American Journal of Botany

American Journal of Botany 99(1): 14–22. 2012.

The cuticle on the gametophyte calyptra matures before the sporophyte cuticle in the moss *Funaria hygrometrica* (Funariaceae)[1]

Jessica M. Budke[2], Bernard Goffinet, and Cynthia S. Jones

Department of Ecology and Evolutionary Biology, University of Connecticut, 75 North Eagleville Road, Unit 3043, Storrs, Connecticut 06269-3043 USA

- *Premise of the study:* In vascular plants, leaf primordia prevent desiccation of the shoot apical meristem. Lacking leaves, the undifferentiated moss sporophyte apex is covered by the calyptra, a cap of maternal gametophyte tissue that is hypothesized to function in desiccation protection. Herein, we compare cuticle development on the calyptra and sporophyte to assess the calyptra's potential to protect the sporophyte from desiccation. As the first comprehensive study of moss sporophyte cuticle development, this research broadens our perspectives on cuticle development and evolution across embryophytes.
- *Methods:* Calyptrae and sporophytes at nine developmental stages were collected from a laboratory-grown population of the moss *Funaria hygrometrica*. Tissues were embedded, sectioned, then examined using transmission electron microscopy. Epidermal cells were measured for thickness of the cuticle layers, cell wall thickness, and lumen size.
- *Key results:* The calyptra cuticle develops precociously and reaches maturity before the sporophyte cuticle. Calyptrae are covered by a four-layered cuticle at all stages, whereas sporophyte cuticle maturation is delayed until sporangium formation. The development and thickening of the sporophyte cuticle occurs in an acropetal wave.
- *Conclusions:* A multilayered calyptra cuticle at the earliest developmental stages is consistent with its ability to protect the immature sporophyte from desiccation. Young sporophytes lack a complex cuticle and thus may require protection, whereas in older sporophytes a mature cuticle develops. The moss calyptra is not a vestigial structure, but rather the calyptra's role in preventing desiccation offers a functional explanation for calyptra retention during the 450 Myr of moss evolution.

Key words: bryophyte; calyptra; cuticle; desiccation prevention; development; electron microscopy; *Funaria hygrometrica*; Funariaceae; sporophyte.

The evolution and elaboration of the plant cuticle was critical for the colonization of land by embryophytes (Kenrick and Crane, 1997). Cuticles protect against an environmental onslaught that includes UV radiation, precipitation, herbivores, and desiccation (Kerstiens, 1996). Meristematic regions, however, are covered by a thin procuticle (Heide-Jørgensen, 1991) that may confer only minor protection for these undifferentiated regions. In vascular plants, whorls of tightly appressed leaf primordia prevent desiccation of the shoot apical meristem. Bryophyte sporophytes, in contrast, lack protective leaf primordia. In mosses, the undifferentiated apex of the sporophyte is covered by the calyptra, a cap of maternal gametophyte tissue that develops from cells of the archegonium and leafy gametophyte below. At maturity, the calyptra is covered by a cuticle that is significantly thicker than the sporophyte cuticle (Budke et al., 2011), which supports the hypothesized role of the calyptra in preventing desiccation of the sporophyte. The next step toward our understanding of moss calyptra function is to determine whether the cuticle on the calyptra has the potential to protect the sporophyte from desiccation in early development, during which the sporophyte apex may be more vulnerable. Herein, we compare cuticle development on the calyptra and sporophyte across a series of developmental stages in the moss *Funaria hygrometrica* Hedw. (Funariaceae) and thus broaden our perspectives on cuticle development and evolution across embryophytes.

Bryophyte gametophytes and sporophytes are covered by a cuticle (e.g., Neinhuis and Jetter, 1995; Pressel et al., 2010) and epicuticular waxes (e.g., Proctor, 1979; Heinrichs et al., 2000; Pressel et al., 2011). Observations of cuticle development in bryophytes are limited to mosses and encompass both morphological changes to the exterior waxes (Koch et al., 2009) and anatomical changes in the cuticle layers of stomata and protonema (Sack and Paolillo, 1983; Cook and Graham, 1998). Moss cuticles transition developmentally from single to multilayered, with the outermost cuticle proper rarely composed of lamellae (Sack and Paolillo, 1983; Cook and Graham, 1998). Despite some similarities in ontogeny, mature moss cuticles are not known to attain the thickness and complexity of layering observed in vascular plants (Jeffree, 2006).

In mosses, with the exception of *Sphagnum*, an apical cell forms the initial sporophyte embryo, including an undifferentiated apical region that will later develop into the sporangium (Goebel, 1905). When the sporophyte is merely a few millimeters tall, the apical cell ceases dividing; at the same time an intercalary meristem, located just below the apical region, is initiated

[1]Manuscript received 30 June 2011; revision accepted 2 December 2011.

