REVIEW ARTICLE

The ecology and physiology of fern gametophytes: A methodological synthesis

Christopher P. Krieg[1]　|　Sally M. Chambers[2]

[1]Department of Botany, University of Wisconsin, Madison, Wisconsin 53703, USA

[2]Department of Botany, Marie Selby Botanical Gardens, Sarasota, Florida 34236, USA

Correspondence

Christopher P. Krieg, 430 Lincoln Drive, Birge Hall, Department of Botany, University of Wisconsin, Madison, Wisconsin 53703, USA.
Email: christopher.p.krieg@gmail.com

Sally M. Chambers, Department of Botany, Marie Selby Botanical Gardens, 1534 Mound Street, Sarasota, Florida 34236, USA.
Email: schambers@selby.org

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Abstract

All green plants alternate between the gametophyte and sporophyte life stages, but only seed-free vascular plants (ferns and lycophytes) have independent, free-living gametophytes. Fern and lycophyte gametophytes are significantly reduced in size and morphological complexity relative to their sporophytic counterparts and have often been overlooked in ecological and physiological studies. Understanding the ecological and physiological factors that directly impact this life stage is of critical importance because the ultimate existence of a sporophyte is dependent upon successful fertilization in the gametophyte generation. Furthermore, previous research has shown that the dual nature of the life cycle and the high dispersibility of spores can result in different geographic patterns between gametophytes and their respective sporophytes. This variation in distribution patterns likely exacerbates the separation of selective pressures acting on gametophyte and sporophyte generations, and can uniquely impact a species' ecology and physiology. Here, we provide a review of historical and contemporary methodologies used to examine ecological and physiological aspects of fern gametophytes, as well as those that allow for comparisons between the two generations. We conclude by suggesting methodological approaches to answer currently outstanding questions. We hope that the information covered herein will serve as a guide to current researchers and stimulate future discoveries in fern gametophyte ecology and physiology.

KEYWORDS

distributions, ferns, fieldwork, gametophytes, physiology, stress tolerance

Ferns are generally defined as seed-free taxa that disperse via spores, contain a vascular system, and have megaphylls. They are sister to all seed plants and the second most diverse lineage of vascular land plants, with roughly 10,500 species currently recognized (PPG I, 2016). Fern species can be found on six of the seven continents, absent only from the extremely cold ecosystems of Antarctica, with the majority of extant diversity concentrated in tropical ecosystems. Not only are ferns highly diverse, they also serve critical roles associated with many aspects of ecosystem health and function (George and Bazzaz, 1999). As with all other lineages of land plants, ferns reproduce via the alternation of generations, whereby gametes are produced by a structure known as a gametophyte, which then unite via fertilization and produce a sporophyte. However, ferns and lycophytes are unique from all other land plants in that the gametophytic generation is capable of living freely and independently from its sporophytic counterpart.

These two life stages differ dramatically from one another in form and function. The sporophyte is the relatively conspicuous life stage for the majority of fern taxa. This life stage produces rhizomes, vascular tissue, a cuticle, stomata, and spores, and can reach heights of 20 m (tree

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Christopher P. Krieg and Sally M. Chambers contributed equally to this work.

ferns, e.g., *Sphaeropteris cooperi* (F. Muell.) R. M. Tryon). Conversely, the gametophytic generation is generally a single cell layer thick, often small (generally <1 cm in length and/or width), and morphologically cryptic, making taxonomic identification at the species level difficult. Fern gametophytes do not produce a cuticle or vascular tissue. These differences in morphological features also lead to different physiological functions and responses to stress. For example, fern sporophytes have evolved a number of anatomical and physiological features that help them regulate water loss; these include waxy cuticles covering frond surfaces that are highly resistant to epidermal conductance to water vapor, and stomata that can open and close in response to variation in atmospheric and soil water availability, which impacts leaf water potentials. In contrast to fern sporophytes, gametophytes do not have xylem, effective waxy cuticles, or stomata, and instead rely on the ability to tolerate and recover from fluctuations in water availability. Fern sporophytes can increase light interception by producing large fronds and growing taller, while fern gametophytes must adapt their photosynthetic machinery to net a positive carbon balance in their prevailing light conditions. In our view, one of the most exciting avenues of research in fern biology is understanding the differences in form and function between life stages and how such differences in optimal conditions for

growth and survival may lead to differences in ecological and distributional patterns (Figure 1).

Here, we review the methodologies that have been used to examine fern gametophyte ecology and physiology, and provide a brief overview of the seminal papers that have led to this field of study. We begin with a historical overview of fern gametophyte ecology and highlight the methodological field and laboratory approaches used to describe the geographic and ecological distribution of fern gametophytes. We then present an overview of fern gametophyte physiology, with an emphasis on the aspects of carbon and water relations that may underlie the geographic and ecological distribution of fern gametophytes and the methods used to study these processes. Lastly, we identify outstanding questions and propose methodological approaches that could be used in the future.

ECOLOGY

Early studies

Fern gametophytes have been referred to as the "handicap" of the fern life cycle (Page, 2002). However, the critical importance of this dynamic life stage and its role in promoting and maintaining fern diversity has long been a topic of study (Stokey, 1951; Atkinson and Stokey, 1964; Cousens, 1981; Haufler and Soltis, 1984). Recent studies have focused on the ecology and physiology associated with the gametophyte phase of the fern life cycle (Sato and Sakai, 1981; Watkins et al., 2007; Pittermann et al., 2013), indicating that the physiological differences between fern sporophytes and gametophytes may result in different ecological patterns and limitations (Figure 1). For example, microhabitat differences can result in gametophyte-only populations that exist only a few feet away from populations producing sporophytes (Ebihara et al., 2019). These fragmented distribution patterns may also be seen in more extreme instances where gametophyte-only populations exist hundreds to thousands of miles away from their sporophyte counterparts (Farrar, 1992; Rumsey and Roberts, 2011; Pinson et al., 2017). It is worth noting that gametophyte-only populations existing at this broad scale are not simply sink populations, but are self-sustaining, often via the production of asexual propagules termed "gemmae."

