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The Poisoned Chalice of Evolution in Water: Physiological Novelty Versus Morphological Simplification in Marsileaceae

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Abstract.—The Marsileaceae is a small family of semi-aquatic ferns displaying numerous traits commonly observed in angiosperms, including heterospory, sophisticated hydraulic architecture, and high rates of atmospheric gas exchange. Despite these similar traits, Marsileaceae is comparatively ecologically limited. Most species are found in *Marsilea* which is sister to *Regnellidium* and *Pilularia*, together these two genera include only seven species. Here we studied the anatomy and physiology of Marsileaceae to better understand the potential constraints on ecological and species diversity in this family. We focused on epidermal anatomy and stomatal responses to changes in light and water availability, which are unique amongst ferns. We found two evolutionary strategies in Marsileaceae, one of morphological simplification, physiological inflexibility, and aquatic specialization in *Pilularia*; which contrasts with a strategy of maximizing photosynthetic carbon gain at the expense of high rates of water loss in *Marsilea* and *Regnellidium*. We conclude that aquatic environments provide evolutionary opportunities for physiological innovation with regard to stomatal function, as well as selective pressures that drive the canalized evolution of highly specialized aquatic forms.

Key Words.—Aquatic ferns, stomata, photosynthesis, *Pilularia*, *Regnellidium*

The Marsileaceae is a small family of semi-aquatic ferns comprising three genera: *Marsilea*, a genus of approximately 70 species, is sister to the monotypic genus *Regnellidium* and *Pilularia*, a genus of approximately 6 species (Pryer, 1999). *Marsilea* appears to have diverged in the late Cretaceous, while fossil evidence for the other two genera date to the Cenozoic (Hermsen *et al*., 2014). This timing indicates that members of the Marsileaceae have not undergone recent and rapid radiations like other families of Polypodiopsida (Schneider *et al*. 2004; Testo and Sundue, 2016), despite a seemingly auspicious beginning with early Marsileaceae strongly resembling some early angiosperms (Hermsen *et al*., 2014; Hermsen, 2019). Here we seek explanations for the limited ecological success of the Marsileaceae family with a particular focus on the genera *Regnellidium* and *Pilularia*.

Members of the Marsileaceae have reproductive and ecological traits that are generally viewed as pathways to dominant land plant forms. For instance, they are among the only heterosporous ferns (Schneider and Pryer, 2002). In the context of land plant evolution, heterospory, which has evolved multiple times, improves resource use efficiency, promotes outcrossing, and has led to the evolution of the seed; this reproductive strategy is often invoked to explain

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the global dominance of seed plants (Bateman and DiMichele, 1994; Qiu, Taylor, and McManus, 2012; Petersen and Burd, 2017). Heterospory may have also contributed to the high species numbers of other non-angiosperm groups, including *Selaginella*, the most diverse lycophyte genus (Petersen and Burd, 2018). In addition to this reproductive novelty, members of the Marsileaceae are unusual for ferns in that they occupy aquatic or semi-aquatic niches. Members of the order Salviniales, including early fossil Marsileaceae, may have been confined to an aquatic environment since the Late Jurassic (Vallati *et al.,* 2017; Hermsen, 2019). Aquatic environments have facilitated innovation and explosive radiations among angiosperm lineages (Du and Wang, 2016), including the monocots (Les, 1995), but not so in Marsileaceae or other aquatic fern families (Mehltreter *et al.*, 2010).

A simple morphological explanation for limited diversity in the Marsileaceae is unlikely because the three genera have remarkably different morphologies. *Marsilea* species have compound leaves with four leaflets that are highly responsive to environmental signals, including a heterophyllous switch mediated by blue light or abscisic acid (Lin and Yang, 1999) and nyctinasty (daily movement of leaflet orientation), a trait not found in any other ferns (Vasco *et al.*, 2013). *Regnellidium* has only two leaflets, which also exhibit heterophylly but less dynamic plasticity and nyctinasty in response to environmental signals (Johnson, 1986). Unlike most ferns, *Pilularia* is unique in producing single filiform leaves (Vasco *et al.*, 2013). This reduction series in leaf morphology is associated with trends in hydraulic architecture. For example, leaf venation is reticulate in *Marsilea*, dichotomizing in *Regnellidium*, and a single trace in *Pilularia* (Johnson, 1986). Vessels have been reported in *Marsilea* and *Regnellidium* roots and some *Marsilea* rhizomes (White, 1961; Schneider and Carlquist, 2000).

