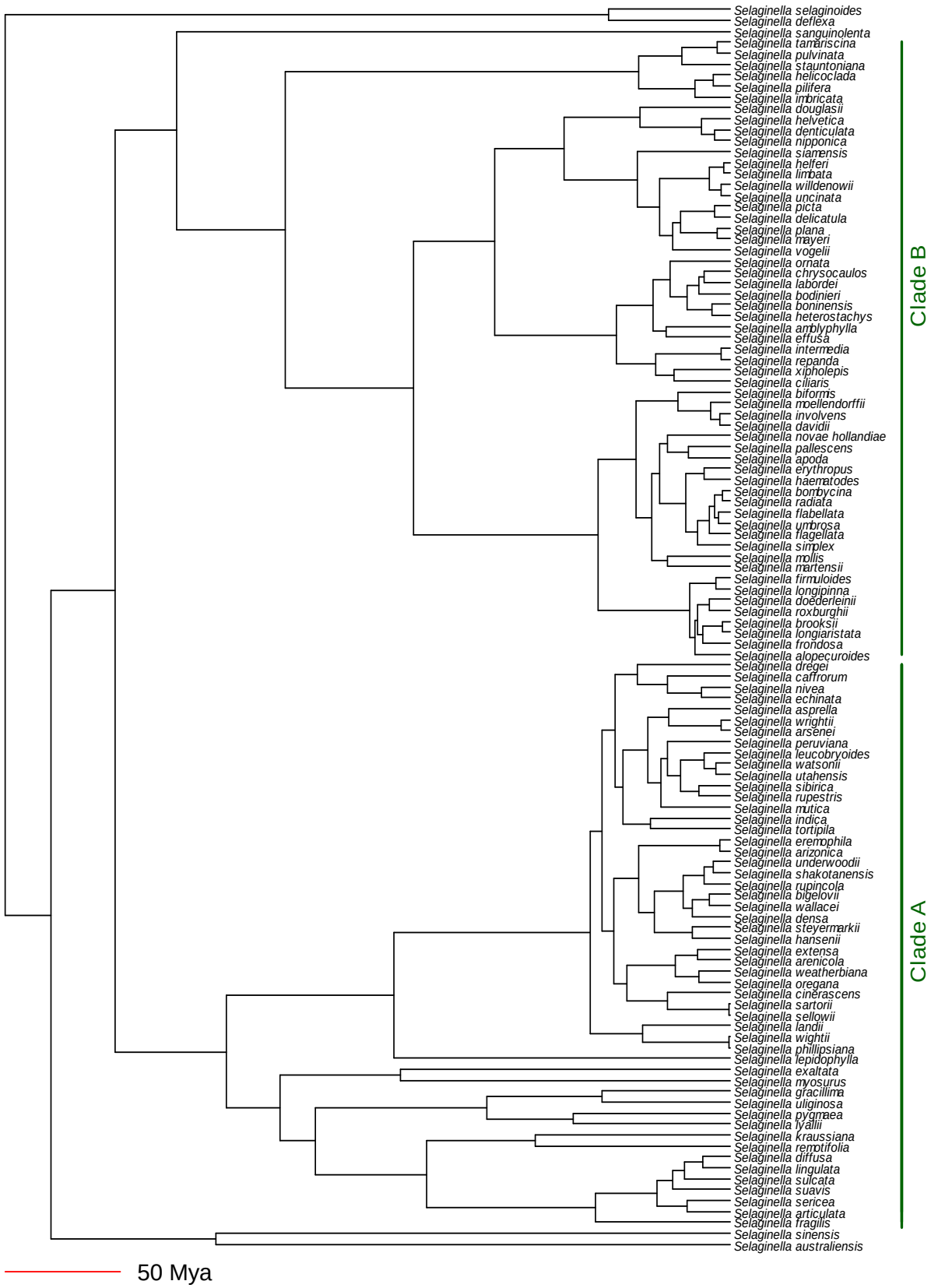

Fig. S1. Fossil calibrated *rbcL* phylogeny for the 114 species of *Selaginella* used in our analysis. The outgroup *Isoetes* has been pruned.

4.0 – Aquatic environments select for more extreme heterospory in the genus *Isoetes*

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4.1 – Abstract

- Heterospory was one of the most important innovations in the history of land plants, although its adaptive origin is poorly understood. Extant species and the fossil record indicate that aquatic habitats are often associated with independent origins of heterospory. Here we explored the adaptive significance of heterospory in the lycopod genus *Isoetes*.
- We measured spore sizes for approximately 40% of the species in *Isoetes* and classified each species into four habitat types that reflect typical water depth and time inundated. A phylogenetic generalized least squares (GLS) ANOVA was calculated in order to test associations of spore size with habitat type.
- *Isoetes* invests significantly more resources into megaspores among species found in Aquatic habitats compared to Ephemeral, Palustral or Terrestrial habitats. However, no difference in megaspore size occurred among other environment types. Habitat type did not affect microspore size.
- Establishment in aquatic habitats is made difficult by light filtering and poor nutrient availability. The degree of heterospory reflects the investment of adult sporophytes in nutrient transfer to the next generation of sporophytes for their early establishment. The

advantage of such investment in aquatic *Isoetes* is a potential model for the advantage of heterospory at its other independent origins.

4.2 – Introduction

Heterospory, the production of unisexual spores of distinct sizes, was one of the most significant evolutionary events in the history of the land plants. However, despite the considerable paleontological evidence documenting the appearance of heterospory in the Devonian, the evolutionary advantage that heterospory offered to early plants is not well understood (Petersen & Burd, 2017). Seed plants are heterosporous and dominate extant plant life: gymnosperms and angiosperms represent approximately 87% of overall land plant species diversity (Chapman, 2009). However, seed plants have reproductively diverged considerably from the first heterosporous plants, in particular through retention of megaspores and megagametophyte development and fertilisation within ovules attached to and nourished by the parent sporophyte (Bewley & Black, 1994). In contrast to the elaborations of the seed, the megaspores of free-sporing heterosporous plants are released straight into the environment from the adult sporophyte before fertilisation occurs. Thus, the megaspores in free-sporing plants have a dispersal function similar to that of a seed, or rather, a seed has a function similar to its predecessor the megaspore. The profound changes that occurred within the extant seed plant lineage make it inappropriate for investigating the ecological origin of heterospory. Instead focus would better be placed on the extant heterosporous Lycopodiales or water fern lineages, Salviniaceae and Marsileaceae (Hermsen *et al.*, 2014; Petersen & Burd, 2017).

The fossil history of the land plants shows that heterospory can be highly advantageous, having evolved independently as many as 11 times (Bateman & DiMichele, 1994). The evolution of heterospory in multiple aquatic groups suggests that there is strong selection for sexual size

dimorphism in free-sporing water plants. For example, extant pteridophytes contain enormous species diversity, yet other than the water ferns, Salviniaceae and Marsileaceae, they are exclusively homosporous (Kenrick & Crane, 1997). The water ferns were not the only origin of heterospory within the pteridophytes. There are examples of heterospory in extinct wetland species of the pteridophyte lineage Equisetales (relatives of extant horsetails) (Bateman & DiMichele, 1994). The possible case of incipient heterospory in *Platyzoma microphyllum* (Pteridaceae), a specialist in ephemeral aquatic habitats, provides another example (Tryon, 1964; Petersen & Burd, 2017). At least circumstantially, evidence for aquatic and wetland origins of heterospory appears to be relatively compelling (Bateman & DiMichele, 1994), thus making these habitats reasonable starting places to investigate the ecological function of heterospory.

In this study, we focused on the free-sporing lycophyte genus *Isoetes*. *Isoetes* is an extant representative of ancient heterosporous plants and resembles some of the earliest heterosporous plants in the fossil record (Kenrick & Crane, 1997). *Isoetes* is a cosmopolitan genus with approximately 250 species (Schneider et al., 2016). Most species are aquatic or emergent, but some occur in terrestrial habitats. Fortuitously, *Isoetes* has an extensive and well understood fossil record indicating an aquatic ancestor (Taylor & Hickey, 1992; Kenrick & Crane, 1997). The fossil ancestor of *Isoetes*, *Isoetites*, exhibited air chambers in its leaves, a feature also observed in extant *Isoetes* and generally considered to provide buoyancy and aeration for aquatic plants (Taylor & Hickey, 1992).

In this study, we used *Isoetes* to investigate the effect of habitat type on the size of microspores and megaspores, a defining feature of heterospory. We expected that low light, complex environments would select for higher nutrient input into individual megaspores. We did not expect to see differences in observed microspore size between habitats due the nature of water dispersal in *Isoetes*.