The concept that fern gametophytes can have distinct distributions (Figure 1) and produce long-standing populations in the absence of a neighboring sporophyte first gained traction in the scientific community in the 1960s. Two studies were published in 1963 documenting the presence of gametophyte-only populations of two different fern species in eastern North America. At the time, these populations could only be confidently identified as members of the tropical genera *Vittaria* Sm. and *Trichomanes* L. (Wagner and Evers, 1963; Wagner and Sharp, 1963; Wagner, 1966). The ability to identify these populations to the taxonomic
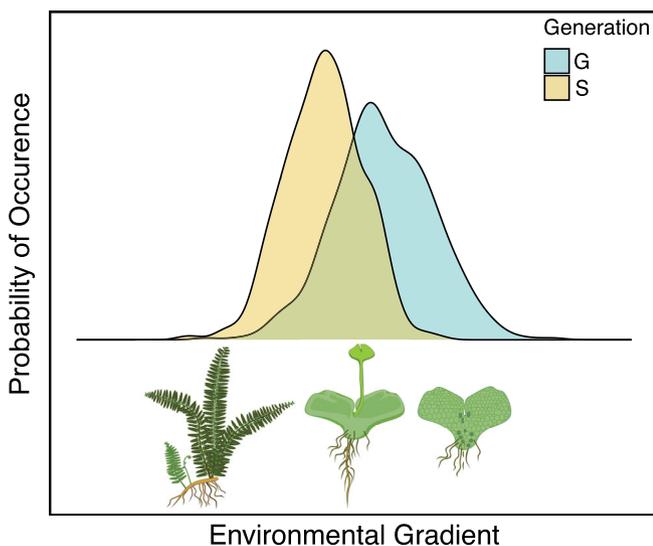
FIGURE 1 Theoretical distribution of the two independent life stages in a single fern species along an environmental gradient. Data were generated to have a Gaussian distribution (i.e., "normal") using the R function *rnorm*, and for each curve to have different means and standard deviations (see Appendix S1 for more detail). Each curve sums to a theoretical 100% of the total probability of occurrence across a given gradient. In this example, the gametophyte stage (G, light blue) and sporophyte stage (S, yellow) differ in two main ways: (1) each life stage has different relative environmental "optimums," indicated by the distance between curve peaks, and (2) the relative environmental breadth of each life stage differs, indicated by the width of each curve (and consequently the height of each curve). We posit that the environmental "optimum" and/or breadth likely differs between life stages in the majority of fern species.

assignment of genus reflects the ribbon and filamentous morphologies that fern gametophytes in these genera exhibit, and also highlights the difficulty in determining a taxonomic assignment at the species level in this cryptic life stage. Briefly, the majority of terrestrial ferns produce the cordate gametophyte often displayed in plant biology textbooks; however, there are currently five recognized morphologies: cordiform, ribbon, strap-shaped, ribbon with gemmae, and strap-shaped with gemmae. The last four morphologies typically belong to epiphytic taxa, whose gametophytes are known to live much longer than a single year in the field (as is typical of terrestrial, cordate gametophytes). For a detailed review of these morphologies, please see Farrar et al. (2008) and Pinson et al. (2017).

In 1967, Farrar published a review of this phenomenon in which he denoted populations composed of solely or mainly gametophytes, and included the observation and identification of a third species, *Moranopteris nimbata* (Jenm.) R. Y. Hirai & J. Prado, whose species-level identification was only possible due to the presence of tiny, juvenile sporophytes (Farrar, 1967). In a subsequent paper, Farrar (1998) further described these taxa, along with a few additional tropical disjuncts displaying the same fragmented distribution. In the late 1990s, the concept had spread internationally to Europe, where independent gametophyte populations were identified based on habitat type and proximity to sporophyte-bearing populations of *Vandenboschia speciosa* (Willd.) G. Kunkel (Rumsey and Roberts, 2011). This finding further suggested that microclimatic conditions may be just as important in creating these disjunct populations as large-scale climatic factors that define a species' geographic range boundary (see Physiology section below).

Field surveys

For locating and studying gametophyte-only populations, researchers of these seminal studies utilized the fact that these tropical relatives and disjuncts all occupied recesses in sandstone escarpments (colloquially known as rock shelters or rock houses) throughout the eastern United States. Using thermometers and, more recently, employing the use of programmable data loggers, researchers determined that these rock shelters buffer seasonal and daily variation in temperature (Farrar, 1998; Stevens and Emery, 2015; Chambers and Emery, 2016). In addition, many of these shelters are located near waterfalls, resulting in environmental conditions that are similar to those of high-elevation tropical forests where the sporophytes of many of these species exist. These observed environmental patterns therefore posed the question as to why these populations were not observed to produce sporophytes, or sporophytes that were able to reach maturity.