The reduced leaf morphologies of *Regnellidium* and *Pilularia*, relative to *Marsilea* and the earliest fossil forms of this genus (Hermsen *et al.*, 2014), have historically been attributed, based on adult morphology, to a progressive loss of leaflets in the *Regnellidium* and *Pilularia* clade, with the stipe being repurposed for photosynthesis in *Pilularia* (Johnson, 1898; Eames, 1936). More recently, Pryer and Hearn (2009) argued that the reductive evolution of leaf form in this family reflects evolution towards heterochrony, specifically neoteny, in sporophyte development. According to this theory, *Regnellidium* and *Pilularia* evolved accelerated and abbreviated developmental programs in response to selection for early and rapid reproduction. The heterochrony hypothesis is supported by the macromorphological trajectory of sporophyte development: juvenile *Marsilea* plants first produce strap-like leaves, then leaves with two leaflets, before finally producing the adult quadrifoliate, palmate leaves. One goal of this paper is to apply micromorphological and physiological observations to test these two hypotheses for leaf evolution in the Marsileaceae: (i) the loss of leaflet and repurpose of stipe for photosynthesis (Johnson, 1898; Eames, 1936) or (ii) developmental heterochrony (Pryer and Hearn, 2009).

From a physiological perspective, the Marsileaceae are notable for the unusually high rates of gas exchange reported in some members. Unlike other homoiohydric ferns, species of *Marsilea*, the best-studied genus of the family, achieve maximum photosynthetic rates and a stomatal conductance rivaling those of highly productive angiosperms (Wu and Kao, 2011; Nadal, Flexas, and Gulías, 2018; Deans *et al.*, 2019; Westbrook and McAdam, 2020a). These high rates of gas exchange likely reflect multiple physiological adaptations linked to evolution in water: aquatic environments offer a unique opportunity for physiological evolution because they effectively remove hydraulic limitations on carbon assimilation (Raven, 1995). The physiological capabilities of *Regnellidium* and *Pilularia* species have received less attention given the limited diversity and distribution of these genera (Pryer, 1999).

Here we investigate anatomical and physiological evolution in Marsileaceae. We compare microanatomical traits of the epidermis of the leaves and stipes of a representative species from each of the three genera of this family. We also compare the magnitude and environmental responsiveness of gas exchange rates in leaves and stipes.

Materials and Methods

We selected three species as representatives of the three genera of Marsileaceae: *Marsilea minuta* R. Br., *Regnellidium diphyllum* Lindm., and *Pilularia globulifera* L. All plants were grown under controlled conditions at 23°C day/18°C night under a natural photoperiod in the glasshouses of Purdue University. All plants were grown in similarly sized containers in the same depth of standing water (~10 cm) above Indiana Miami topsoil (a fine-loamy, mixed, active, mesic Oxyaquic Hapludalf), watered daily, and given monthly applications of complete liquid fertilizer (Jack's Classic Petunia feed, N:P:K 20:6:22, applied as a solution of 50 ppm N according to label (JR Peters Inc. Allentown, PA)). All material for anatomical and physiological assays was harvested from leaves that had emerged above the water level.

For anatomical measurements, we cleared tissue segments (~1 cm^2) in an aqueous 3.75% NaOCl solution, stained for 5 minutes with aqueous dilute solutions of safranin or toluidine blue solution, and rinsed with water. Images were captured on a light microscope (ZEISS Axio Imager 2 with Axiocam 506 camera controlled with Zen 2.3) using a LD Plan-NEOFLUAR 20x objective. ImageJ (version 1.5.2) was used to measure stomatal density on both surfaces of leaf blades (n=17 leaves; 0.09 mm^2 field of view). To create traced images of leaf and stipe epidermal anatomy, we took images with a 0.31 mm^2 field of view at 20x magnification and traced cells in ACD Canvas (v9.0.3).

A LI-6800 Portable Photosynthesis System (Li-Cor, Lincoln, NE) was used for gas exchange measurements. Conditions in the cuvette were maintained for the duration of the experiment with air temperature set at 22°C, CO_2 concentration at 400 μmol mol^{-1}, flow rate at 500 μmol s^{-1}, and fan speed at 10,000 r.p.m. The light environment included 90% red light with a maximum of 40 μmol m^{-2} s^{-1} blue light. To test for responses to changes in light intensity, leaves were exposed to step changes between 0 μmol m^{-2} s^{-1} and either 1000 μmol m^{-2} s^{-1} or 1500 μmol m^{-2} s^{-1}, for between 30 and 130 minutes depending on how long it took stomata to reach a new steady-state. In all trials, the vapor pressure deficit between the leaf and the atmosphere (VPD) was set and regulated by the LI-6800. VPD was set to 1.2 kPa in light response trials and underwent step changes in VPD response trials. All data were automatically logged every 30 seconds and gas exchange data were corrected for leaf or stipe area in the cuvette. Area in the cuvette was quantified from images of the leaf or stipe in the chamber using ImageJ. Transitional spikes and VPD equilibration periods were removed from gas exchange traces for clarity.