4.3 – Materials and Methods

Spore size: Megaspore sizes (*n* = 60 species) and microspore sizes (*n* = 55 species) of *Isoetes* species were collected from literature and measured directly from herbarium sheets in the United States National Herbarium (US), Smithsonian Institution, Washington, D.C., in 2014 (Table 1). For the 26 species that were assessed directly, megaspores and microspores were removed from strobili and placed in distilled water on a slide and photomicrographed with a calibrated scale bar. Spore diameter was measured using the software ImageJ 1.47 (Rasband 1997) and the average diameter for each species was calculated. The number of diameter measurements was lowest in *Isoetes hawaiiensis* (*n* = 1) and *I. melanospora* (*n* = 3) but all other species had four or more megaspores measured. Microspore count was lowest in *I. hawaiiensis* (*n* = 3) but all other species had over 8 microspores measured (*I. abyssinica* and *I. coromandelina* had no microspores available on herbarium sheets). The remaining spore size data for 34 species was retrieved from published literature (Table 1). The average size was available for nearly all 34 species on the phylogeny except for *I. hypsophila* where only a single measurement was available (Table 1).

Habitat data: The habitat type in which species occurred was selected using information collected from herbarium sheets and published literature. *Isoetes* habitat preference was well documented in the literature, and many species exist only in extremely small aquatic-island populations. Habitat classifications are classifications of the water availability not the growth habit of the plant *i.e.* Aquatic does not refer to a plant growing underwater. Four habitat assignments were created for *Isoetes*: Aquatic (*n* = 25), Ephemeral (*n* = 23), Terrestrial (*n* = 8), and Palustral (*n* = 4). Aquatic habitats have species that were completely submerged or emergent in or around permenant water bodies. Ephemeral habitats have species that were plants that grew completely submerged, or emergent on the edge of non-permenant or seasonal water bodies/pools. Terrestrial habitats have species that were defined as plants that were reported as either not to be Aquatic (*i.e.* fully terrestrial), or 'rarely emergent' but the preferred environment was Terrestrial. Palustral

habitats have species that were plants located in swamp-wetland type environments, where they were rarely (if ever) submerged, but always within persistent wetlands.

Table 1. *Isoetes* habitat categorisation, spore size data, and size source. The spore data has either standard deviations (preferable) or size range where available. Rarely some species only had one average value in the literature and where the true mean was not supplied the mean was estimated from the range values. Data from the United States National Herbarium (US) was directly collected by us.

		x̄ Spore Size (µm) with SD or Range		Sample Size (n)		
Species	Habitat	Megaspore	Microspore	Megaspore	Microspore	Size Source
Isoetes abyssinica Chiovenda	Ephemeral	431 ± 13.8	*NA*	n= 6	-	United States National Herbarium (US)
Isoetes alpine Kirk	Aquatic	464 ± 16.1	28 ± 1.7	n= 5	n= 9	United States National Herbarium (US)
Isoetes anatolica Prada & Rolleri	Ephemeral	700 (600-800)	23 (21-25)	-	-	Prada & Rolleri (2005)
Isoetes asiatica (Mak.) Mak.	Aquatic	390 (380-400)	27 (24-30)	-	-	Huang et al. (1992)
Isoetes australis S. Williams	Ephemeral	425 (350-500)	30 (27-33)	-	-	Johnson (1984)
Isoetes brevicula E.R.L. Johnson	Ephemeral	350 (300-400)	29 (27-30)	-	-	Johnson (1984)
Isoetes butleri Engelm.	Terrestrial	617 ± 53.6	27 ± 1.4	n= 5	n= 12	United States National Herbarium (US)
Isoetes capensis Duthie	Ephemeral	475 (380-570)	30 (24-36)	-	-	Duthie (1929)
Isoetes caroli E.R.L. Johnson	Ephemeral	375 (350-400)	42 (30-53)	-	-	Johnson (1984)
Isoetes coreana Y.H. Chung & H.K. Choi	Aquatic	420 (355-484)	35 (31-38)	-	-	Choi et al.(2008)
Isoetes coromandelina L.f. subsp. *coromandelina*	Ephemeral	523 ± 54	*NA*	n= 5	-	United States National Herbarium (US)
Isoetes coromandelina L.f subsp. *macrotuberculata*	Ephemeral	475 (420-530)	*NA*	-	-	Kim et al. (2010)
Isoetes cubana Engelm. Ex Bak.	Ephemeral	345 (290-400)	29 (25-33)	-	-	Hickey (1981)
Isoetes dixitei Shende	Aquatic	525 (440-610)	31 (16-45)	-	-	Srivastava et al. (1992)
Isoetes drummondii A.Braun	Ephemeral	350 (300-400)	32 (30-33)	-	-	Johnson (1984)
Isoetes durieui Bory	Terrestrial	690 ± 42.5	36 ± 2.9	n= 11	n= 10	United States National Herbarium (US)
Isoetes echinospora Dur.	Aquatic	344 ± 19.4	25 ± 1.4	n= 7	n= 17	United States National Herbarium (US)
Isoetes engelmannii A. Braun	Aquatic	454 ± 38.9	24 ± 2.5	n= 6	n= 8	United States National Herbarium (US)
Isoetes flaccida A. Braun	Aquatic	318 ± 11.4	24 ± 1.5	n= 4	n= 17	United States National Herbarium (US)
Isoetes habbemensis Alston	Aquatic	551 (507-594)	43	-	-	Croft (1980)
Isoetes hallasanensis H.K. Choi, Ch. Kim & J. Jung	Aquatic	410 (356-464)	29 (26-31)	-	-	Choi, Jung & Kim (2008)

Isoetes hawaiiensis W.C. Taylor & W.H. Wagner	Aquatic	554	75 ± 0.6	n= 1	n= 2	United States National Herbarium (US)
Isoetes histrix Bory	Terrestrial	497 ± 35.7	24 ± 1.5	n= 6	n= 11	United States National Herbarium (US)
Isoetes howellii Engelm.	Aquatic	433 ± 14.4	29 ± 1.7	n= 5	n= 12	United States National Herbarium (US)
Isoetes hypsophila Hand.-Mazz	Ephemeral	320	17 (15-18)	-	-	Palmer (1927)
Isoetes inflate E.R.L. Johnson	Ephemeral	400 (300-500)	33 (30-36)	-	-	Johnson (1984)
Isoetes jamaicensis Hickey	Ephemeral	398 (320-440)	36 (30-40)	-	-	Hickey (1981)
Isoetes japonica A.Br.	Aquatic	530 ± 20.9	30 ± 2	n= 5	n= 11	United States National Herbarium (US)
Isoetes jejuensis H.K. Choi, Ch. Kim & J. Jung	Palustral	375 (325-425)	29 (26-32)	-	-	Choi et al.(2008)
Isoetes laosiensis C. Kim & H.K. Choi	Aquatic	595 ± 36.9	35 ± 2.2	-	-	Kim, Bounphanmy, Sun and Choi (2010)
Isoetes libanotica Musselman, Bolin & R.D. Bray	Ephemeral	339 ± 5.2	27 ± 0.4	-	-	Bolin, Bray and Musselman (2011)
Isoetes lithophila N. Pfeiff	Ephemeral	354 ± 26.7	23 ± 1.5	n= 4	n= 13	United States National Herbarium (US)
Isoetes malinverniana Cesati & De Not.	Ephemeral	529 ± 25	28 ± 1.7	n= 5	n =13	United States National Herbarium (US)
Isoetes maritima Underw.	Aquatic	475 (350-600)	35 (30-40)	-	-	Löve (1962)
Isoetes melanopoda J. Gay	Terrestrial	317 ± 39.1	25 ± 1.8	n= 10	n= 17	United States National Herbarium (US)
Isoetes melanospora Engelm.	Ephemeral	308 ± 12.4	25 ± 1.2	n= 3	n= 22	United States National Herbarium (US)
Isoetes minima A.A. Eaton	Terrestrial	320 (290-350)	29 (26-31)	-	-	Frye and Jackson (1913)
Isoetes muelleri A. Braun	Aquatic	561 ± 34.6	32 (30-33)	n= 4	-	United States National Herbarium (US) (Megaspore); Johnson (1984)(Microspore)
Isoetes muricata Dur.	Aquatic	479 ± 54.4	32 ± 3.1	n= 6	n= 10	United States National Herbarium (US)
Isoetes nuttallii A.Br.	Terrestrial	348 ± 17.1	23 ± 2.1	n= 7	n= 22	United States National Herbarium (US)
Isoetes olympica A.Br.	Ephemeral	405 (360-450)	*NA*	-	-	Bolin, Bray, and Musselman (2011)
Isoetes orcuttii A.A. Eaton	Ephemeral	367 ± 16.2	24 ± 2.8	n= 5	n= 22	United States National Herbarium (US)
Isoetes orientalis Hong Liu & Q.F. Wang	Palustral	420 (350-450)	22 (19-29)	-	-	Hong, Qing-Feng and Taylor (2005)
Isoetes panamensis Maxon & C.V Morton	Aquatic	413 ± 35	32 ± 1.7	n= 5	n= 17	United States National Herbarium (US)
Isoetes philippinensis Merrill & Perry	Aquatic	420 (385-455)	28 (25-30)	-	-	Merrill and Perry (1940)
Isoetes prototypus D.M. Britton	Aquatic	500 (425-575)	28 (24-32)	-	-	Britton and Goltz (1991)
Isoetes pseudojaponica M. Takamiya, Misturu Watanabe & K. Ono	Aquatic	467 (374-600)	32 (26-38)	-	-	Takamiya, Watanabe and Ono (1998)
Isoetes pusilla C.R. Marsden & Chinnock	Aquatic	390 (345-435)	31 (28-33)	-	-	Chinnock (1993)
Isoetes setacea Lam.	Ephemeral	700 (600-800)	28 (25-30)	-	-	Robert et al. (1973)
Isoetes sinensis Palmer	Aquatic	375 (360-390)	29 (27-30)	-	-	Huang, Chen & Li (1992)
Isoetes stellenbossiensis A.V. Duthie	Terrestrial	520 (450-590)	34 (32-36)	-	-	Duthie (1929)
Isoetes stephansenii A.V. Duthie	Ephemeral	533 (450-615)	32 (28-36)	-	-	Duthie (1929)
Isoetes stevensii J.R. Croft	Aquatic	516 ± 19.5	32 ± 2.7	n= 4	n= 11	United States National Herbarium (US)
Isoetes storkii T.C. Palmer	Aquatic	485 ± 25.9	28 ± 1.9	n= 5	n= 8	United States National Herbarium (US)