While field surveys focusing on unique geologic features have been useful in developing the concept of gametophyte-only populations and locating them in temperate ecosystems, identifying independent populations of fern gametophytes is exceptionally difficult in many plant communities. Modern studies seeking to identify and examine fern gametophytes utilize a methodology known as DNA barcoding. In this approach, field-collected gametophytes are subjected to the sequencing of one or two genes, the results of which are compared to a database of sequenced sporophytes that include the regional fern species pool. Utilizing this methodology, contemporary studies have been able to identify fern gametophytes to the species level (Duffy et al., 2015), and this approach has been used successfully in multiple tropical locations in Asia, the Pacific, and Central America (Ebihara et al., 2013; Nitta et al., 2017, 2020; Park et al., 2020). Using DNA barcoding to study fern gametophytes and their distributions, particularly in the tropics, clarifies our understanding of species distribution patterns and just how far spores can travel and still develop into healthy gametophytes (see Synthesis and Future Directions).

Researchers only began using metabarcoding techniques to identify and survey fern gametophyte populations roughly a decade ago. In one study, de Groot et al. (2011) used soil samples from two locations in Europe to grow fern gametophytes in a greenhouse, and subsequently identify them using DNA barcoding. In Japan, Ebihara et al. (2010) first published on the concept of building a robust barcode library comprising all sporophytes reported for the country, and followed up with a study comparing the barcodes of unidentified gametophytes to this library for identification (Ebihara et al., 2013). To thoroughly sample fern gametophytes, Ebihara et al. (2013) developed a strategy whereby 25-cm^2 quadrats were subdivided into grids of 25 squares. Within each square, gametophytes were binned based on morphology, and one collection was made of each morphological type observed per square. Each field-collected gametophyte was then identified using DNA barcoding techniques. Nitta et al. (2017) followed a similar approach on the islands of Mo'orea and Tahiti, but placed gametophyte sampling plots within larger sporophyte sampling plots. Importantly, this paper also explicitly discusses the surveying of epiphytic gametophytes at a height of 2 m, which was conducted on trees within the larger sporophyte sampling quadrats. Although DNA barcoding has proven to be a powerful approach in the identification of fern gametophytes to species, there are still instances where difficulties may arise. This typically occurs when the sporophytes of a given study location have not been fully surveyed and thus their sequences are not available for genetic comparison to the unidentified gametophyte. Other difficulties, such as loci selection, may present additional hurdles that researchers must overcome (see Nitta and Chambers, 2022; Quinlan et al., 2022; Wu et al., 2022).

Laboratory and greenhouse studies

Prior to the invention of DNA barcoding, most studies examining ecological and physiological responses in ferns

were conducted in a laboratory setting using gametophytes grown from spores (Caponetti et al., 1982; Watkins et al., 2007; Testo and Watkins, 2013; Sessa et al., 2016). Common garden studies conducted in greenhouses and neighboring field locations have also proven to be effective in characterizing the environmental conditions that permit growth and those that prevent it (i.e., lethal thresholds) (Farrar, 1998) and in examining gametophyte plasticity (Greer and McCarthy, 1999). In some cases, these early studies yielded data that were only attributable to a particular genus, as the identity of field-collected gametophytes could not be confirmed given their cryptic morphology (e.g., Figure 9.5 in Farrar et al., 2008). Laboratory-based studies can be particularly powerful when used to isolate and manipulate a single environmental factor such as temperature, light intensity, or relative humidity. Such studies are often conducted in growth chambers to keep all other environmental factors constant. They can also be used to examine specific questions relating to mating systems and fitness, which in ferns can be tested by pairing or isolating gametophytes to determine how much inbreeding impacts sporophyte production (Haufler et al., 2016; Sessa et al., 2016). From these important studies, we have learned that some gametophytes, despite their frail appearance and lack of a cuticle, are quite robust and can tolerate desiccation better (Watkins et al., 2007) and survive colder temperatures than their sporophytic counterparts (Farrar, 1998).

While laboratory-based studies have addressed a number of questions relating to fern gametophyte ecology, many fern species are difficult to grow from spores. Additionally, many ecological questions cannot be addressed using lab-reared gametophytes and must be conducted in the field. Furthermore, laboratory conditions have been shown to alter patterns associated with development and sexuality, with laboratory-grown gametophytes being shown to produce more males than females (Ranker and Houston, 2002). To this end, field-based studies are extremely powerful, and the advent of DNA barcoding to identify gametophytes to species truly opens the door for addressing more of these important ecological questions. These studies can cover a broad range of topics including sex expression and fertilization, local adaptation, geographic range dynamics (such as contraction and expansion), dispersal potential, and stress tolerance. To date, such field-based studies have only been conducted in the gametophyte-only fern *Vittaria appalachiana* Farrar & Mickel (Stevens and Emery, 2015; Chambers and Emery, 2016).

