INTRODUCTION

Methodologies in gametophyte biology

All land plants reproduce following a pattern known as the alternation of generations, whereby fertilization occurs in the sexual gametophyte stage, which results in an asexual sporophyte stage. In the ~470-million-year history of terrestrial plants, there has been a slow but remarkable shift in the way different lineages alternate between these generations. Bryophytes closely align with their algal ancestors in growing primarily as gametophytes; the short-lived sporophytes they produce are most often nutritionally dependent on their haploid counterparts and senesce shortly after dispersing their spores. Seed plants evolved the opposite tactic, with large, dominant sporophytes and microscopic, nutritionally dependent gametophytes. Ferns and lycophytes reside at the center of this life-history spectrum, with free-living sporophytes and gametophytes that are not nutritionally or structurally dependent on one another.

Each generation is arguably equal in its importance to the long-term survival of species, yet most of the scientific inquiry in vascular plants has focused primarily on sporophytes. In noting this imbalance for ferns, Richard Holtum (1938) wrote, “We are accustomed to see and marvel at the great varied form and adaptation of the sporophytes...but indeed, there must be nearly as much variety of adaptation among the gametophytes.” The study of gametophytes has since become less and less relegated to the sidelines of plant biology. Indeed, several recent studies have used gametophytes and gametophyte-dominant lineages as a unique system for plant biology using biotechnological approaches. However, because of their relatively small size and cryptic morphology, there is still much to learn about the ecology, physiological resilience, and astounding evolutionary innovations of gametophytes. The 15 papers in this special issue, “Methods in Gametophyte Biology,” illustrate the breadth and importance of gametophyte biology while highlighting the novel tools and techniques researchers are leveraging to answer fundamental questions regarding the haploid generation.

Given its haploid nature, the gametophyte generation likely experiences selective pressures differently than its sporophytic counterpart. Yet, these pressures undoubtedly affect the sporophyte generation due to pleiotropic effects between the two life cycle stages. To address this gap in understanding, Sorojsrisom et al. (2022) developed a Python wrapper, shadie (Simulating Haploid–Diploid Evolution), which allows users to run SLIM 3 (Haller and Messer, 2019) simulations to examine evolutionary patterns in plant life cycles. This offers researchers a platform for better understanding how selection in gametophytes has affected all lineages of land plants. By providing a flexible and global simulator for constructing evolutionary models, Sorojsrisom et al. open the field to an understudied key aspect of the biphasic life cycles of land plants.

A second article in this special issue broadly focusing on all embryophytes examines gametophyte ultrastructure and mechanisms of spore and pollen dispersal. Mitchell et al. (2022) review the use of high-speed videography and microscopy techniques to further our understanding of the biomechanical mechanisms of spore and pollen release in plants. They show that studies of dispersal biomechanics are often only focused on specific taxa, in part due to a lack of suitable and broadly available methodologies. The authors suggest that methods combining high frame-rate videos, which can be slowed for detailed study, are particularly powerful when combined with advanced microscopy. This methodology has the potential to allow for the examination of processes occurring at scales as fine as the subcellular level. This perspective, therefore, encourages a broader application of these approaches in studying processes associated with plant dispersal and reproductive biology, making this review a timely contribution to gametophyte research.

The emergence of Anthoceros agrestis Paton as a hornwort model species fills a critical scientific gap and allows for genomic and physiological comparisons that promise answers to longstanding evolutionary questions. However, their slow growth rates have hampered the development of necessary genetic tools to examine such questions. Gunadi et al. (2022) present a detailed protocol for in vitro growth and show how various media supplements benefit rapid hornwort biomass production. In doing so, the study reports an innovative and substantial technological achievement. As a guideline specifically targeted to hornwort biology, these in vitro protocols have the potential to advance developmental genetic studies of hornwort traits. Growing hornworts axenically, faster, and in more significant quantities will speed future studies into the biology of these enigmatic bryophytes.

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While rapidly growing hornwort gametophytes in vitro is an essential step in examining evolutionary patterns among land plants, the same can be said for developing a protocol that allows for genetic transformation. Researchers may rapidly alter a plant's genome by working with protoplasts, as there is no cell wall to block DNA transformation. Because the establishment of the model hornwort *A. agrestis* has arisen relatively recently (Szövényi et al., 2015), protocols for working with hornwort protoplasts have not yet been established. Building on their previous work, Neubauer et al. (2022) fill this gap in scientific knowledge by providing the first step-by-step protocol for the transformation of protoplasts in *A. agrestis*. This study paves the way for the investigation of protein function, localization, and interactions in vivo and facilitates CRISPR/Cas9 genome editing in hornworts.