Isoetes subinermis (Genn.) Cesca & Peruzzi	Terrestrial	440 (320-560)	*NA*	-	-	Cesca and Peruzzi (2001)
Isoetes taiwanensis De Vol	Palustral	312 ± 17.4	21 ± 1.7	n= 5	n= 9	United States National Herbarium (US)
Isoetes toximontana Musselman & J.P. Roux	Ephemeral	298 (275-320)	25	-	-	Musselman and Roux (2002)
Isoetes valida (Engelm.) Clute	Palustral	335 ± 10	21 ± 1.7	n= 5	n= 15	United States National Herbarium (US)
Isoetes velata A.Br.	Ephemeral	514 ± 18.6	25 ± 1.9	n= 5	n= 10	United States National Herbarium (US)
Isoetes yunguiensis Q.F. Wang & W.C. Taylor	Aquatic	390 (340-430)	23 (20-25)	-	-	Qing-Feng, Xing, Taylor and Zhao-Rong (2002)

Phylogenetic tree: The 5.8S gene data (including ITS1 and ITS2) were collected from Genbank in November, 2015. Sixty four species of *Isoetes* and two sister lineage species of *Selaginella* as the outgroup were used to create the gene tree for the trait analysis. ClustalW (Larkin *et al.,* 2007) was used to align the gene data and the alignment was visually inspected for algorithm mistakes in MEGA7 (Kumar *et al.,* 2016). The initial model, GTR+G+I, was selected by AIC in jModelTest software version 2.1.1 (Darriba *et al.*, 2012). The hypothesis of a molecular clock was tested using PAUP (Swofford, 2002); however, a strict clock was rejected by a likelihood ratio test (chi-dist: $df = 1$, $P < 0.0001$). The Bayesian phylogenetic package BEAST software version 1.8.4 (Drummond *et al.,* 2012) was selected for bulding the tree. Four MCMC runs were setup in BEAUTi (BEAST package) with a lognormal relaxed clock, GTR+G+I substituion model and birth-death with incomplete sampling tree model (Stadler, 2009). Each of the four MCMC chains were run for 20 million steps from random starting seeds. All four runs were inspected in Tracer software version 1.6 (Rambaut *et al.,* 2014) and converged well. The runs were combined then analysed in TreeAnnotator (BEAST package) to produce a maximum clade credibility tree.

Statistical analysis: To compare spore size among habitat types, we used a phylogenetic generalized least squares (GLS) ANOVA by performing a generalized least squares regression with phylogenetic correlation of the residuals and using the categorical habitat as the independent variable. The covariance matrix was provided by the 'corBrownian' function in the ape package (Paradis *et al.,* 2004) and the analysis was implemented in the nlme package (Pinheiro *et al.,* 2017) in R version 3.3.2 (R Core Team, 2016). This method is analagous to the pGLS method described in

Rohlf (2001) and demonstrated to have more statistical power (Revell, 2013) than the phylogenetic ANOVA of Garland et al. (1993). The habitat categories were tested against megaspore and microspore diameter using a pruned phylogenetic tree (original tree tips, $n = 66$) for a total of 60 species for megaspores and 55 species for microspores.

4.4 – Results

Isoetes species that were growing in the Aquatic habitat type had significantly larger megaspores than those in any other habitat (Table 2) (comparison of Aquatic mean to means of Ephemeral: $t = –2.42$, $df = 60$, $P = 0.019$; Palustral: $t = –2.10$, $df = 60$, $P = 0.04$; Terrestrial: $t = –3.10$, $df = 60$, $P = 0.003$). The phylogenetically adjusted mean megaspore diameter for the Aquatic group (515 μm) was 1.14 times that of the Ephemeral group (451 μm), 1.16 times that of the Palustral group (444 μm), and 1.26 times that of the Terrestrial group (409 μm), implying volume differences by factors of 1.49, 1.56, and 2.00, respectively. The phylogeny showed that *Isoetes* species grouped tightly with species of the same habitat type, *i.e.* there appears to be a degree of niche conservatism (Figure 1). Ephemeral species were expected to have been larger than terrestrial due to the short time span available for growth, although no difference was observed ($t = 1.74$, $P = 0.088$). The Terrestrial and Palustral results showed no difference in megaspore size. The microspores of *Isoetes* showed no difference in size among habitat types.

Table 2. Phylogenetically adjusted mean spore diameter (μm) and pGLS ANOVA comparisons of means across habitat types. Means sharing the same letter within a spore type are not significantly different ($p > 0.05$).

	Aquatic	Ephemeral	Palustral	Terrestrial
Megaspores	515^{a}	451^{b}	444^{b}	409^{b}
Microspores	31^{a}	29^{a}	28^{a}	28^{a}

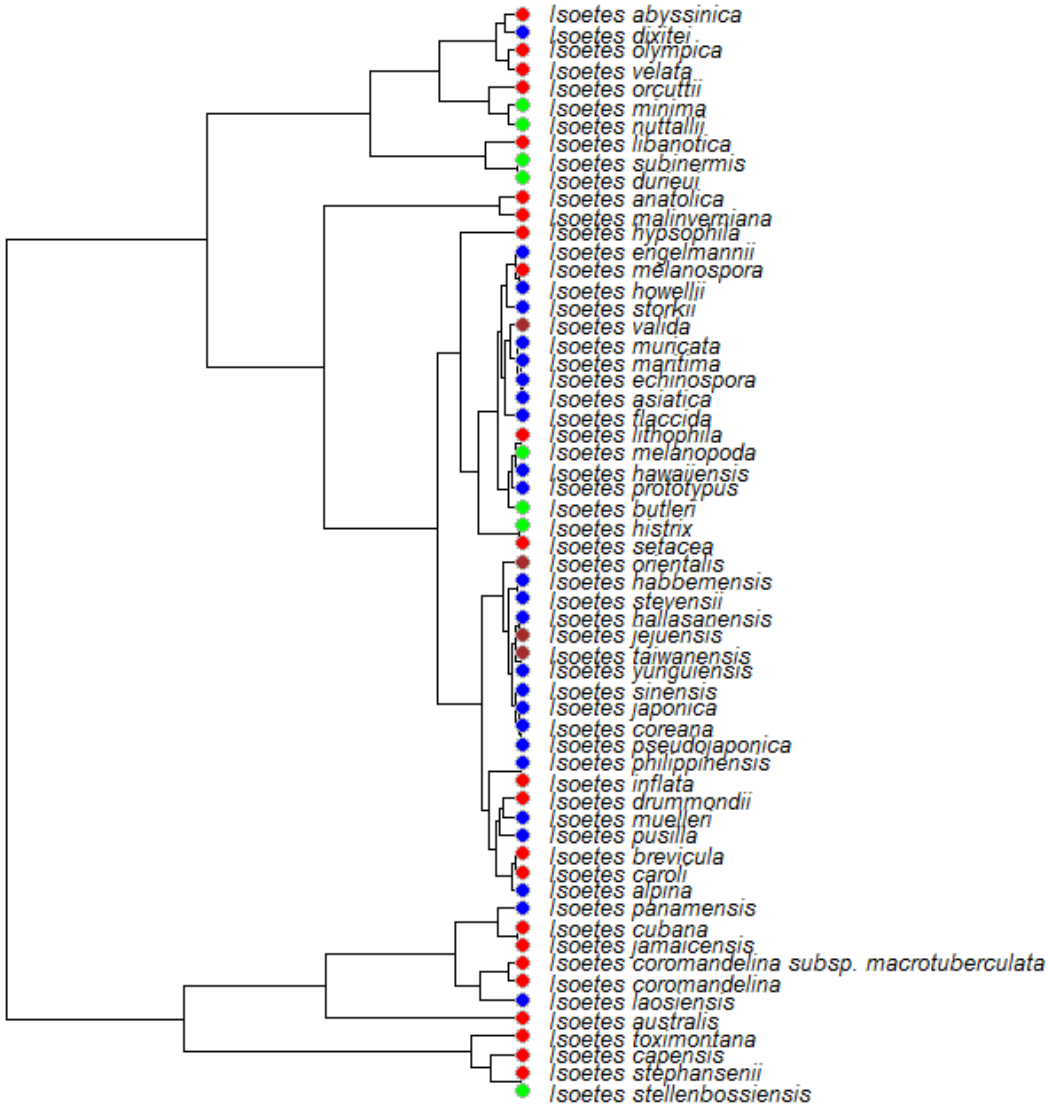

Figure 1. *Isoetes* 5.8S-ITS gene tree with *Selaginella* outgroup. Blue represents Aquatic habitats, red represents Ephemeral habitats, brown represents Palustral habitats, and green represents Terrestrial habitats.