PHYSIOLOGY

Carbon: growth and light use

In ferns, the traits that maximize growth and fitness in a given environment are split between the gametophyte and sporophyte generation. Given that plants need a positive carbon balance for growth and reproduction, differences in the optimal environment for photosynthesis between sporophyte and gametophyte generations may directly impact their respective distributions (Figure 1). Therefore, understanding the variation in light requirements and photosynthetic responses to environmental variation across ecologically and phylogenetically diverse species is critical to characterizing the adaptive life history and growth strategies (but see Farrar and Johnson [2022] and Rimgailė-Voicik and Naujalis [2022] for discussion of the special case of subterranean gametophyte ecology). Indeed, research in vascular plant sporophytes, including fern sporophytes, has discovered key traits related to photosynthetic capacity (e.g., the quantum yield of photosynthesis [Φ] and photosystem II [ΦPSII], light compensation point [LCP], maximum rate of carboxylation by RuBisCO [V_{cmax}], and the maximum rate of electron transport of photosynthesis [J_{max}]) to be key drivers of species growth strategies (Poorter et al., 1990; Wright et al., 2002) and correlated with aspects of a species' ecological niche (Choy-Sin and Suan, 1974; Brach et al., 1993; Hietz and Briones, 2001; Saldaña et al., 2005; Ali et al., 2015). Although in some respects research in fern gametophytes is decades behind that in sporophytes, investigators have long been interested in how carbon fixation by fern gametophytes may influence gametophyte growth rates, and how gametophyte growth responses to different environments may differ from those of their sporophytic counterparts. Since at least the early 1950s, researchers started laying the foundation to understand gametophyte photosynthetic biology, and some of the earliest work on the photobiology of fern gametophytes established that gametophyte growth rates are strongly impacted by light intensity (Mohr, 1956; Miller and Miller, 1961; Donaher and Partanen, 1971) and ecological gradients (Näf, 1953). To understand the potential for photosynthetic variation in fern gametophytes, early studies estimated carbon fixation by the change in dry mass and/or protein content over time (e.g., Hotta and Osawa, 1958; Mohr and Ohlenroth, 1962). These early studies were among the first to demonstrate that fern gametophytes are not physiologically restricted to inhabit extremely low light conditions, but could acclimate to different light conditions and had the capacity for phenotypic plasticity in carbon- and growth-related traits (Figure 2). More recent studies have confirmed that the mechanisms by which many fern gametophytes adapt to light intensity are similar to those used by sporophytes of ferns and seed plants (e.g., increasing concentrations of photoprotective pigments with increasing light intensity; Farrar et al., 2008; Fernández-Marín et al., 2012).

By the late 1970s, three main tools were being used to characterize the extent to which environmental conditions impact carbon- and growth-related traits of fern gametophytes. These tools included infrared gas analyzers (IRGA), polarographic O_2 electrode systems, and fluorometers. Each of these tools can be used to assess the photosynthetic physiology of fern gametophytes. Briefly, an IRGA
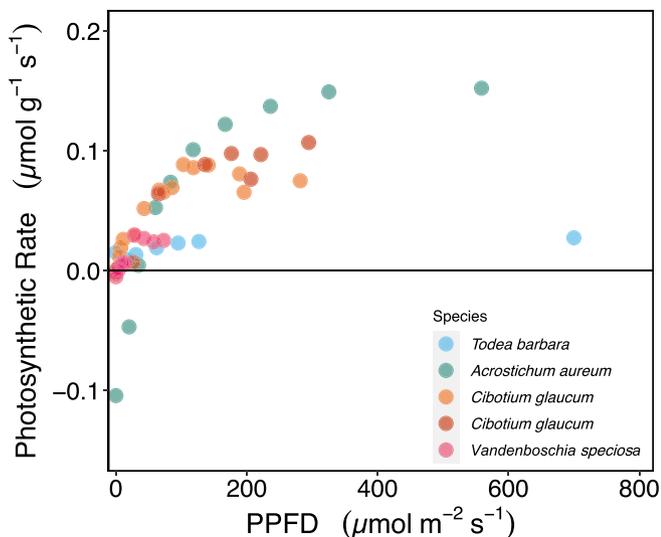
FIGURE 2 Visualization of published experimental data showing variation in photosynthetic responses to changes in light intensity (photosynthetic photon flux density [PPFD]) among gametophytes of four fern species: *Todea barbara* (Hagar and Freeberg, 1980), *Acrostichum aureum* (Li and Ong, 1998), *Cibotium glaucum* (Friend, 1974), and *Vandenboschia speciosa* (Johnson et al., 2000); see Appendix S1 for more detail.

estimates the rate of net CO_2 flux by measuring the CO_2 concentration of air entering a chamber containing plant tissue (e.g., a leaf in a chamber), measuring the CO_2 concentration of air exiting the chamber, and using a mass-balance equation to solve for the amount of CO_2 uptake or release per unit plant material (e.g., area or mass) per unit time (Field et al., 2000). Polarographic O_2 electrode systems measure the change in oxygen concentrations caused by photosynthetic activity (see Appendix S1 for more detail) by measuring the effects of oxygen gas on the electrical current between an anode and a cathode (González et al., 2001). A fluorometer is commonly used to estimate the capacity of photosystem II (PSII) to shuttle light energy to photochemical processes by exposing plant tissue to supersaturating light intensities and quantifying the proportion of light energy that is dissipated as heat energy, re-emitted as fluorescence, or absorbed by PSII for photochemical processes (see Maxwell and Johnson, 2000; also see Water section for more uses of a fluorometer). These tools have increased our understanding of photosynthetic capacity and the thresholds for positive carbon gain of fern gametophytes in various light levels and stress conditions. For example, Friend (1974) provided one of the most thorough examinations of photosynthetic plasticity we have available today. In this study, Friend used gas analyzers to measure the changes in carbon- and growth-related traits, such as photosynthetic capacity, growth rates, and morphology, in response to different growth temperature and light conditions. The results characterized the different optimal growth conditions for *Cibotium glaucum* (Sm.) Hook. & Arn. gametophytes and sporophytes, including light

compensation points and estimated lethal light intensities (Friend, 1974).