Two additional articles in this special issue focus on reproduction and genotypic patterns in bryophytes, specifically mosses. For dioecious mosses, sex is determined by either a *U* chromosome (female) or a *V* chromosome (male). Determination of sex in many mosses has previously been based on gamete production, making it difficult to accurately assess sex ratios in the field, as sterile mosses cannot be categorized. Focusing on the species *Syntrichia caninervis* Mitt. as a study system, Ekwealor et al. (2022) provide a method for the sex classification of sterile shoots. Using a restriction fragment length polymorphism (RFLP) approach, the authors identified a locus on the sex chromosomes which they then used to develop targeted PCR primers to screen non-expressing shoots. The authors are optimistic that these primers will work broadly across the genus *Syntrichia* Brid., allowing for a broader understanding of sex distribution in dioecious mosses.

Although all mosses require a thin layer of water for sperm to swim to eggs, recent findings have shown that microarthropods facilitate outcrossing in some species (Cronberg et al., 2006). It was subsequently discovered that these microarthropods were being attracted to biogenic volatile organic compounds (BVOCs) (Rosenstiel et al., 2012), a variety of which have now been characterized and are thought to play a role in reproductive isolation leading to high rates of diversification. Here, Brennan et al. (2022) review four methodologies available to rapidly collect and quantify moss BVOCs—with the ability to sample multiple mosses simultaneously, enabling the creation of a BVOC “fingerprint”—and describe a new gas-chromatography technique to identify volatiles collected in the field or a laboratory setting.

The remaining articles in this issue focus on the gametophytic generation of ferns and lycophytes. Both lineages are unique among extant land plants in that both the gametophytic and sporophytic generations grow independently of one another. For lycophytes and a subset of fern lineages, the gametophytes are aholophyllous and subterranean, making them extremely difficult to locate and study. Rimgaile-Voicik and Naujalis (2022) review methods previously employed to locate and isolate aholophyllous gametophytes. They provide a succinct guide for the detection of subterranean gametophytes and best practices for gametophyte extraction, which vary according to the goals of each project. The paper culminates in a discussion of the scientific questions revolving around subterranean gametophyte population development, which may be addressed when employing the recommended methods.

Farrar and Johnson (2022) also focus on approaches associated with studying subterranean gametophytes, providing a refined technique for the recovery of gametophytes through soil centrifugation. This approach also allows investigators to obtain small, juvenile sporophytes and intact gemmae. Gemmae are delicate, asexual reproductive structures that grow from the margin of fern gametophytes belonging to certain genera; they detach easily and are therefore especially difficult to study among species that have subterranean life stages. The authors ultimately hope that this approach allows for further study relating to population dynamics, responses to stress, and species conservation.

Subterranean gametophytes are entirely dependent on fungal associations for the acquisition of nutrients, yet to date, few studies have been conducted that characterize these fungal communities. It is currently unknown whether dark septate endophytes (DSE), which are common in environments where plants are subject to high amounts of physiological stress, are present in the aholophyllous, subterranean gametophytes of ferns. Chen et al. (2022) provide a workflow for sequencing the mycobiome of a single gametophyte sample using a metabarcoding technique, demonstrating for the first time that DSEs are present in subterranean gametophytes and are likely integral components of their microbiome.

Fern and lycophyte gametophytes are relatively diminutive in size and often nondescript, lacking the morphological characters necessary for taxonomic identification. Nitta and Chambers (2022) review the use of DNA barcoding methodologies to identify cryptic fern gametophytes. By comparing short stretches of gametophyte DNA to a reference sporophyte database, DNA barcoding allows for taxonomic assignment among field-collected fern gametophytes. In their review, Nitta and Chambers discuss a broad assembly of literature on molecular identification of fern gametophytes, which are applicable to lycophytes as well. They cover specific applications of DNA barcoding technology and provide a glimpse at future possibilities for field studies of ferns.