4.5 – Discussion

The presence of more extreme heterospory among species in Aquatic environments presented here supports a central element of the model for the evolution of heterospory by Haig & Westoby, (1988). They proposed that isospores began to evolve to larger size to provide assistance with early establishment in an increasingly complex Devonian environment. The fossil evidence

supports a correlation between increasing environmental complexity and spore size. Chaloner (1967) effectively illustrated that spores diversified in size through the Devonian and this diversification correlated with an increase in complexity and diversity of the early land plants. Large isospores offer greater advantage to female function, but offer little advantage to male function. Thus, size increases continued to occur until at a point it was advantageous to produce smaller, sex-distinct spores. Indeed, Haig & Westoby suggested that the size decrease from large isospores to small microspores was the primary evolutionary novelty of heterospory. As *Isoetes* is an ancient genus the exact selection that led to the initial evolution of heterospory in its lineage is difficult to pinpoint. Nevertheless, extant plants provide good evidence that complex, especially aquatic, environments continue to select for more extreme heterospory.

Terrestrial and macrophytic aquatic plants both experience resource limitation in their environments. Light limitation is exaggerated by light filtering in the water column (Barrat-Segretain, 1996; Ralph *et al.,* 2007; Bornette & Puijalon, 2011) and in aquatic environments dissolved carbon dioxide can be extremely limited (Keeley, 1998). *Isoetes* often inhabit very nutrient poor waters where growth and establishment is difficult (Bornette & Puijalon, 2011). The *Isoetes* of non-permanent aquatic habitats are mostly alleviated from aquatic nutrient stressors. The difficulty establishing in aquatic environments is supported by the occurrence of larger megaspores in the aquatic group. Previous work with a sample of 10 species of *Isoetes* noted, in contrast to our findings, that terrestrial species had larger spores than aquatic species and suggested that adult aquatic *Isoetes* are nutrient limited, thus invest fewer resources into individual megaspores (Cox and Hickey, 1984). However, this conjecture overlooks the fundamental role of size-dependent fitness functions and size-fecundity trade-offs (Smith and Fretwell, 1970) rather than total amount of reproductive resources in shaping selection on propagule size. Land plants tend to adopt a strategy where they input *more* energy into individual propagules as environments become more nutrient poor and competitive (Salisbury, 1974; Quero *et al.,* 2007; Moles *et al.,* 2007). In the terrestrial heterosporous sister lineage to *Isoetes*, *Selaginella*, the competitiveness of a habitat,

measured through leaf area index (inferred as light availability) and net primary productivity, influences megaspore and microspore size (Petersen & Burd, *unpublished*). In addition, the pattern of allocating more resources to establishing propagules in nutrient limited environments is not exclusive to land plants. Togashi *et al.* (2012) identified a similar pattern in gamete resource allocation in marine green algae. In deeper light restricted water, green algae placed more nutrients within individual female gametes. Indeed, in deep water, marked anisogamy was preferred, but in shallow environments, selection for isogamy or slight anisogamy was preferred.

One criticism that may arise is that the spore size does not necessarily reflect nutrient stores. However, the megaspores of free-sporing heterosporous plants contain substantial nutrient stores from the developing sporophyte (Campbell, 1891; La Motte, 1937; Robert, 1971; Schneider & Pryer, 2002). *Isoetes* megaspores from different environment types showed no internal difference, with most volume being occupied by nutrient stores, and a small area occupied by cells comprising the gametophyte (Campbell, 1891; La Motte, 1937). It should be noted that in some of the water ferns megaspores contain flotation mechanisms, which could contribute to the overall size of the spores. Nonetheless, for example, *Marsilea* also has distinct starch reserves for the development of the sporophyte in addition to floatation mechanisms (Schneider & Pryer, 2002). Nutrients found in the free sporing heterosporous plants megaspores are kept for the development of the sporophyte; the gametophyte does not exploit them (Petersen & Burd, 2017). Thus, we consider that size is an appropriate indication of nutrition input from adult sporophytes into their propagules.

Seed plants that grow in arid habitats often have larger seeds than plants growing in other open-canopy vegetation types (Jurado & Westoby, 1992). Arid environments tend to have wet, but short growth seasons (*i.e.* higher risk of drought preventing establishment). Aquatic-ephemeral habitats are similar in this regard. The plants have only a relatively short period of time to establish in an environment that is both competitive and likely to be nutrient poor. Thus, a larger nutrient store would allow juveniles to grow consistently after germination. We expected to see a size difference in Ephemeral species compared to at least those of Terrestrial habit. Perhaps the analogy

of Ephemeral *Isoetes* megaspores and arid seeds in angiosperms is less clear due to some species being non-arid Ephemerals, *e.g. I. hypsophila* growing in seasonal alpine ponds. Nonetheless, the effect of Ephemeral habitats may be much clearer in a study that encompassed the whole genus.

The microspores of *Isoetes* exhibited no differences in size among any habitat types. We have observed change in microspore size among species of the sister lineage *Selaginella* that are related to habitat foliage density, which we associate with microspore filtration by foliage (Petersen & Burd, *unpublished*). Aquatic *Isoetes* are less likely to be affected by microspore filtration as their spores are usually released within water (Cox and Hickey, 1984). Even Terrestrial *Isoetes* appear to rely on water events for the majority of spore dispersal (Mahabalé, 1968). There may be other effects acting on the size of aquatic microspores, *e.g.*, possibly microspores have more trouble finding megaspores in aquatic environments. *Selaginella* also includes some arid species that would likely not experience much, if any, filtration effect. These arid species contrast well with forest species, making a pattern distinctly observable (Petersen & Burd, *unpublished*). Spiders' webs, that have been identified as pollen filters (Bera *et al.*, 2002; Song *et al.*, 2007), might be common around wetlands, but they are unlikely to affect aquatic species that release into the water body. The particular factor that influences microspore size in *Isoetes* is difficult to interpret from our data and results. Further investigation within this topic would be necessary.

In conclusion, *Isoetes* provides insight into the ecological advantage of heterospory. Aquatic habitats present additional nutrient restriction and competition compared to those of Terrestrial habitat. Larger spores filled with nutrients would give young sporophytes an early stable growth advantage and plants in more complex environments often provide more nutrients to their megaspores. Heterospory and the ancestors of *Isoetes*, likely appeared within the Devonian when habitats became more complex and the heterosporous lycopods were often found around wetlands (Kenrick and Crane, 1997). *Isoetes* has occasionally moved into Terrestrial type environments but the fossil record suggests their ancestors have been historically aquatic (Kenrick and Crane, 1997). *Isoetes* may not respond to Ephemeral habitats as strongly as seed plants due to reproductive

restrictions, but this may be better resolved in the future with an analysis of a larger subset of the genus, which may disentangle any actual biological difference between Ephemeral and Terrestrial species.

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5.0 – The enigma of sex allocation in *Selaginella*

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5.1 – Abstract

Background and Aims The division of resource investment between male and female functions is poorly known for land plants other than angiosperms. The ancient lycophyte genus *Selaginella* is similar in some ways to angiosperms (in heterospory and sex allocation that rests in the sporophyte generation, for example) but lacks the post-fertilization maternal investments that angiosperms make via fruit and seed tissues. One would therefore expect *Selaginella* to have sex allocation values less female-biased than in flowering plants and closer to the theoretical prediction of equal investment in male and female functions. Nothing is currently known of sex allocation in the genus, so even the simplest of predictions have not been tested.

Methods Volumetric measurements of microsporangial and megasporangial investment were made in 14 species of *Selaginella* from four continents. In five of these species the length of the main above-ground axis of each plant was measured to determine if sex allocation is related to plant size.

Key Results Of the 14 species, 13 showed male-biased allocations, often extreme, in population means and among the great majority of individual plants. There was some indication from the five species with axis length measurements that relative male allocation might be related to the release height of spores, but this evidence is preliminary.

Conclusions Sex allocation in *Selaginella* provides a phylogenetic touchstone showing how the innovations of fruit and seed investment in the angiosperm life cycle lead to typically female

biased allocations in that lineage. Moreover, the male bias we found in *Selaginella* requires an evolutionary explanation. The bias was often greater than what would occur from the mere absence of seed and fruit investments, and thus poses a challenge to sex allocation theory. It is possible that differences between microspores and megaspores in their dispersal ecology create selective effects that favour male-biased sexual allocation. This hypothesis remains tentative.