Discoveries of distinct physiological responses to environmental conditions, optimal growth conditions, and lethal thresholds between fern gametophytes and sporophytes shed light on the potential mechanisms that underlie differentiated ecological and distributional patterns (Sato and Sakai, 1981). Such comparisons of photosynthetic biology between generations can illuminate other unique biological features of fern species. For example, several studies showed that sporophytes of *Pyrrosia longifolia* (Burm. f.) C. V. Morton have evolved the Crassulacean acid metabolism (CAM) photosynthetic pathway, a highly water-efficient strategy to capturing and fixing carbon often seen in sporophytes of desert plant species (Cushman, 2001; Kluge and Ting, 2012). In 1995, Martin et al. used an IRGA (in conjunction with other tools) to characterize the photosynthetic pathway of *Pyrrosia longifolia* gametophytes, and demonstrated that this species of fern transitions from C_3 photosynthesis in the gametophyte stage to CAM in the sporophyte stage (Martin et al., 1995). Many epiphytic fern species have been shown to exhibit CAM photosynthesis (Martin et al., 2005), yet the adaptive value of this transition in these species has not been well demonstrated and is ripe for experimentation.

The environmental conditions that bound a species' ability to perform photosynthesis under stress are critical for understanding the mechanisms underlying species' distributions, yet only a few studies have examined this in fern gametophytes. In particular, gas-exchange systems have been used to characterize photosynthetic responses to stressors like saline soils. In 1998, Li and Ong used O_2 electrode systems to understand the photosynthetic response of fern gametophytes belonging to the salt marsh species *Acrostichum aureum* L. to a series of salt treatments (Li and Ong, 1998). Although high salt concentrations can impose drought-like conditions on living cells, Li and Ong found that gametophytes of this species performed better (higher photosynthetic capacity and efficiency) in the presence of salt than without. More work is needed to understand the extent to which photosynthetic sensitivity to salinity of fern gametophytes may be an important predictor of distributional patterns and how gametophytic adaptations to salinity differ from sporophytes. In addition to soil and substrate properties like salinity, variation in temperature can substantially impact photosynthetic carbon gain. In sporophytes, species generally have an optimum temperature to achieve maximum photosynthetic rates, which generally decrease at relatively lower and higher temperatures (Farquhar et al., 1980; Bernacchi et al., 2009). Some research has suggested that fern sporophytes and gametophytes may share similar response mechanisms. For example, Johnson et al. (2000) used a fluorometer and O_2 electrode system to compare the photobiology (e.g., light use efficiency, capacity of PSII, respiration) to changes in light and temperature in gametophytes and sporophytes of

Vandenboschia speciosa in the northernmost parts of its range (Britain). They found evidence that the gametophyte and sporophyte responded similarly by decreasing photosynthetic processes with decreasing temperatures and increasing photosynthetic rates with increasing light intensity. However, they also noted large differences in respiration rates and maximum photosynthetic capacities. The authors hypothesized that higher respiration rates in the sporophyte could be required to build and maintain more complex plant structures and non-photosynthetic tissues, unlike in gametophytes, which may help explain why some extremely low-light habitats were found to support gametophyte-only populations. Despite the value of these data, little is known about the interactions between light level and temperature on the respiratory costs of growth strategies at the limits of species' distributions in fern sporophytes and gametophytes.

Water: stress tolerance and avoidance

For sexually reproducing fern species, water availability is thought to be a critical driver of establishment, as fertilization occurs with liquid water through which flagellated sperm can swim to archegonia (Sato, 1992). Although tolerance to extreme desiccation in fern sporophytes is relatively rare (Proctor and Pence, 2002; Kessler and Siorak, 2007), there are comparatively fewer studies of gametophyte water relations relative to sporophytes. The earliest studies on fern gametophyte water relations date back to at least the early 1900s. Pickett (1913, 1914) was the first to test hypotheses about the extent to which fern gametophytes might withstand and survive desiccation similar to and even beyond that experienced in its natural habitat. To understand if desiccation tolerance (DT) might be an adaptation to limited water availability in native habitats, Pickett observed and compared the growth and survival of gametophytes from two species of *Asplenium* L. that differ in the water availability in their native habitats, *A. rhizophyllum* L. and *A platyneuron* (L.) Britton, Sterns & Pogg. Gametophytes of both species were exposed to combinations of high and low light intensity (full sun to partial sun), warm and cool temperatures (25–16°C), and ambient humidity or desiccation, which was generated by passing air through chemical desiccants such as glycerin or sulphuric acid. Pickett found that the species from more xeric sites (*A. rhizophyllum*) had moderately higher survival rates under extreme artificial desiccation treatments compared to the relatively more mesic species (*A. platyneuron*) (i.e., surviving an average of about 8–9% longer under treatment conditions before complete death). Pickett then continued to explore DT in a broader range of fern species using similar approaches (i.e., manipulating exposure to ambient air and desiccated air using chemical desiccants), including *Myriopteris gracillima* (D. C. Eaton) J. Sm., *Pellaea atropurpurea* (L.) Link, *Pellaea glabella* Mett. ex Kuhn, *Polypodium vulgare* L., and other species

(Pickett and Manuel, 1926; Pickett, 1931). These early studies were the first to show natural variation in responses to water stress and that some fern gametophytes, despite their delicate appearance, can be robust in response to abiotic stress.

Fast forward to the early 2000s when this work was revived by Watkins et al. after more than 70 years. Watkins published a paper describing the adaptive value of DT in tropical fern gametophytes and linking variation in DT to habitat preferences (Watkins et al., 2007). The primary method to manipulate plant water availability employed in these DT studies was the use of various salt solutions that act as desiccants to bring the air around a gametophyte to a known relative humidity, and which draw water from the gametophyte's tissue (see Appendix S1). An important distinction between the early desiccation studies and modern desiccation studies is the availability and concurrent use of tools that measure the impact of desiccation on photosynthetic activity. Watkins et al. (2007) used a fluorometer to assess the capacity of gametophyte photosystems to capture light and transmit electrons through photosynthetic machinery (e.g., PSII; Genty et al., 1989; Seaton and Walker, 1990) in healthy, desiccated, and recovered gametophytes. In short, the salt method and fluorometer can be used in combination to understand the degree of metabolic recovery after desiccation and the lower limits of desiccation from which a species can still recover a level of metabolic activity similar to pre-desiccation levels. Contemporary papers have used this combination of tools to characterize variation in DT across species, including between tropical epiphytic and terrestrial species, and to compare populations (Watkins et al., 2007; Testo and Watkins, 2013; Chambers et al., 2017). Many more population-level studies are needed to fully understand the dynamic ecological and physiological patterns displayed by fern gametophytes.