The authors thank L. Lewis and two anonymous reviewers for helpful comments on earlier versions of the manuscript. This research was supported by grants from the National Science Foundation (DEB-0919284 to B.G. and C.S.J.), H. N. Andrews Endowment Fund to the Connecticut Museum of Natural History, International Association of Bryologists, Microscopical Society of America, and Botanical Society of America. Assistance from the faculty and staff of the University of Connecticut Electron Microscopy Facility is appreciated. They also thank J. Machnicki and V. Kask for help with figure illustrations. This research represents a portion of the doctoral dissertation of J.M.B. at the University of Connecticut.

[2]Author for correspondence (e-mail: jessica.budke@uconn.edu)

doi:10.3732/ajb.1100311

Fig. 1. Sporophyte development of *Funaria hygrometrica* topped by calyptrae. Black arrows indicate the apex of the sporophyte beneath the calyptra. White arrows indicate the bottom edge of the calyptrae. (A) Stage 1, calyptra formation with sporophyte as a small embryo inside. (B) Stage 2, calyptra with inflated base formed and attached to leafy gametophyte. (C) Stage 3, calyptra detached from leafy gametophyte, sporophyte early spear-shaped <15 mm tall. (D–I) Inset, sporophyte apices. (D) Stage 4, sporophyte late spear-shaped >20 mm tall. (E) Stage 5, sporophyte capsule expanding. (F) Stage 6, sporophyte capsule filling the calyptra's inflated base. (G) Stage 7, calyptra on top half of capsule, sporophyte annulus visible and green. (H) Stage 8, calyptra on top third of capsule, sporophyte annulus pigmented pink. (I) Stage 9, calyptra on very apex of capsule or absent, sporophyte peristome teeth visible with annulus pigmented red. Bars = 1 mm. *Abbreviations:* R, calyptra rostrum; IB, calyptra inflated base.

to form the seta (French and Paolillo, 1975a). Following seta development, near the end of sporophyte development, the dormant apical region differentiates into a spore-filled sporangium. Early in development, the gametophyte tissue surrounding the moss sporophyte detaches at the base to form the calyptra (Janzen, 1917). This cap of maternal gametophyte tissue covers the apex of the sporophyte throughout its development and is necessary for normal sporangial and spore formation (French and Paolillo, 1975b). Indeed, young sporophytes grown without calyptra survived only when kept at high humidity (Bopp, 1954; French and Paolillo, 1975b). In the absence of a calyptra, upward water movement within the sporophyte is accelerated (Bopp and Stehle, 1957), likely due to increased transpiration. This observation suggests that the calyptra reduces water loss and thus prevents desiccation of the undifferentiated apical region and actively dividing seta meristem of the immature sporophyte.

Consistent with the role of desiccation protection is the presence of a multilayered cuticle on the mature calyptra that is significantly thicker than that covering the leafy gametophyte or sporophyte capsule (Budke et al., 2011). Herein we address the following hypotheses: (1) If the calyptra is able to protect the sporophyte from desiccation during early development, we predict that the calyptra cuticle undergoes precocious development and reaches maturity early relative to sporophyte development. (2) If the sporophyte requires protection from desiccation during early development, we predict that the sporophyte cuticle will show delayed maturation until late sporangium formation. Here we document both the developmental maturation of the cuticle and the underlying epidermal cells relative to overall morphological development of the sporophyte to assess the potential for desiccation protection of the sporophyte by the calyptra in *Funaria hygrometrica*. This study is the first comprehensive analysis of moss sporophyte cuticle development.

MATERIALS AND METHODS

Study taxon—*Funaria hygrometrica* is a cosmopolitan moss species found in disturbed habitats (Miller and Miller, 2007). Choosing a taxon that is representative of all mosses is challenging due to the wide variation in both sporophyte and calyptra morphology across the approximately 12 500 moss species (Crosby et al., 1999) and even within the Funariaceae (Liu et al., in press). *Funaria hygrometrica* has a capsule that is operculate (i.e., lidded) with an arthrodontous peristome atop a seta that may reach 5 cm in length. Morphologically, the calyptra is divided into a narrow rostrum above a wider inflated base (Fig. 1D inset). These sporophyte and calyptra features are found in a wide array of moss taxa, with *F. hygrometrica* representing one of the more complex morphologies. Despite differences in mature morphology, the vast majority of moss sporophytes transition from a small spear-shaped sporophyte that is completely

TABLE 1. Calyptra and sporophyte developmental stages of *Funaria hygrometrica* prior to capsule dehiscence. Images of these stages are presented in Fig. 1.