Results

We first investigated the epidermal anatomies of the lamina and stipes in *Marsilea* and *Regnellidium* and compared them to the epidermal anatomy of *Pilularia* leaves. We found that the elongated, rectangular epidermal cells of *Pilularia* leaves were nearly indistinguishable from those observed on the stipes of *Marsilea* and *Regnellidium* (Fig. 1). In contrast, the lamina epidermal pavement cells of *Marsilea* and *Regnellidium* were characteristically wavy in outline (Fig. 1). Total stomatal densities (Fig. 2A) were very high in the amphistomatic lamina of *Marsilea* (311 stomata mm^{-2}) and *Regnellidium* (431 stomata mm^{-2}). The lamina of *Marsilea* and *Regnellidium* were not evenly amphistomatic, with the majority of stomata often found on the adaxial surface (Fig. 2A). Relative to *Marsilea* and *Regnellidium* lamina, *Pilularia* leaves had a gradient of lower stomatal densities, ranging from 22 stomata mm^{-2} at the base of the leaf (lower 25% of the leaf) to 115 stomata mm^{-2} on the top of the leaf (the 25% of the leaf closest to the tip) (Fig. 2A). Stomatal densities in *Marsilea* stipes were low (40 stomata mm^{-2}) without a gradient along the length of the stipe. In *Regnellidium* stipes, stomatal densities were too low to quantify: we never observed more than two stomata in a field of view (Fig. 1; Fig. 2A). *Regnellidium* stipe stomata were primarily found in the upper 25% of the stipe.

This pattern in stomatal density correlated with steady-state rates of atmospheric gas exchange under high light across leaves and stipes of all species (Fig. 2B–2C). The lamina of *Marsilea* and *Regnellidium* had the highest photosynthetic rates (10.79 μmol m^{-2} s^{-1} and 13.17 μmol m^{-2} s^{-1}, respectively) and stomatal conductances (0.244 mol m^{-2} s^{-1} and 0.222 mol m^{-2} s^{-1}, respectively). In contrast, *Pilularia* leaves achieved modest rates of assimilation, 3.17 μmol m^{-2} s^{-1}, and stomatal conductance, 0.057 mol m^{-2} s^{-1}. *Marsilea* stipes recorded comparable rates of gas exchange as *Pilularia* leaves, with a mean assimilation rate of 3.55 μmol m^{-2} s^{-1} and stomatal conductance of 0.063 mol m^{-2} s^{-1}. We found that that stipes of *Regnellidium* did not photosynthesize (-0.76 μmol m^{-2} s^{-1}), although they did lose water at a measurable conductance of 0.065 mol m^{-2} s^{-1}. Taken together, these data indicate a strong correlation between stomatal density and maximum rates of photosynthesis across organs and species in Marsileaceae.