An expansion of the applicability of DNA barcoding is reviewed by Wu et al. (2022), focusing on tissue-direct PCR (TD-PCR). This method forgoes the typical DNA extraction protocol and directly amplifies fern gametophyte tissue using either species-specific or universal PCR primers. The TD-PCR protocol was designed with the aim of saving researchers time and money, and successfully amplifies DNA from tissue amounts too small for traditional DNA extraction protocols. Wu et al. then focus on two case studies to document the utility of TD-PCR in addressing broad ecological questions associated with fern gametophytes.
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The inability to confidently identify most fern gametophytes has led to a lack of information regarding field-based ecological and physiological patterns. However, with advances in DNA barcoding, as reviewed by Nitta and Chambers (2022), many of these studies may now be possible. Krieg and Chambers (2022) provide an overview of the historical research and previously employed methods used to study fern gametophyte ecology and physiology over the past century. They specifically focus on the advantages and limitations of laboratory and greenhouse studies, highlighting the potential field studies that become possible as fern gametophytes are unambiguously identified through DNA barcoding techniques.

Quinlan et al. (2022) provide an excellent example of how DNA barcoding and TD-PCR may be applied to address some of the broad ecological questions highlighted by Krieg and Chambers (2022). Focusing on a tree fern common in Taiwan, Allophila podophylla Hook., Quinlan et al. successfully examined phenological patterns. They developed a protocol whereby investigators can examine the relationship between spore maturation and release, gametophyte establishment, and the development of a juvenile sporophyte. Using a plot-based design, the authors systematically examined subplots every two months. While the authors did sequence all gametophytes collected within the sampled subplot from their first visit, they subsequently developed species-specific markers so that only gametophytes of A. podophylla amplified, allowing researchers to avoid the expense associated with sequencing during subsequent surveys.

A major question regarding gametophytes is how well they respond to stressful conditions like desiccation or freezing compared to sporophytes. As reviewed in Krieg and Chambers (2022), much of the previous work on fern gametophyte ecology has occurred in laboratory or greenhouse settings. Schneller and Farrar (2022) also highlight this in their study but provide a way to examine stress responses over time in natural, undisturbed habitats. Focusing on seasonal changes in a temperate habitat, Schneller and Farrar developed a method where investigators may use fixed-distance photography to monitor the longevity and growth rates of fern gametophytes in response to stressful environmental conditions. The approach has the benefit of being non-invasive and may be programmed to allow for rapid computer analysis, providing both qualitative and quantitative evaluations.

While field-based studies are particularly lacking in the fern gametophyte literature, laboratory studies are still powerful ways to conduct manipulative experiments. There are a limited number of studies examining competitive interactions among fern gametophytes, even though this is likely a common occurrence. One reason there are so few studies in this area is because of the current difficulty in quantifying the factors that would be impacted in a competitive environment, such as plant size. Hornych et al. (2022) outline an approach that utilizes the free software program Easy Leaf Area (Easlon and Bloom, 2014) to rapidly quantify gametophyte area. The authors employed this approach to examine the competitive interactions among three species, two of which are apomictic. The results were comparable to more time-consuming image analysis approaches. This methodology is thus applicable for rapidly examining the area of large numbers of gametophytes and is a useful approach for examining several poorly understood ecological processes associated with fern gametophytes.

The articles published in this special issue point to the scientific community’s increased understanding of the importance of the gametophytic generation. After all, without gametophytes, there would be no sporophytes. Each study in and of itself is a micro-review, providing investigators with pertinent background information and historical accounts of previously employed methodologies. The novel methods and approaches proposed within each paper will undoubtedly advance our understanding of the gametophytic life stage, thereby providing a holistic understanding of land plant evolution, ecology, and physiology.

AUTHOR CONTRIBUTIONS

S.M.C. prepared the first draft of the manuscript. J.B.P and S.W. provided select article summaries and provided reviewing and editing assistance. All authors approved the final version of the manuscript.

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REFERENCES

Brennan, D. L., L. M. Kollar, S. Kiel, T. Deakova, A. Laguerre, S. F. McDaniel, S. M. Eppley, et al. 2022. Measuring volatile
emissions from moss gametophytes: A review of methodologies and new applications. *Applications in Plant Sciences* 10(2): e11468.

Chen, K.-H., Q.-Y. Xie, C.-C. Chang, and L.-Y. Kuo. 2022. Mycobiome detection from a single subterranean gametophyte using metabarcoding techniques. *Applications in Plant Sciences* 10(2): e11461.