5.2 – Introduction

In contrast to our reasonably ample knowledge of sex allocation in angiosperms (Goldman and Willson, 1986; Campbell, 2000; de Jong and Klinkhamer, 2005), we know little about the sexual division of reproductive investment in free-sporing land plants: liverworts, mosses, hornworts, the early diverging vascular lineage of lycophytes, and the ferns and related lineages (monilophytes) (Judd *et al*., 2008). Life histories in these lineages, simpler in many ways than in flowering plants, make them potentially interesting models for probing the theory of sex allocation (Charnov, 1982; West, 2009).

Sex allocation in land plants is closely tied to the alternation of generations, a fundamental innovation underlying the ecological success of land plants in the terrestrial environment (Niklas and Kutschera, 2009). In some plant life histories, sex allocation occurs in the gametophyte generation. This is true of about half the species in the non-vascular lineages—liverworts, mosses, hornworts—and in the vast majority of monilophytes (Tryon and Tryon, 1982; Wyatt and Anderson, 1984). Sporophytes of these species produce spores of a small, unimodal size (*isospores*) that give rise to bisexual gametophytes. Such spores have no unique sexual identity. Only in the development of the bisexual gametophytes does the opportunity arise to invest resources in specifically male or female functions (in antheridia and archegonia).

In contrast, sex allocation resides in the sporophyte generation of those species in which obligately unisexual gametophytes arise from separate male and female spores. In the non-vascular

lineages (McDaniel *et al.*, 2012), these spores are morphologically isospores (or with slight size difference: Vitt, 1968) but there is a chromosomal basis to sexual identity (Immler and Otto, 2015), and spore mother cells necessarily give rise to two female and two male spores in a meiotic tetrad (Allen, 1917; McLetchie, 1992). Gametophytes grown from isolated spores produce nearly equal sex ratios in some but not all species (Newton, 1972; Stark et al., 2010). Sexual differences in spore abortion (Longton and Greene, 1979; McLetchie 1992) may alter sporophytic sex allocation from strict equality between male and female investment.

Among vascular plants, unisexual spores are associated with heterospory, a key innovation in the evolutionary history of land plants (Petersen and Burd, 2017). Small, male microspores and large, female megaspores differ greatly in size and are produced in separate sporangia in the heterosporous vascular plants (Petersen and Burd, 2017). Angiosperms, for example, determine sex allocation by the production of microsporangia (anthers), megasporangia (ovules), and the associated floral and extra-floral structures that assist male and female function. One important prediction of sex allocation theory has been abundantly demonstrated in angiosperms. Self-pollination results in a highly structured mating arena of competition among sibling pollen grains that creates selection for decreased allocation to male function (Charlesworth and Charlesworth, 1981; de Jong *et al*., 1999). Shifts toward greater relative female allocation with increasing rates of self-fertilization are, indeed, among the best documented aspects of sexual allocation in flowering plants (Goldman and Willson, 1986; Campbell, 2000; de Jong and Klinkhamer, 2005).

Outside the seed plants, the only heterosporous vascular lineages are two closely related families of leptosporangiate water ferns, Marsileaceae and Salviniaceae, and two closely related monotypic families within the lycophytes, Selaginellaceae and Isoetaceae (Tryon and Tryon 1982). As in flowering plants, sporophytes of these lineages invest in the production of microspores with specifically male function, and megaspores with female function. They can adjust sexual allocation through the size and number of microsporangia and megasporangia produced. In contrast to angiosperms, however, megaspores are not retained on the parent plant in these free-sporing

lineages, and so their life cycle provides no opportunity for post-fertilization maternal investments in secondary structures like seeds and fruits. This simple but important life-history difference makes the free-sporing heterosporous plants useful contrasts to the angiosperms for exploring sex allocation. Here, we focus our attention on *Selaginella*.

Seed and fruit maturation consumes much of a flowering plant's maternal investment (Burd and Head, 1992; Day and Aarssen, 1997; Baker *et al*., 2005) and so estimates of sex allocation at the end of a reproductive season tend to be more female-biased than the corresponding allocations measured at anthesis (Cruden and Lyon, 1985; Goldman and Willson, 1986; Ågren and Schemske, 1995). Female-biased allocation at the fruiting stage is common among flowering plants (Campbell, 1992; Klinkhamer *et al*., 1997; Baker *et al*., 2005). For example, six outcrossing species examined by Cruden and Lyon (1985) devoted 81–93 percent of their total reproductive biomass to female function.

In *Selaginella*, paternal and maternal sexual investments yield microspores and megaspores, which are dispersed from the sporophyte. Gametophyte development, gamete production and mating then occur in the environment away from the parent sporophyte. The absence of megaspore retention precludes any sporophytic maternal investments beyond the reserves originally placed in the megaspores. In the absence of seed and fruit investment, we would expect *Selaginella* to show less female bias in sexual investment than is typically the case in angiosperms. Indeed, spore dispersal and external mating should create fairly homogeneous mating opportunities throughout populations of *Selaginella*, so that they conform to the touchstone prediction of sex allocation theory that hermaphrodites should allocate half their reproductive investment to each sex function in a well-mixed mating arena (Charnov, 1982; West, 2009). Confirmation of these simple predictions would reveal something of the reproductive ecology of free-sporing plants and at the same time provide a phylogenetic reference point for understanding the sex allocation patterns of angiosperms.

Sex allocation appears not to have been measured previously in *Selaginella*. We examined fourteen species of *Selaginella* with a diverse array of growth habits native to four continents. In five of these species we also measured the main axis length of individual plants to see if sex allocation varied with plant size. Of the fourteen species, a pronounced male-biased allocation occurred in thirteen of them. While this result confirms our expectation that the evolutionary innovations associated with seeds and fruits tend to give angiosperms their characteristic female biased investment, it also presents the issue of explaining the pervasive and sometimes extreme male biased investment in *Selaginella*. Wind dispersal differences between megaspores and microspores may be implicated, although our evidence of a plant size effect on sex allocation is equivocal on this point. We consider other factors that may play a role in *Selaginella* sex allocation that should also be relevant for sex allocation theory in general.

5.3 – Materials and Methods

Species and locations

We sampled 14 *Selaginella* species with a variety of growth forms from a variety of habitats, including temperate eucalypt forest in south eastern Australia, rainforest in north eastern Australia, wet dipterocarp forest in Peninsular Malaysia and Borneo, wet lowland forest in Central America, along with species of various tropical and sub-tropical origins occurring in the Singapore Botanic Gardens (Table 1). Sampling took place between November 2010 and August 2015. The species we examined represent a phylogenetically as well as geographically broad sample. The majority-rule consensus tree from a Bayesian analysis of nuclear and plastid genes by Weststrand and Korall (2016) had a basally diverging lineage of two species that is sister to the remainder of the genus, itself divided into two diverse clades labelled A and B. In a Bayesian analysis of *rbcL* sequences from additional species, including 12 of the 14 species examined here, we recovered this

basic topology (K. Petersen and M. Burd, unpublished). Within clade A, the Australian *S. uliginosa* occurs in the subclade ericitorum, and the neotropical *S. arthritica* and South African *S. kraussiana* in subclade gymnogynum. Four subclades within clade B are represented: the southeast Asian species *S. willdenowii*, *S. mayeri*, and *S. plana* in one, *S. longipinna* and *S. frondosa* in a second, and the South American species *S. erythropus* and *S. haematodes* in the third, with *S. intermedia* in a fourth. We have no information on the phylogenetic position of *S. padangensis* and *S. brisbanensis*. In the absence of complete phylogenetic information for all fourteen species, we present our results here without a phylogenetic comparative framework, and defer such an analysis for the future and a larger data set.

Measurements

We estimated sex allocation for individual plants from two components, a count of the number of microsporangia and megasporangia in a sample of strobili from a plant, and measurements of the volume of megasporangia and microsporangia. Sporangium contents are completely converted to spores (Gifford and Foster, 1989; Morbelli and Rowley, 1993), so the volume of a spore sac will reflect resource investment by the sporophyte. In 2011, we checked the biochemical composition of mature spores of *S. uliginosa* through Fourier-transform infrared spectroscopy. Spectra for microspores and megaspores were nearly identical (unpublished data), implying equivalent material composition and thus equal costs per unit volume for each sex function. We assume similar equivalence of the micro- and megaspore contents in the other species.

For most species we sampled 30 plants per species, more from three Australian species for which we could sample multiple populations (Table 1). Single plants may produce hundreds of strobili, each containing dozens of sporangia. On each plant we removed ten haphazardly selected strobili (five for *S. uliginosa* and *S. kraussiana*) and counted the number of megasporangia and microsporangia on each under a dissecting microscope. The type of spore sac is readily

distinguished by the presence of a single large tetrad of spores within megasporangia, and if there were any doubt, we simply crushed the sac to determine if it contained four large spores or many tiny ones. Even immature spore sacs could be distinguished in this way.