SYNTHESIS AND FUTURE DIRECTIONS

Despite the paramount contributions of early works on fern gametophyte ecology and physiology, compared to other areas of fern biology, relatively few studies have compared gametophyte and sporophyte distributions, and even fewer have explored the physiological mechanisms that underlie ecological patterns in both generations. With the applicability of novel technologies and new methodologies, we believe this area of research is poised for exploration and discovery. Below, we outline a series of major questions that remain poorly understood in fern gametophyte ecology and physiology and suggest paths forward to gain answers and insights.

> Q1: *How common is it for the gametophyte and sporophyte generations to substantially differ in geographic range? What are the main environmental axes that unite and separate the realized niche between generations?*

Although several studies show fern sporophytes and gametophytes can exist in different geographic locations, few studies have directly compared their distributions and the environmental components that may be driving these differences. Two studies have utilized elevational gradients and the natural changes in environment that accompany these elevational changes to begin to address these questions. In Tahiti and Mo'orea, Nitta et al. (2017) determined that sporophyte diversity follows the common ecological pattern of mid-elevation peaks, whereby diversity is highest at middle elevations relative to low and high elevations. Conversely, gametophyte diversity was consistent across the examined elevational gradients. A study conducted by Pinson et al. (2022) on the island of Oahu, Hawaii, that examined the distribution of gametophytes and sporophytes of *Callistopteris baldwinii* (D. C. Eaton) Copel. examined the role of microclimatic trends in temperature and relative humidity across an elevational gradient in which gametophytes grow at low elevations and sporophytes at high. This study concluded that these microclimatic factors do differ significantly between life stages, although these differences are relatively small and may not be the primary drivers in the inability of gametophyte populations to produce sporophytes. Other environmental factors may also be important drivers of population establishment and growth, including the primary source of water for most plants (i.e., soil water content) and the primary driver of plant water loss (vapor pressure deficit), as well as the primary source of energy (i.e., light). The interactions among stressors (termed "poly-stress") may also be important predictors of species ecology and distribution (Sack, 2004; Laanisto and Niinemets, 2015; McCulloh et al., unpublished data). To our knowledge, no study has examined the impacts of soil water content, vapor pressure deficit, and light on the distribution of any species of fern gametophyte.

Several tools exist to understand the amount of macro- and micro-environmental overlap and separation between individuals and populations of both generations. First, researchers would need to characterize the geographic distribution of gametophyte and sporophyte individuals. Although many sources are available for sporophyte location data from previous fieldwork (e.g., the Global Biodiversity Information Facility [GBIF], https://www.gbif.org), few data exist for the location of fern species in the gametophyte stage. Extensive fieldwork, the use of species occurrence databases, and the use of DNA barcoding could be used to characterize the geographic distribution of a species' gametophyte and sporophyte generation. Once the geographic distribution of generations is spatially characterized, environmental data can be compared to reveal the factors that best correlate with species occurrences. Coarse spatial environmental data are available from popular sources like WorldClim (https://www.worldclim.org) and Chelsa (https://chelsa-climate.org) at a resolution of about 1 km^2. Other sources of spatial data of similar resolutions for relative humidity, temperature, vapor pressure deficit,

and soil water content include PRISM Climate Group (https://prism.oregonstate.edu/) and TerraClimate (https://www.climatologylab.org/terraclimate.html). Microclimate data can be gathered using deployable sensors and data loggers such as iButtons (iButtonLink Technology, Whitewater, Wisconsin, USA), or sensors from ONSET (Onset Computer Corporation, Bourne, Massachusetts, USA) or METER (METER Group, Pullman, Washington, USA). If microclimate factors have a stronger impact on species growth and survival than macroclimate, these and similar devices may be particularly critical to characterize the environmental factors that correlate with species occurrences in the gametophyte stage.

Once environmental data are collected and geographic distributions are characterized, statistical associations between the occurrence of an individual and the prevailing environmental conditions can be detected with a variety of approaches including machine learning and traditional statistical models (i.e., species distribution models; see Townsend Peterson et al., 2011). Environmental factors that strongly correlate with the spatial occurrence of species are likely to impose selective pressures on function and physiology; thus, the output from these environmental analyses can be used to inform studies of the physiological mechanisms that underlie distribution patterns. Moreover, the analysis of ecological niche space is an area of rapid methodological development. By coupling DNA barcoding, environmental data collection (at both the macro- and micro-environmental levels), and the latest analytical approaches to characterize the ecological niche, the ability to characterize how fern gametophytes share similar aspects of their ecological niche with their sporophytic counterparts, and how they may be distinct, has never been more accessible to researchers.