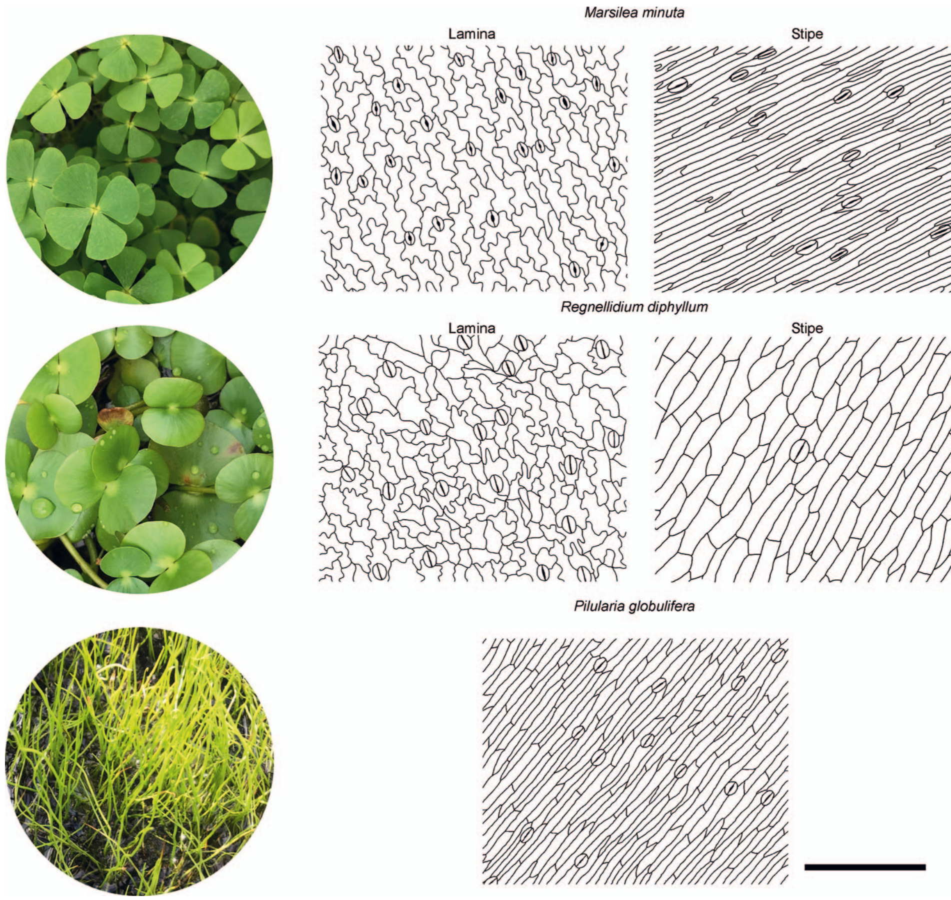

FIG. 1. Images of representative Marsileaceae species from each of the three genera. Drawings of the epidermal anatomy of lamina and stipes of representative species at x20 magnification (250 μm scale bar applies to all cellular-scale tracings).

We next tested stomatal responsiveness to changes in light environments. There was no appreciable difference between light and dark stomatal conductances for *Regnellidium* stipes, which were essentially non-photosynthetic and bore very few stomata (Fig. 2B; Fig. 2C). We found that stomata closed in response to darkness in the lamina and stipes of *Marsilea* and the lamina of *Regnellidium* (Fig. 2C). In contrast, stomata of *Pilularia* leaves remained open in darkness (Fig. 2C). We confirmed this finding by tracking photosynthetic rates and stomatal conductance during transitions from light to darkness in all tissue types. Stomata in lamina of *Marsilea* and *Regnellidium* closed rapidly as photosynthesis ceased in response to darkness; however, stomata of *Marsilea* responded more quickly (Fig. 3). In *Pilularia* leaves, photosynthesis ceased in darkness but stomata did not close (Fig. 4A). In contrast the stomata of *Marsilea* stipes, which had similar initial rates of leaf

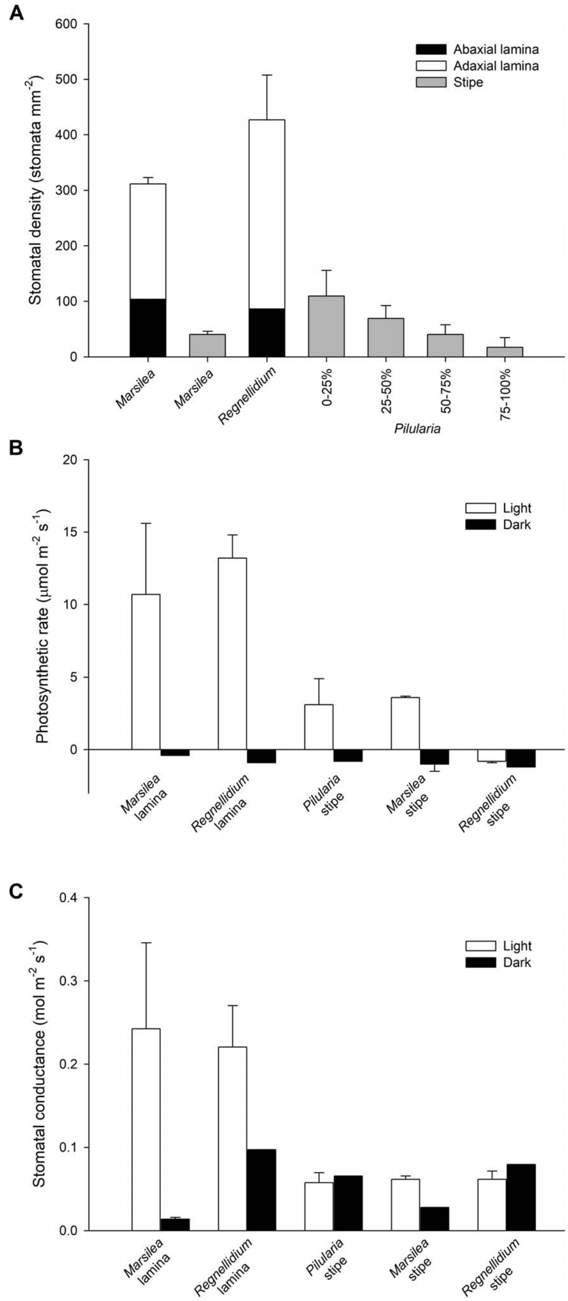

FIG. 2. Mean stomatal density and rates of gas exchange across species of Marsileaceae. (A) Mean stomatal density of *Marsilea minuta* lamina and stipe, *Regnellidium diphyllum* lamina (the extremely low stipe density is not shown), and *Pilularia globulifera* leaves sampled at several locations from the tip (0%) to base (100%). SD given where available. Measurements from adaxial surfaces, abaxial surfaces, and filiform leaves or stipes are shown in white, black, and grey, respectively. (B) Mean photosynthetic rate and (C) stomatal conductance in lamina and stipes of Marsileaceae species under 1500 μmol m^{-2} s^{-1} of light (white) or darkness (black), with SD where available.