Sporangial volumes were calculated from linear measurements made directly with an adjustable ocular micrometer on a dissecting microscope or from calibrated digital micrographs. Volume calculation required different approaches for micro- and megasporangia, and occasionally idiosyncratic variations for particular species depending on the shape of the sporangia. (1) Microsporangia are ellipsoid to reniform, depending on species. For ellipsoid sporangia, we measured the major axis (length) and minor axis (height) of the transverse profile of the sporangium, and calculated the volume as an ellipsoid of rotation about the major axis. That is, for a microsporangium with length $2w$ and height $2x$, we took its volume to be $V_0 = 4\pi w x^2/3$. For reniform shapes, we made micrographs of both transverse and tangential profiles. We divided the transverse face into ten sections of equal width along the major axis and determined the area of each section. We multiplied this area by the depth of the section measured on the tangential face to obtain a volume for the section. The summed volumes of the ten sections gave the whole microsporangium volume. (2) Megasporangia are broadly tetragonal, following the arrangement of the four meiotic products, and megaspores are approximately spherical. It was simpler to measure individual spore volumes and then multiply by four to yield an estimate of megasporangium volume. Individual megaspore volumes were calculated as an ellipsoid of rotation of the spore profile about its major axis (major and minor axes were usually nearly equal, so megaspores differed only marginally from spheres). That is, a megaspore with a major diameter $2y$ and minor diameter $2z$ would have its volume calculated as $V_s = 4\pi y z^2/3$, and the megasporangium as $V_1 = 4V_s$.

Sporangium volumes V_0 and V_1 were calculated in this way for 30–50 mature sporangia of each type per species, from which we determined mean volumes for the species. We took the product of the mean sporangial volume and the count of the sporangia of that type on an individual plant to obtain a volumetric estimate of resource allocation to male or female function for the plant.

Individual sex allocation was quantified as the proportion of microsporangial volume relative to total volume of all sporangia. That is, a plant that produced N_0 microsporangia and N_1 megasporangia in its sample of strobili would have a sex allocation $N_0V_0/(N_0V_0 + N_1V_1)$, i.e., M/(M + F), so that equal allocation corresponds to a value of 0.5.

We attempted to detect any dependence of sex allocation on vegetative size for a subset of the species in our sample. As an estimate of plant size, we measured the total length of the main axis of *S. uliginosa*, *S. kraussiana*, *S. willdenowii*, *S. intermedia*, and *S. plana*. Main axis length is only an approximate basis for comparison among these five species, given substantial differences in growth habit among them (Table 1). We use axis length as an indicative measure here, but better study of the effect of vegetative size or resource status on sex allocation is needed for *Selaginella*, as for most plant species.

Analysis

The main objective of the analysis was to test whether sex allocation deviated significantly from equal male and female investment for individual plants and for population means. Because sex allocation was determined for individual plants from a subsample of strobili, we calculated studentized 99% confidence intervals (CI) for the sex allocation of each plant from 1000 bootstrap samples of its strobili. A 99% CI that did not include 0.5 was considered to indicate significant departure from equal allocation. At the population level, we calculated the mean sex allocation among the plants in each sample, and determined the studentized 99% CI for population means from 1000 bootstrap samples of the individual plants. Bootstrapping was carried out with the R package “boot” (Canty, 2002).

To assess the relationship of sex allocation to plant vegetative size, we first normalized the axis length measurements to each species’ maximum, because the range of this metric was vastly incommensurate among species, and then tested whether there were homogeneous slopes for this

relation using analysis of covariance with a plant size × species interaction term. This interaction was significant, indicating heterogeneity in the sex allocation-plant size relationship among species. We therefore conducted a separate regression analyses for each species. The analysis of covariance and regression analyses were calculated using the R base package, ver. 3.3.1 (R Core Team, 2016).

Table 1. Species descriptions, sample sizes and sampling locations

Species	Habit and typical maximum size	*N*	Sample Sites
S. uliginosa	Erect and delicate, lightly branched stems *c*. 20 cm high	50	Victoria, Australia
S. kraussiana	Prostrate and heavily branched,	150	Victoria, Australia
S. willdenowii	Scrambling climber, main axis up to 700 cm	30	Ampang, Selangor, Malaysia
S. intermedia	Low, suberect to *c*. 25 cm high, densely foliaged	30	Gunung Mulu, Sarawak, Malaysia
S. plana	Frondose, heavily branched, to *c*. 70 cm high	30	Gunung Mulu, Sarawak, Malaysia
S. haematodes	Frondose and densely foliaged	30	Barro Colorado Island, Panama
S. arthritica	Frondose	30	Barro Colorado Island, Panama
S. australiensis	Prostrate, creeping, sometimes growing on trees	30	Wooroonooran and Barron Gorge, Queensland, Australia
S. brisbanensis	Prostrate, delicate, heavily branched	30	Wooroonooran, Queensland, Australia
S. longipinna	Frondose	60	Wooroonooran, Queensland, Australia
S. padangensis	Somewhat frondose, heavily branched	30	Singapore Botanic Gardens, Singapore
S. erythropus	Somewhat frondose, ventral surface distinctly red	30	Singapore Botanic Gardens, Singapore
S. mayeri	Prostrate, spreading	30	Singapore Botanic Gardens, Singapore
S. frondosa	Frondose, basal stem reddish	30	Singapore Botanic Gardens, Singapore

5.4 – Results

Nearly every species had a mean population sex allocation that was significantly male biased (Table 2), as did most individual plants (Fig. 1). Only *S. longipinna*, a frondose species of tropical northern Australia, had nearly equal mean sex allocation and a substantial number of individual plants with female biased allocation. Among the remaining species, male bias was sometimes extreme. Six species invested 80% or more of their sporangial investment in microspores, and it was not uncommon to find individual plants that produced no megaspores among the strobili we sampled. An occasional plant with significantly female biased allocation occurred in *S. padangensis*, *S. erythropus*, and *S. haematodes*, but these exceptions did not obscure the evident pattern of male bias in these species, and in the sample at large (Fig. 1).

The differences among species in sex allocation were due to variation in both the sizes and numbers of male and female sporangia they produced. There was, nonetheless, greater variation in numbers than sizes. Megasporangia ranged from less than half the size of microsporangia (*S. intermedia*) to 13.7 times the size of microsporangia (*S. australiensis*), while the number of microsporangia ranged from approximately three times (*S. brisbanensis*) to over 57 times (*S. padangensis*) the number of megasporangia (Table 2). Because both contribute to species differences in sex allocation, neither the size nor number ratio was systematically related to sex allocation (Fig. 2).

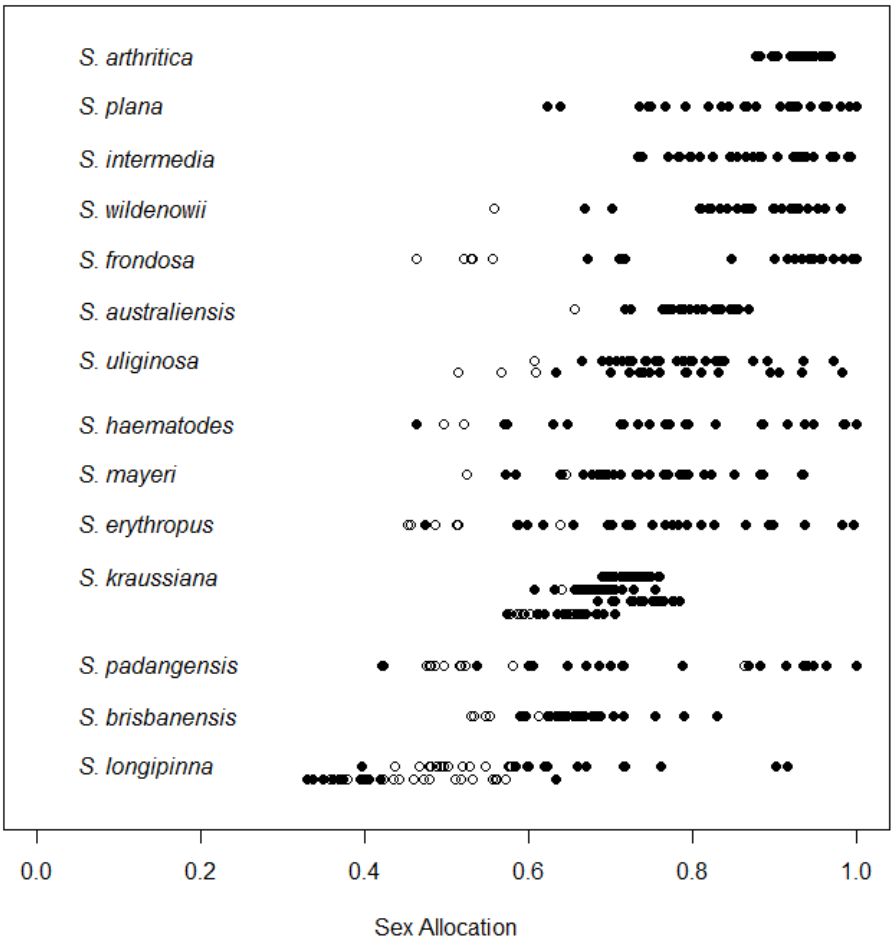

Fig. 1. Sex allocation (proportion of male investment measured as microsporangial fraction of total sporangial volume) in fourteen species of *Selaginella*. Values above 0.5 indicate an investment bias toward male function, and below 0.5 an investment bias toward female function. Each point represents an individual plant. Plants with 99% confidence intervals for sex allocation that include 0.5 are shown in open circles; plants with significantly sex-biased allocations are shown in filled circles. Geographically distinct populations of *S. longipinna*, *S. kraussiana*, and *S. uliginosa* are shown on separate lines.