Q2: *How do the physiological functions of fern gametophytes compare to their sporophyte counterparts?*

Overall, many basic aspects of fern physiology remain unknown or poorly characterized, particularly for the gametophyte generation. For example, Sakamaki and Ino (1999, 2007) estimated that approximately 40% of a gametophyte's resources are translocated to emerging sporophytes in *Thelypteris palustris* (Salisb.) Schott. The extent to which this allocation differs between species with different habitat preferences and/or life histories remains unknown. If photosynthetic capacity drives growth strategies in gametophytes similar to other sporophyte-dominated species, photosynthetic characteristics may help predict a species' niche position by helping define their growth strategy (Grime, 1977, 1988; Craine, 2005; Jabot and Pottier, 2012). To our knowledge, no study has compared the natural variation in gametophytic photosynthetic response to gradients of light intensity across a broad range of ecologically and phylogenetically diverse species (e.g., light response curves; Figure 2). Therefore, it is not surprising how little is known about the ecological

significance of variation in photosynthetic capacity in fern gametophytes. Furthermore, future research could take a similar approach to Sakamaki and Ino (1999, 2007) across a broader range of species and additionally compare the growth strategies of gametophytes to their sporophyte counterparts.

If photosynthetic physiology is similarly linked to growth strategies in fern gametophytes and their sporophytic counterparts, are stress responses similar as well? Do drought-tolerant sporophytes tend to have drought-tolerant gametophytes? So far, we lack comparative physiology data across broad ecological and phylogenetic scales to assess whether trends in the physiology of fern gametophytes are similar to those in sporophytes. In particular, we have been unable to compile and present data from published studies on the DT of fern gametophytes because there is no universal metric or standard to rank a species' desiccation tolerance. For example, studies that have performed DT experiments on fern gametophytes vary in the intensity of the desiccation treatment by using different salt solutions that achieve different tissue water potentials. They also vary in the time (e.g., minutes to hours or days) and method of recovery treatments (e.g., how gametophytes are rehydrated). To date, all fern gametophyte DT studies present their data relative to a measurement taken in a healthy hydrated state and ranked against other measurements within the data set. There is no commonly used metric in fern gametophyte DT studies that use salt solutions to compare the DT of species across data sets. We propose that DT studies adopt the set of metrics that have long been used to study sporophyte plant–water relations, in addition to the currently used protocols. For example, several metrics in plant–water relations, such as the turgor loss point (Ψ_{tlp}; i.e., the water potential at which the pressure potential = 0 MPa), osmotic potential at full turgor (Ψ_o), and bulk elastic modulus (ε) of cells (Tyree and Hammel, 1972), have shown strong correlations with aspects of a species' ecological niche and tolerance to water stress (Lenz et al., 2006; Baltzer et al., 2008; Bartlett et al., 2012; Li et al., 2018; Zhu et al., 2018). Recent methodological developments to generate pressure-volume curves for fern gametophytes using screen-caged thermocouple psychrometers now allow the calculation of these parameters (Krieg et al., 2019). Although published Ψ_{tlp} data are available for some fern sporophyte species, the extent to which these parameters are correlated between fern gametophytes and sporophytes across broad scales remains unknown. Furthermore, no study has combined these methods (salt method and pressure-volume curves) to thoroughly describe the physiological consequences of fern gametophyte desiccation relative to a gametophyte's turgor loss point.

Q3: *What are the drivers of ecological and physiological variation across a species' geographic range and how might these be impacting the distribution and evolution of fern taxa?*

The relative contributions of environmental and genotypic variation on phenotypic variation (i.e., trait = genotype + environment) is a classic framework of study in biology. This framework has been applied in numerous organismal systems to understand the fundamental evolutionary and ecological drivers of physiological variation across landscapes (Mobley et al., 2011; Lima et al., 2012; He et al., 2013; Shinn et al., 2015). To date, the combination of field and laboratory methods to answer such questions across populations of fern gametophytes has been used sparingly (Chambers and Emery, 2016, 2018). This likely reflects the difficulty in locating and identifying fern gametophytes prior to the availability of DNA barcoding, and in making accurate measurements of carbon- and/or water-related physiological traits. However, this approach has been shown to be a powerful method to integrate both observed ecological patterns and physiological limitations. To begin to understand the relative contributions of genetic and environmental factors on gametophyte and sporophyte phenotypes, researchers would need to employ a multi-step, cross-disciplinary approach. For example, the first step would likely be to employ extensive fieldwork to delineate the realized distribution of a species' gametophyte generation, using DNA barcoding. Once identified to species, detailed genetic analyses may be completed to examine population genetic structure. Investigators may choose to use traditional approaches such as allozymes, microsatellite loci, inter-simple sequence repeat (ISSR) markers, or mtDNA (Culley and Wolfe, 2001; Balloux and Lugon-Moulin, 2002; Manel et al., 2003; Chapuis and Estoup, 2007; Holsinger and Weir, 2009). Alternatively, they may try more modern molecular approaches, such as ddRADSeq; however, such studies in ferns are limited due to their large and complex genomes (Rowe et al., 2018; Kinosian et al., 2020; Pelosi and Sessa, 2021).

Extensive fieldwork would also be required to make field measurements of standing physiological variation and microclimate conditions in populations. Several works have been published that outline an extensive list of methods commonly employed in plant ecophysiology (e.g., Pearcy et al., 1989; Roger, 2001; Pérez-Harguindeguy et al., 2013) (see Physiology section). Macro- and micro-environmental data can be gathered from a variety of sources and field-deployable devices (see Physiology section). At this point, researchers would be able to partition the impact of genetic identity and environmental conditions on phenotypic variation using any of several multivariate modeling approaches that have been used in other systems (Brommer, 2011; Karhunen et al., 2013; Da Silva and Da Silva, 2018). One accessible approach we recommend is to calculate the relative phenotypic variation within and among populations (PST), which assumes genetic divergence is a neutral process and that measured traits have an additive genetic basis to estimate the genetic divergence of quantitative traits (QST) (Wright, 1952; Spitze, 1993). Such an approach would significantly contribute to understanding the evolution of morphological and physiological traits across

generations (Sorojsrisom et al., 2022). To date, no study has related the genetic variation within and/or among natural populations to variation in ecophysiological traits of fern gametophytes.