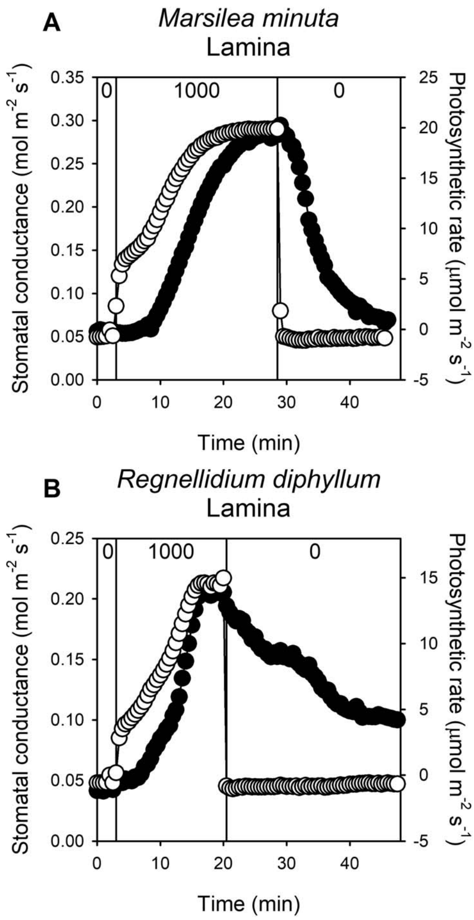

FIG. 3. The representative response of stomatal conductance (black) and photosynthetic rate (white) to step changes in light intensity (denoted by vertical lines and values) in a representative lamina of (A) *Marsilea minuta* and (B) *Regnellidium diphyllum* (one trace is shown from three replicates). Light intensity transitions were 0–1000–0 μmol m^{-2} s^{-1}.

gas exchange, closed in response to darkness (Fig. 4B). Our results indicate that stomatal closure in response to darkness occurs in *Marsilea* and *Regnellidium* lamina, and *Marsilea* stipes, but this response has been lost in *Pilularia*.

Finally, we tested whether the stomata of *Pilularia* were responsive to changes in leaf water status by imposing step changes in vapor pressure difference between the leaf and atmosphere (VPD). We found that the stomata of *Pilularia* leaves, like the stomata of *Marsilea* stipes, were responsive to changes in VPD (Fig. 5). Stomata closed on exposure to high VPD and opened without any hysteresis when re-exposed to low VPD (Fig. 5).

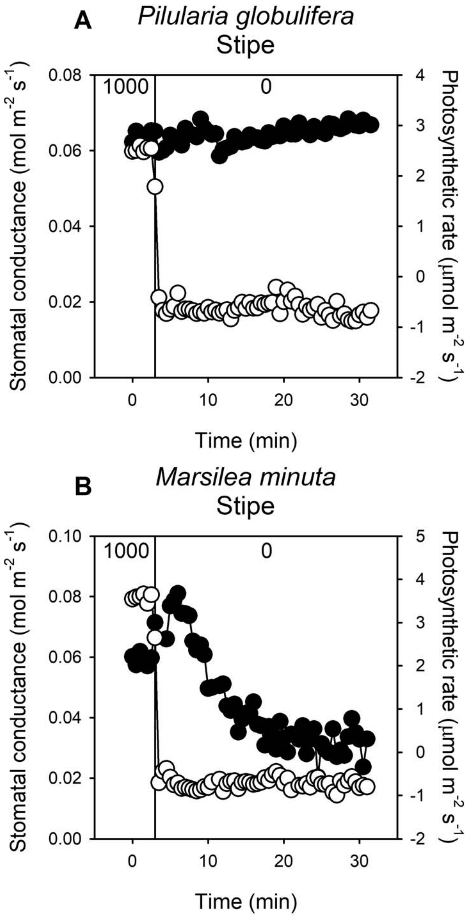

Fig. 4. The response of stomatal conductance (black) and photosynthetic rate (white) to a decrease in light intensity (marked by vertical line) in (A) a representative stipe of *Pilularia globulifera* and (B) stipe of *Marsilea minuta* (one trace is shown from three replicates). Light intensity was lowered from 1000–0 μmol m^{-2} s^{-1}.