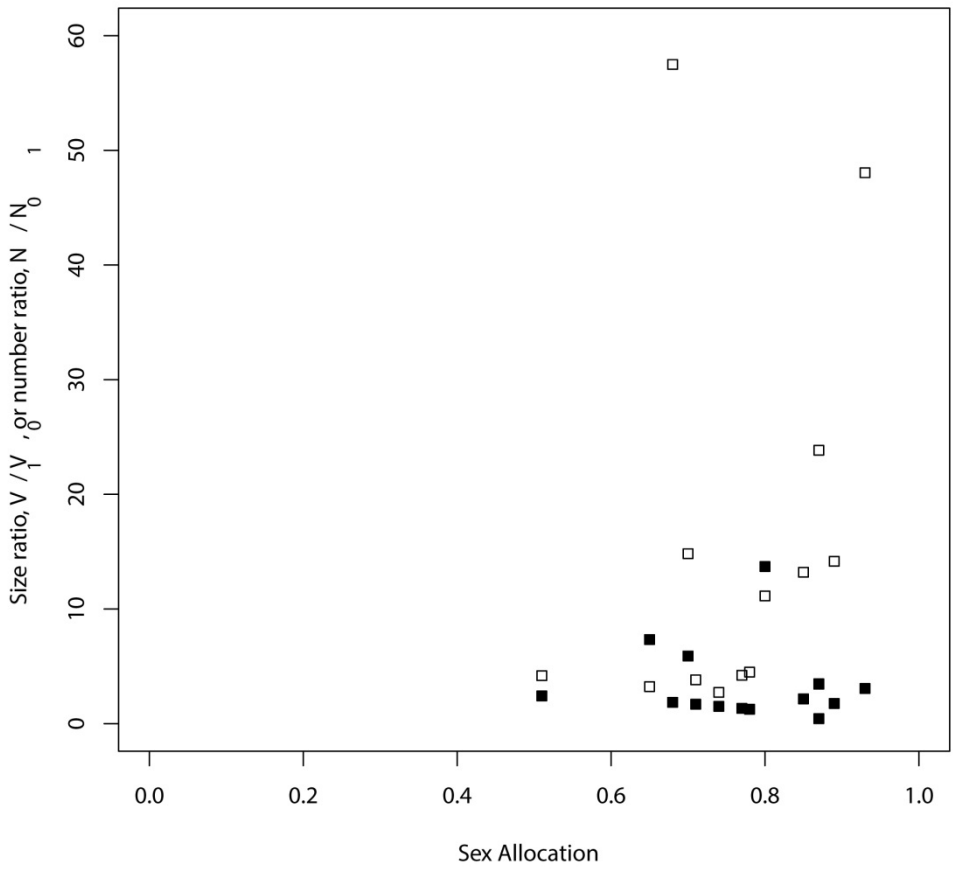

Fig. 2. Relationship of species mean sex allocation to sporangial size ratio (mean megasporangium volume relative to mean microsporangium volume, V_1/V_0) (filled symbols), or to sporangial number ratio (mean number of microsporangia relative to mean number of megasporangia, N_0/N_1) (open symbols).

Sex allocation bore little relation to vegetative size in the species we measured. Homogeneity of slopes among species was strongly rejected (species × normalized axis length interaction: $F_{4, 260} = 7.63$, $P = 0.000008$), requiring separate regression for each species. The slope of the relationship between sex allocation and axis length was significantly different from zero in two species, *S. kraussiana* and *S. plana*, but the relationships were not strong, accounting for only about 6% of the variance in sex allocation in *S. kraussiana* and about 30% in *S. plana* (Table 3). In both cases, male allocation increased with increasing axis length. *Selaginella kraussiana* and *S.*

plana differ in plant habit and typical height, so the connection between male allocation and plant size in these species does not appear to follow from a distinctive morphology relative to the other species.

Table 2. Numbers and sizes of megasporangia and microsporangia, and population sex allocation for the study species. N_0, N_1: number of microsporangia or megasporangia, respectively, per plant. V_0, V_1: volume of microsporangium or megasporangium, respectively. Values are means ± 1 SD. Sex allocation is measured as the proportion of the total volume of sporangia used for male function (i.e., microsporangial volume). Thus, values above 0.5 represent male-biased allocation. Confidence intervals were obtained by bootstrapping (see Methods).

Species	N_0	N_1	V_0 (mm^3)	V_1 (mm^3)	Sex allocation	
					mean	99% CI
S. uliginosa	57.1 ± 23.5	12.7 ± 7.6	0.113 ± 0.046	0.142 ± 0.064	0.78	(0.67, 0.89)
S. kraussiana	74.1 ± 11.3	5 ± 0.0	0.136 ± 0.033	0.802 ± 0.227	0.70	(0.65, 0.75)
S. willdenowii	357.7 ± 102.1	15.0 ± 8.0	0.106 ± 0.028	0.369 ± 0.062	0.87	(0.78, 0.94)
S. intermedia	572.2 ± 194.2	166.3 ± 89.7	0.128 ± 0.011	0.056 ± 0.02	0.87	(0.82, 0.94)
S. plana	868.0 ± 169.2	61.3 ± 58.2	0.071 ± 0.013	0.125 ± 0.049	0.89	(0.82, 0.94)
S. haematodes	302.1 ± 87.6	71.6 ± 50.1	0.018 ± 0.005	0.024 ± 0.005	0.77	(0.71, 0.84)
S. arthritica	480.5 ± 181.7	10 ± 0.0	0.029 ± 0.017	0.089 ± 0.04	0.93	(0.91, 0.94)
S. australiensis	563.4 ± 156.7	9.8 ± 0.4	0.017 ± 0.006	0.233 ± 0.113	0.80	(0.73, 0.84)
S. brisbanensis	116.2 ± 22.8	8.8 ± 1.9	0.018 ± 0.004	0.132 ± 0.045	0.65	(0.56, 0.77)
S. longipinna	270.3 ± 95.3	99.1 ± 45.8	0.024 ± 0.008	0.058 ± 0.012	0.51	(0.42, 0.66)
S. padangensis	611.0 ± 267.0	159.9 ± 106.4	0.057 ± 0.015	0.106 ± 0.027	0.68	(0.60, 0.77)
S. erythropus	314.7 ± 140.7	97.5 ± 97.6	0.023 ± 0.007	0.039 ± 0.012	0.71	(0.62, 0.81)
S. mayeri	452.1 ± 174.9	108.0 ± 51.6	0.057 ± 0.007	0.086 ± 0.03	0.74	(0.64, 0.86)
S. frondosa	572.6 ± 208.0	51.4 ± 68.1	0.019 ± 0.004	0.041 ± 0.008	0.85	(0.78, 0.94)

Table 3. Regression relationships between sex allocation and vegetative size (relativized main axis length) of individual plants.

Species	Slope	*F*	d.f.	*P*	R^2
S. uliginosa	–0.00030	1.70	1, 48	0.198	0.034
S. kraussiana	0.00013	7.60	1, 128	0.007	0.056
S. willdenowii	–0.00001	0.43	1, 28	0.517	0.015
S. intermedia	–0.00024	3.26	1, 28	0.081	0.104
S. plana	0.00038	12.10	1, 28	0.002	0.302

5.5 – Discussion

In flowering plants, maternal tissues such as fruits and arils or maternally supported tissue such as endosperm are a principal source of female-biased sex allocation (Burd and Head, 1992; Day and Aarssen, 1997; Baker *et al*., 2005). Because the *Selaginella* life cycle lacks these features, we expected to find a more balanced division of resource allocation between male and female function. Indeed, no species in our sample had female biased allocation. To the contrary, 13 of the 14 species showed a statistically significant and substantial male bias, with male investment often exceeding female by a factor of two or more (Table 2). This bias is more extreme than can be accounted for merely by the absence of fruit and seed investment (Goldman and Willson, 1986; Ågren and Schemske, 1995; Baker *et al*., 2005). For example, only 6 of 13 xenogamous angiosperm species examined by Cruden and Lyon (1985) had a mean male allocation based on stamen and pistil biomass that exceeded 0.67 at the floral stage, while this was true of 12 of the 14 *Selaginella* species we examined. Thus, our results confirm one simple expectation but raise the question of what accounts for the male bias among *Selaginella* species.