Q4: *How will geographic ranges shift in response to Earth's changing climate?*

We have little empirical data examining how the geographic ranges of fern species will respond with regard to the changing climate. Given the critical importance of the gametophyte generation in fern establishment, we feel this should be a primary area of research in fern biology and that researchers could use several of the aforementioned techniques (e.g., field surveys, DNA barcoding, and physiological tools) to address these and similar questions. For those taxa capable of producing long-lived gametophytes existing in geographic areas with exceptionally heterogeneous environments, is it possible that some species may shift their populations into refugia, as is believed to be the case with the previously mentioned independent gametophytes located in sandstone rock recesses (see Ecology: Field surveys, above). In instances such as these, the ability for these recesses to buffer daily and seasonal temperature variation suggests that they should be surveyed to determine how and if fern ranges are shifting. These locations may also act as "stepping stones," for geographic range expansion and natural recruitment.

The use of common garden experimental designs and transplant studies may elucidate ecophysiological patterns associated with local adaptation or dispersal limitation, both of which would hinder the ability of a species to shift its geographic range to track the changing climate (Kawecki and Ebert, 2004; Parmesan, 2006; Valladares et al., 2014). Prior to experimentation, investigators may choose to first propagate gametophytes from appropriate sporophytes, or to field-collect and DNA barcode gametophytes that would be suitable for those that produce clonal populations. Transplanting gametophytes into new areas, especially those that differ from the sporophyte range, would allow researchers to test whether gametophytes can grow beyond the species' current geographic range.

Aside from quantitative experiments, researchers could employ the use of computational methodologies to model species distributions and project them into the future. The additional gametophyte identifications made possible by DNA barcoding will allow researchers to paint a holistic picture of fern distributions, and even project how the distributions of both life stages may shift in response to the changing climate. This, however, will require further field surveys to identify the full extent of both life stages. It will also be important to account for seasonality when conducting such surveys, as it has been shown that gametophyte abundance correlates with the timing of spore dispersal and gametophyte phenology (Quinlan et al., 2022; Schneller and Farrar, 2022). Additionally, the tracking of any known gametophyte populations and measurement of seasonal changes in population growth, survival, and sporophyte production can help to narrow the season and the type of environmental conditions that occur in that time (e.g., Sato, 1982, 1992), which may offer the best chances of finding new populations.

CONCLUDING REMARKS

Understanding the ecological and physiological factors that directly impact the gametophyte life stage is of critical importance because the ultimate existence of a sporophyte is dependent upon successful fertilization in the gametophyte generation. In the past 100 years, researchers have discovered and affirmed the importance of gametophyte ecology and physiology as critical for holistically understanding fern biology. We find that there are two main research avenues in particular that have had a significant impact in the field, and find that there are natural extensions of each that are relevant for imperative future studies (Q1–4).

First, field studies support the concept that fern gametophytes can occupy geographic spaces in which a sporophyte cannot be produced or survive to maturity (see Ecology section), yet the extent to which this is commonplace and the environmental factors that are most relevant remain poorly understood. We proposed that researchers combine established and recent methodologies to characterize how fern gametophytes share similar aspects of their ecological niche with their sporophytic counterparts, and how they may be distinct, with a particular focus on the abiotic conditions that drive species distributions (Q1). Second, manipulative and laboratory studies show natural variation in physiological responses to the environment (e.g., Figure 2) and that some fern gametophytes can be robust in response to stressful conditions (e.g., desiccation tolerance; see Physiology section). Despite these findings, few studies compare the physiological responses of fern gametophytes to those of their sporophyte counterparts, thus limiting our understanding of the adaptive physiology that may be similar between independent generations, and the adaptive physiology that may be divergent. We proposed that researchers examine carbon- and growth-related traits (e.g., photosynthesis) and water-related traits (e.g., desiccation tolerance) in both generations, using a comparable set of metrics that allow for analyses across data sets (Q2).

We further extended these two main avenues of research to combine genetic, physiological, and ecological methodologies to understand range dynamics and genotype × environment interactions in ferns, with a focus on the gametophytic generation. In particular, the genetic and environmental underpinnings of physiological responses that strongly impact species distributions remain unknown for any fern species. We believe the future of fern gametophyte ecology and physiology research should seek to understand the relative contributions of genetic and environmental factors on gametophyte and sporophyte

phenotypes across landscapes (Q3). Finally, we acknowledge the current threat of climate change to global biodiversity. However, because we have little empirical data examining how the geographic ranges of fern sporophytes will respond to the changing climate, and no joint data for each independent life stage of any fern species, we lack the ability to make robust predictions into the future for any fern species. We believe the future of fern gametophyte ecology and physiology research should combine established and recently developed methodologies characterizing the ecological and physiological processes that drive the distributions of fern gametophytes and sporophytes to make better predictions about how species may respond to our changing climate (Q4). Ultimately, we hope that the information, direction, and ideas presented in this review serve as a guide for investigators moving forward to examine fern ecology, physiology, and conservation.

AUTHOR CONTRIBUTIONS

C.P.K. and S.M.C. contributed equally to all aspects of the manuscript, including conception of the manuscript content, data analysis, and writing. Both authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Fern gametophyte ecology and physiology data acquisition and preparation.

Appendix S2. Reference table and conversions for using the salt method in desiccation tolerance studies.

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