Discussion

Reduction, not heterochrony, drives leaf evolution in Marsileaceae.—Our analysis of epidermal cell shape across aerial grown *Pilularia* leaves revealed a striking correspondence with the stipes of *Marsilea* and *Regnellidium*. The apparent homology between the elongated, rectangular cells of these three tissues strongly supports the classic argument that *Pilularia* leaves are stipes without leaflets (Johnson, 1898) and not a larger juvenile leaf retained into reproductive maturity (Pryer and Hearn, 2009). Consequently, we now refer to *Pilularia* leaves as stipes. Although there exists interspecific variation in *Marsilea* in lamina epidermal cell shape, the shape we report here in *Marsilea minuta* has strong congruency with the anatomy reported in a broad diversity

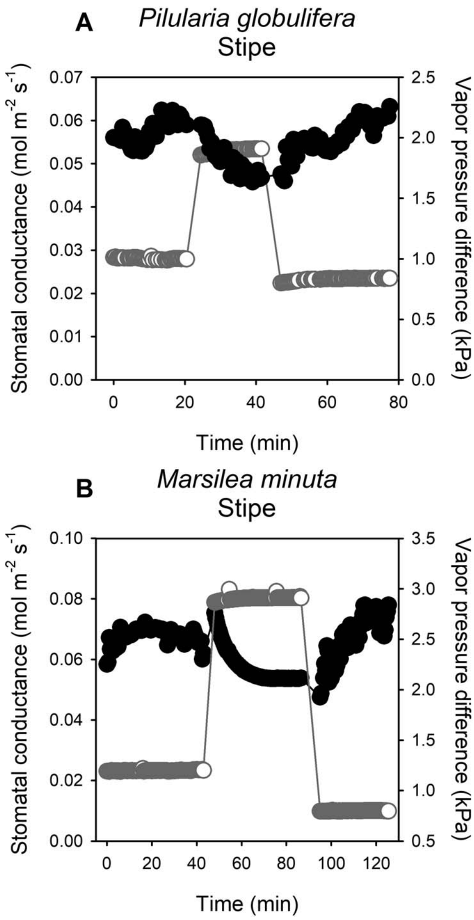

Fig. 5. The response of stomatal conductance (black) to step transitions in vapor pressure difference (gray, VPD) in (A) a representative stipe of *Pilularia globulifera* and (B) stipe of *Marsilea minuta* (one trace is shown from three replicates). VPD equilibration periods have been deleted (gray lines).

of species from this genus (Mickel and Votava, 1971). The elongated epidermal cells of stipes may reflect a shared developmental ontogeny, that being apical, rather than lateral cell division.

We integrated novel and established data on stomatal densities across Marsileaceae and found that mean stomatal densities fell into three categories. *Marsilea* and *Regnellidium* lamina have high stomatal densities, particularly on the adaxial surface (Fig. 2). Adaxial stomata, which allow for atmospheric gas exchange in leaves floating on the surface of water, are common in aquatic angiosperms (Kaul, 1976). Interspecific and intraspecific variation in the presence and density of abaxial stomata in *Marsilea* has been linked to water availability (Mickel and Votava, 1971). Our stomatal density counts in

Marsilea minuta are similar to those reported across the genus *Marsilea* (Mickel and Votava, 1971). *Pilularia* and *Marsilea* stipes form a second category with much lower stomatal densities (Fig. 2). Finally, *Regnellidium* stipes have only occasional stomata (Johnson and Chrysler, 1938). This pattern was mirrored by the relative rates of atmospheric gas exchange in these tissues under high light conditions (Fig. 2). We measured high rates of gas exchange in *Marsilea* and *Regnellidium* lamina and much lower rates in *Pilularia* and *Marsilea* stipes. *Regnellidium* stipes did not photosynthesize at measurable rates. The correlation between stomatal density and atmospheric gas exchange not only reinforces the concept of morphological homology between *Marsilea* and *Pilularia* stipes but also suggests that stomatal density may serve as a key limitation to atmospheric gas exchange in this group. The diversity in stomatal numbers across lamina and stipe in this family may provide an exciting future model system in which to investigate the potential genetic regulators of stomatal development in ferns.

Aquatic specialization in Pilularia.—The Cretaceous ecological radiation and dominance of angiosperms has been attributed, in part, to evolutionary innovations related to leaf hydraulics to facilitate high rates of photosynthesis (Brodribb and Feild, 2010), but as evidenced by the evolutionary history of other fern lineages for which diversification has been attributed to an epiphytic growth habit (Schuettpelz and Pryer, 2009), not to mention non-fern groups (Bond, 1989), not all evolutionarily successful strategies involve high rates of gas exchange. In the case of *Pilularia*, the loss of highly photosynthetic lamina may have supported other traits of adaptive value, particularly in an aquatic environment. Most clearly, cylindrical stipes which have a high surface area to volume ratios (Chambers *et al.*, 2008; Givnish *et al.*, 2018). This trait is highly advantageous for underwater photosynthesis because it partially offsets the considerably slower diffusion of CO_2 in water (approximately 10,000x slower) than in air (Nobel, 1999). Pryer and Hearn (2009) suggested a second advantage of this reduced leaf form in *Pilularia*: that it facilitates early and efficient reproduction. Although this reproductive hypothesis does not necessarily require heterochrony (which is not supported by our anatomical data) as the proximate developmental explanation, future investigations are needed to test whether intergeneric changes in life cycle speed occur across Marsileaceae