Although it is rare in angiosperms, male biased sex allocation has been reported in *Andropogon gerardii* and *Sorghastrum nutans* (Poaceae), which had 60–90% male allocation for most investment currencies, including biomass (McKone *et al*., 1998). The male investment bias in these two species arises, in all likelihood, from wind pollination and passive dispersal of seeds. When one sex function has more restricted dispersal than the other, selection favours investment in the better-dispersing sex (Bulmer & Taylor, 1980; West, 2009; Fromhage and Kokko, 2010). Wind pollination and passive seed dispersal are likely to afford better dispersal opportunity to male than to female function (Fromhage and Kokko, 2010; Pickup and Barrett, 2012). Accordingly, male allocation increases with plant height in several wind-pollinated species because pollen dispersal is greatly enhanced by elevated release height (McKone and Tonkyn, 1986; Burd and Allen, 1988; Solomon, 1989; Bickel and Freeman, 1993; Fox, 1993).

Does this effect occur in *Selaginella*? In principle it could. Microspores of *Selaginella* are typically 18–60 μm in diameter, while megaspores range from 200–1033 μm in diameter (Tryon and Lugardon, 1991). The smaller, lighter microspores generally disperse further than megaspores (Filippini-DiGiorgi *et al*., 1997), setting the stage for selection favouring allocation to male function. However, we found only modest evidence of an effect of plant height on sex allocation within species (Table 3). *Selaginella kraussiana* and *S. intermedia* have prostrate growth, so that the length of the main plant axis would not be related to release height of spores. Curiously, male allocation increased with increasing axis length in *S. kraussiana*, but the relationship was weak (Table 3). *Selaginella willdenowii* has a main axis that can reach several meters in length, but it is a scrambling climber on surrounding plants, and its axis length may also be poorly related to spore release height. Only *S. plana* and *S. uliginosa* have erect growth forms in which the main axis length would be reflect height above ground, but *S. uliginosa* is diminutive and may have too little height variation to reveal the expected effect of spore dispersal on sex allocation. In *S. plana* we found a statistically significant positive relationship between main axis length and male allocation (Table 3), in support of the hypothesized effect of wind dispersal on sex allocation. Thus, the

evidence from intraspecific variation suggests, but only tentatively, that male-female dispersal differences affect sex allocation in at least some *Selaginella* species.

Dispersal of microspores and megaspores may differ generally within the genus whether or not individual plant size has a strong effect, of course. It remains possible, therefore, that differences in spore dispersal explain the apparently widespread male allocation bias in *Selaginella*, but this is an open hypothesis. The apparent extent of male-biased allocation within the genus is consistent with such an effect, but much additional investigation would be needed to substantiate it.

We have some qualitative indication that the male bias we found for most of the species in our sample is widespread among *Selaginella* species generally. As part of our continuing study of *Selaginella* ecology, we examined specimens of over 100 species at the U.S. National Herbarium of the Smithsonian Institution in order to extract and photomicrograph microspores and megaspores. As we searched specimens for sporangia that had retained spores, we noticed that it was very frequently difficult to find megasporangia, although microsporangia were abundant, implying that these specimens had male-biased sexual allocation. This pattern was not universal, however. Specimens from occasional species did bear abundant megasporangia. The enigmatic variation in sex allocation within and among *Selaginella* species, and differences with the angiosperms, are interesting aspects of land plant biology in their own right, but will also provide useful empirical models for probing the theory of sex allocation.

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6.0 – Thesis Conclusion

Heterospory was one of the most innovative changes to the land plants. It allowed for selection to act on sexes independently. Heterospory meant that sporophytes were able to provide more nutrients to establishing sporophytes and produce smaller better dispersing microspores. The independent selection on female function allowed for the evolution of the seed, and the connection between heterospory and the seed has dominated the thinking of heterospory in literature. This thesis synthesised the past 160 years of heterospory discussion (chapter 2), providing evidence that the adaptive advantage of heterospory is a major knowledge gap in plant science. There are few propositions made for the adaptive nature of heterospory. Inbreeding avoidance has most often been raised (Sporne, 1962; Steeves, 1983; Kar & Dilcher, 2002; Qiu, Taylor & McManus, 2012), but the evidence from homosporous lycophytes does not support this argument (Haufler & Soltis, 1984; Holsinger, 1987; Soltis & Soltis, 1987, 1990, 1992; Pryor *et al.*, 2001). Haig and Westoby (1988) provide the best theoretical explanation for the evolution of heterospory and this thesis predominantly aimed to test its assumptions.

The theory for the origin of heterospory by Haig & Westoby (1988), explained in detail in chapter 2, is the most complete theory available and was used as a basis for the empirical tests in this thesis. Research from seed plants and even anisogamy suggest support for assumptions in their paper. The change of size and selection for megaspore and microspores suggested by Haig and Westoby (1988) are supported by the fossil record. However, in this thesis the adaptive significance of heterospory was tested, that is, how does it provide advantage to plants? Haig and Westoby suggested that heterospory appeared in a time where habitat complexity increased and establishment became difficult. Indeed, in the seed plants evidence shows that larger seeds are produced in complex habitats (Moles *et al.*, 2005), and larger seeds are advantageous for establishment in hazardous environments (Moles & Westoby, 2004). Likewise evidence from anisogamy in green algae shows dark, deeper waters select for more extreme anisogamy (Togashi, Cox & Bartelt,

2007). The evidence from other plant and algae taxa, as an analogy, appear to support the original assumptions of Haig & Westoby (1988).

The first empirical evidence for the advantage offered by heterospory in terrestrial free-sporing plants is provided in chapter 3. *Selaginella* invests in much larger megaspores in very shaded and fast growing environments, similar to what is observed in seeds. These environments are particularly difficult to establish in, but a megaspore with a larger nutrient store could enable consistent growth to a larger size where the young sporophyte is then self-sufficient. This pattern is not universal within the genus, as some species produce small megaspores in shaded environments. These small megaspore species are likely taking advantage of a much different establishment niche than large megaspore species. However, *Selaginella* never produce very large megaspores in very open environments where it is likely less competitive to establish. *Isoetes* in chapter 4 also supports the hypothesis that heterospory is advantageous in competitive, complex environments. *Isoetes* is mostly aquatic, or often closely associated with water, and evidence in this thesis suggests that aquatic environments are more difficult to establish in. Aquatic habitats in particular are light stressed, the deeper the water the more difficult it is to obtain light nutrients (Bornette & Puijalon, 2011). Aquatic species have larger megaspores than other habitat types. The results seen in *Isoetes* are comparable to the selection for larger female gametes observed in anisogamous marine green algae (Togashi, Cox & Bartelt, 2007). Thus, this thesis provides the first substantial evidence from two genera of free-sporing heterosporous plants that heterospory is selected for in competitive, complex environments. These results agree with the assumptions of Haig and Westoby (1988).

The selection that would be acting on microspores was less clear, but a pattern arose where *Selaginella* microspores decreased in size as habitats became more shaded and productive. A hypothesis for this decrease in size is more difficult to specify as no other study has ever noted this pattern in free-sporing heterosporous plants. Two potentially relevant effects are noted in angiosperm pollens. Larger pollen grains of have better surface:area ratios and may help with protection from desiccation (Zhang *et al*., 2010; Ejsmond *et al*., 2011), and pollen in forested

environments are subject to filtering effects from spider webs, bark and leaves (Tauber, 1967; Waateringe, 1998; Bera *et al.*, 2002; Song *et al.*, 2007) . These two effects alone could explain the pattern observed in *Selaginella*, but careful analysis of this genus in the future may be required to confidently disentangle the selection occurring here.

Chapter 5 examines a consequence of heterospory evolution, namely sex allocation. How much control a plant has over sex allocation is closely connected to the alternation of generations. Plants that have sporophyte dominant life cycles and are heterosporous have switched sex allocation control from the gametophyte to the sporophyte. *Selaginella*, and lycophytes in general, are a novel group for sex allocation investigation. As *Selaginella* is a free-sporing plant it lacks the required investment that angiosperms have in female function. Sex allocation has been extensively investigated in angiosperms (Goldman and Willson, 1986; Campbell, 2000; de Jong and Klinkhamer, 2005). Angiosperms are considerably female biased with their sex allocation. Female biased allocation is associated with the large investment required to produce fruit, seed and the associated organs, *i.e.*, pistils. Lacking these investment requirements it would be expected that sex allocation would have equal contribution to both sexes. However, this thesis shows that *Selaginella* is in fact highly male biased. The male bias observed in *Selaginella* doesn't appear to be strongly tied to wind dispersal, something observed in some angiosperm Poaceae (McKone *et al*., 1998), but it is difficult to disentangle ecological purpose for male bias in *Selaginella* without further study.

The core question of this thesis was: why did heterospory evolve? The evidence provided here suggests heterospory is an adaptation to help with establishment in uncertain and complex environments.

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