Cronberg, N., R. Natcheva, and K. Hedlund. 2006. Microarthropods mediate sperm transfer in mosses. *Science* 313: 1255.

Easlon, H. M., and A. J. Bloom. 2014. Easy Leaf Area: Automated digital image analysis for rapid and accurate measurement of leaf area. *Applications in Plant Sciences* 2: 1400033.

Ekwolor, J. T. B., S. D. Benjamin, J. Z. Jomsky, M. A. Bowker, L. R. Stark, D. N. McLetchie, B. D. Mishler, and K. M. Fisher. 2022. Genotypic restriction fragment length polymorphisms. *Applications in Plant Sciences* 10(2): e11467.

Farrar, D. R., and C. L. Johnson. 2022. Methodologies for soil extraction and conservation analysis of ferns and lycophytes with belowground gametophytes. *Applications in Plant Sciences* 10(2): e11469.

Gunadi, A., F.-W. Li, and J. Van Eck. 2022. Accelerating gametophytic growth in the model hornwort *Anthoceros agrestis*. *Applications in Plant Sciences* 10(2): e11460.

Haller, B. C., and P. W. Messer. 2019. SLiM 3: Forward genetic simulations beyond the Wright–Fisher model. *Molecular Biology and Evolution* 36(3): 632–637.

Holttum, R. E. 1938. The ecology of tropical pteridophytes. In F. Verdoorn [ed.], Manual of pteridology, 421–422. Springer, Dordrecht, the Netherlands.

Hornych, O., L. Černochová, A. Lisner, and L. Ekrt. 2022. An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area. *Applications in Plant Sciences* 10(2): e11466.

Krieg, C. P., and S. M. Chambers. 2022. The ecology and physiology of fern gametophytes: A methodological synthesis. *Applications in Plant Sciences* 10(2): e11464.

Mitchell, N., N. P. Piatczyc, D. D. Wang, and J. Edwards. 2022. High-speed video and plant ultrastructure define mechanisms of gametophyte dispersal. *Applications in Plant Sciences* 10(2): e11463.

Neubauer, A., S. Ruaud, M. Waller, E. Frangedakis, F.-W. Li, S. J. Nötzold, S. Wicke, et al. 2022. Step-by-step protocol for the isolation and transient transformation of hornwort protoplasts. *Applications in Plant Sciences* 10(2): e11456.

Nitta, J. H., and S. M. Chambers. 2022. Identifying cryptic fern gametophytes using DNA barcoding: A review. *Applications in Plant Sciences* 10(2): e11465.

Quinlan, A., P.-H. Lee, T.-Y. Tang, Y.-M. Huang, W.-L. Chiou, and L.-Y. Kuo. 2022. Providing the missing links in fern life history: Insights from a phenological survey of the gametophyte stage. *Applications in Plant Sciences* 10(2): e11473.

Ringailė-Voicik, R., and J. R. Naujalis. 2022. Techniques for locating and analyzing subterranean *Lycopodium* and *Diphasiastrum* gametophytes in the field. *Applications in Plant Sciences* 10(2): e11458.

Rosenstiel, T. N., E. E. Shortlidge, A. N. Melnychenko, J. F. Pankow, and S. M. Eppley. 2012. Sex-specific volatile compounds influence microarthropod-mediated fertilization of moss. *Nature* 489: 431–433.

Schneller, J. I., and D. R. Farrar. 2022. Photographic analysis of field-monitored fern gametophyte development and response to environmental stress. *Applications in Plant Sciences* 10(2): e11470.

Sorojsrisom, E. S., B. C. Haller, B. A. Ambrose, and D. A. R. Eaton. 2022. Selection on the gametophyte: Modeling alternation of generations in plants. *Applications in Plant Sciences* 10(2): e11472.

Szövényi, P., E. Frangedakis, M. Ricca, D. Quandt, S. Wicke, and J. A. Langdale. 2015. Establishment of *Anthoceros agrestis* as a model species for studying the biology of hornworts. *BMC Plant Biology* 15(1): 98.

Wu, Y.-H., Y.-T. Ke, Y.-Y. Chan, G.-J. Wang, and L.-Y. Kuo. 2022. Integrating tissue-direct PCR into genetic identification: An upgraded molecular ecology approach to survey fern gametophytes in the field. *Applications in Plant Sciences* 10(2): e11462.

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