Environmental responsiveness and diversity in Marsileaceae.—Our results highlight the importance of physiological flexibility as an adaptive trait. *Marsilea* is extremely responsive both morphologically and physiologically to environmental signals on both short and long timescales, whereas *Regnellidium* and *Pilularia* are less responsive. This pattern holds true for well-characterized traits, such as leaf form plasticity (Johnson, 1986) and nyctinasty (Gomez, 1981), and also helps contextualize the anatomical data reported here. For instance, *Marsilea* lamina stomata closed rapidly in response to darkness, while *Regnellidium* lamina stomata closed at slower rates, more reminiscent of other ferns (McAdam and Brodribb, 2012). Rapid stomatal responses to light transitions better promote water use efficiency (McAusland *et al.*, 2016), an essential trait for most land plants. We also found that the stomata on *Pilularia* stipes respond to VPD but not to light. The VPD response probably reflects simple hydropassive mechanics: ancestral stomata in vascular plants functioned as simple passive valves in response to changes in leaf water status (Brodribb and McAdam, 2011). This function persists even in highly modified fern stomata, such as those of the exclusively aquatic *Azolla*, which is in the same order as the Marsileaceae (Busby and Gunning, 1984). The loss of a stomatal response to light in *Pilularia* is one of only a few cases in which such a dramatic change to core vascular land plant stomatal function has been documented; a change reminiscent of the absence of active stomatal responses in bryophytes (Kubásek *et al.,* 2021). Our work suggests that stomatal function in this fern family has undergone considerable adaptive radiation, making Marsileaceae unique amongst land plants and emphasizing the potential importance of stomatal evolution in influencing land plant ecology and evolution.

Despite being non-photosynthetic, the stipe of *Regnellidium* was found to lose water at a considerable rate. This finding suggests that the cuticle on the typically submerged stipe of *Regnellidium* is highly permeable to water. It is possible that all Marsileaceae cuticle permits significant evaporation, as is suggested by the high rates of water loss from leaves in the dark. This effect has been observed before: the cuticle, a critical adaptation preventing dehydration in land plants, may be reduced, maintained, or co-opted for other functions in lineages that have secondarily returned to an aquatic ecology (Riederer and Muller, 2008). At least in *Marsilea*, cuticular thickness is a plastic trait associated with the heterophyllous switch (Gaudet, 1964). Future work should investigate cuticular permeance and chemistry across the blades and stipes of Marsileaceae.

The two paths for evolution in water.—Our dataset is consistent with a model in which the earliest Marsileaceae had highly photosynthetic lamina and moderately photosynthetic stipes, with the latter function being lost in *Regnellidium* but retained in *Pilularia* which instead has lost lamina. This model demonstrates that Marsileaceae evolution has, in some groups, involved the development and progressive loss of adaptations for facilitating and regulating high rates of atmospheric gas exchange. The adaptations found in *Marsilea*, such as vessels (Schneider and Carlquist, 2000), high stomatal densities (Mickel and Votava, 1971), and sensitive stomatal control mechanisms (current study), are more characteristic of angiosperms than other ferns (Westbrook and McAdam 2020a; Westbrook and McAdam 2020b). In fact, the remarkable resemblance between *Marsilea* and some of the (unassigned) earliest-diverging angiosperms (Hermsen *et al.*, 2014; Hermsen, 2019) suggests extensive convergence.

Given the convergence between angiosperms and some Marsileaceae, angiosperms may provide models of possible evolutionary trajectories for this family. Some of the earliest angiosperms may have emerged from aquatic environments, which served as a cradle for evolutionary novelties by relaxing constraints on hydraulic architecture and water use efficiency (Coiffard, Gomez, and Thevenard, 2007; Soltis *et al.*, 2018).

The monocots, a cosmopolitan and extremely successful group with aquatic origins, provide an excellent example of a group of angiosperms that have exploited adaptations that evolved in an aquatic environment in terrestrial settings (Les, 1995; Givnish *et al.*, 2018). In contrast, other groups of aquatic angiosperms have remained restricted to aquatic environments and have failed to undergo large adaptive radiations. For instance, the *Ceratophyllaceae* has a long fossil record and is closely related to the monocots (Doyle and Endress, 2018), but has a suite of reduced morphologies and has never invaded terrestrial environments during their long evolutionary history (Gomez *et al.*, 2015). We propose that the Marsileaceae followed a similar trajectory, with some members undergoing increasing morphological reduction and ecological restriction.

One method of investigating the restrictions on the Marsileaceae family involves comparing the evolutionary histories of *Marsilea* with that of the genera *Regnellidium* and *Pilularia. Marsilea* has radiated most successfully in slightly drier environments (Nagalingum *et al.*, 2007), adopting semi-aquatic lifestyles and undergoing habitat-dependent selection for morphological traits (Wu and Kao, 2011). In contrast, *Regnellidium* and *Pilularia* have diversified very little (Pryer, 1999) across long periods of evolutionary time (Hermsen, 2019). We argue that the physiological factors reported here, particularly the limited movement of lamina in *Regnellidium* and reduced photosynthetic capacity through a loss of lamina in *Pilularia*, could explain this difference. Other factors may also be involved, such as the large amount of aerenchyma in the *Regnellidium* and *Pilularia* stipe (Johnson and Chrysler, 1938; Johnson, 1898), which would hamper emergent growth, and suggests increased adaptation to an aquatic environment compared to *Marsilea*.

Another method of investigating the restrictions on the Marsileaceae family involves comparisons to other aquatic ferns. With scattered exceptions, most aquatic ferns belong to the Salviniaceae, a group of limited diversity that represents the only other family in the order Salviniales (Mehltreter *et al.*, 2010). *Azolla*, a fully aquatic member of this group, deserves special mention for achieving ecological dominance in the Arctic Ocean during the Middle Eocene (Speelman *et al.*, 2009). *Azolla* has the highest growth rates of modern ferns (Speelman *et al.*, 2009). Like the derived Marsileaceae examined here, *Azolla* evolved a physiological profile unconstrained by selection for water conservation and became highly adapted to a narrow ecological niche. This strategy proved very successful in an ocean with relatively fresh and warm surface waters (Brinkhuis *et al.*, 2006). This environment no longer exists, and modern *Azolla* — much like *Regnellidium* and *Pilularia* — is now limited in its distribution.

Why doesn't Marsilea *outcompete angiosperms?*—What determines whether an aquatic lineage invades terrestrial landscapes or remains restricted to water? From a physiological perspective, aquatic environments are evolutionarily permissive and likely to allow novel adaptations, such as high vein and stomatal densities or vessels, that facilitate high rates of atmospheric gas exchange (Soltis *et al.*, 2018). While all of these traits provide fitness benefits on land if water loss can be controlled, they cannot be the only prerequisites for the move out of water; otherwise, the sophisticated hydraulic architecture (Mickel and Votava, 1971; Schneider and Carlquist, 2000) and high rates of leaf gas exchange (Deans *et al.*, 2019; Westbrook and McAdam, 2020a; Westbrook and McAdam, 2020b) of *Marsilea* would have established this genus in fully terrestrial habitats. We propose that the absence of metabolic stomatal responses to changes in water availability, which would limit excessive water loss during periods of water deficit, may explain why *Marsilea* is restricted to an amphibious habit (Westbrook and McAdam, 2020b).

In seed plants, water stress triggers the synthesis of abscisic acid (ABA), which closes stomata and activates other responses facilitating drought tolerance and survival in dry environments (Walton, 1980). Ferns, like lycophytes and bryophytes, do not exhibit ABA-mediated stomatal closure in response to water stress (Brodribb and McAdam, 2011; Westbrook and McAdam, 2020b; Kubásek *et al.,* 2021). Fern stomata instead react to hydraulic changes through exclusively hydropassive mechanisms, which are generally less efficient than ABA-based mechanisms (McAdam and Brodribb, 2013). Consequently, high rates of atmospheric gas exchange come at the cost of rapid water loss during drought. For *Marsilea*, a highly productive genus, this connection means that a crucial physiological advantage is necessarily accompanied by a major physiological drawback. This handicap may explain why, despite being one of the few homoiohydric fern groups to rival angiosperm photosynthetic rates, the Marsileaceae remain limited in their diversity and occupy a narrow range of ecological niches (Westbrook and McAdam, 2020b).

Conclusion.—Two distinct patterns emerge from the evolutionary history of the Marsileaceae. *Marsilea*, the genus with the most sophisticated physiological capabilities (Johnson, 1986), is the most diverse (Nagalingum *et al.*, 2007). In contrast, the genera *Regnellidium* and especially *Pilularia*, demonstrate a trend towards increasing morphological simplification and specialization for aquatic habitats (Johnson, 1898; Johnson and Chrysler, 1938). The resulting forms are unable to invade terrestrial environments without evolving mechanisms to increase water conservation, which would probably involve regaining their physiological flexibility and (in the case of *Pilularia*) photosynthetic capacity. Consequently, these fern genera are likely canalized on an evolutionary trajectory that erases physiological convergences with angiosperms seen in *Marsilea* (Westbrook and McAdam, 2020b). Furthermore, our work emphasizes the importance of aquatic species as cradles for evolution in stomatal function, which could be potentially useful systems in which to screen for novel variants in plant water use regulation.

Acknowledgements

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