Stage	Calyptra	Sporophyte
1	Formation, epigonium	Small embryo
2	Formed and attached to leafy gametophyte	Small embryo
3	Detached from gametophyte, covering apex	Early spear-shaped <15 mm tall
4	Detached from gametophyte, covering apex	Late spear-shaped >20 mm tall
5	Detached from gametophyte, covering apex	Capsule expanding
6	Detached from gametophyte, covering apex	Capsule filling calyptra's inflated base
7	Covering top 1/2 of capsule	Annulus visible and green
8	Covering top 1/3 of capsule	Annulus pink, meiosis occurs (Garner and Paolillo, 1973)
9	Covering the very apex or absent	Peristome teeth visible, annulus red

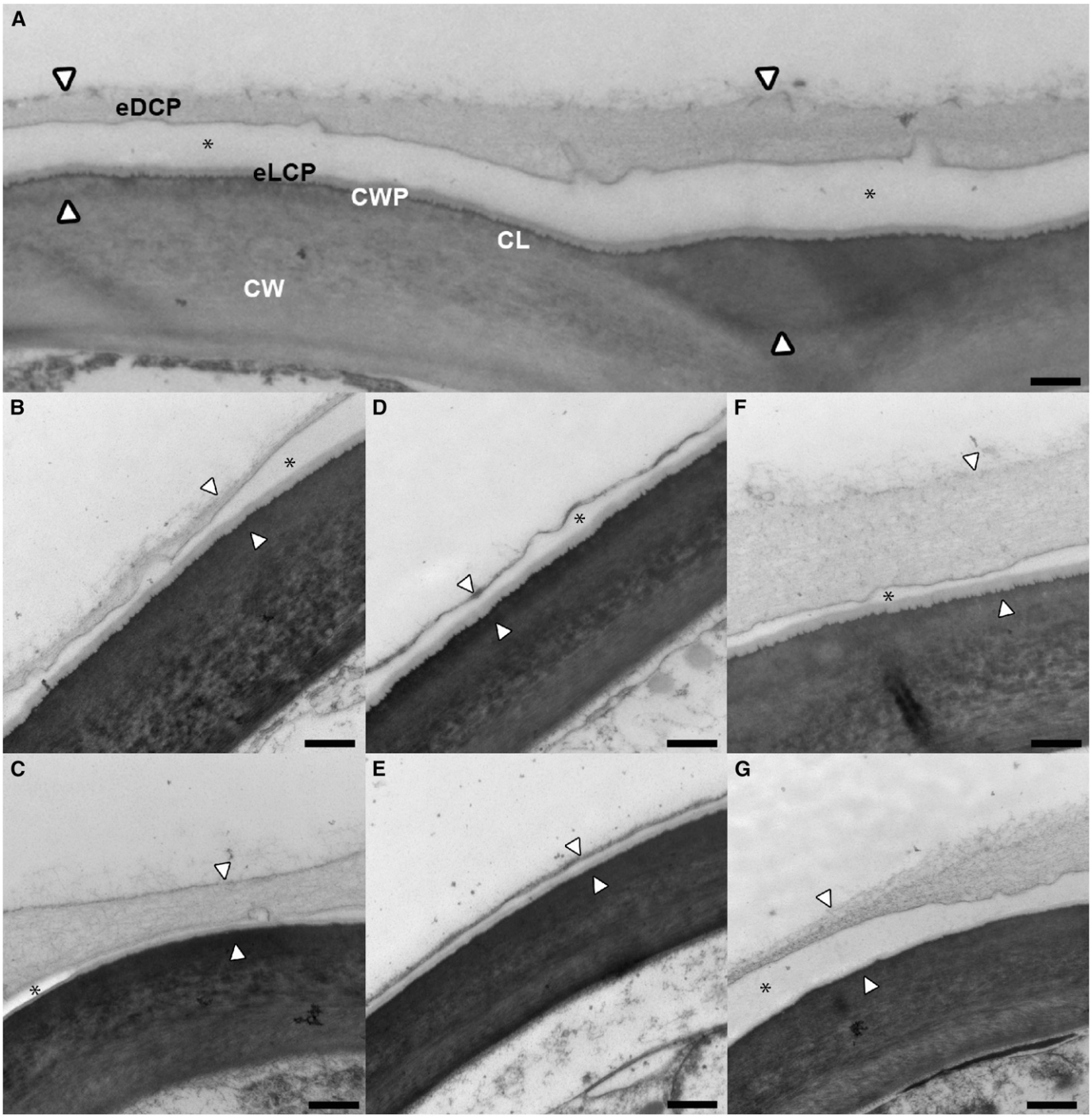

Fig. 2. Transmission electron micrographs of transverse sections through *Funaria hygrometrica* calyptrae. Inner and outer edges of cuticle are indicated by arrowheads; the asterisk (*) indicates artifactual separation of the layers. All developmental stages of the calyptra have a cuticle that includes a cuticular layer, cell wall projections of the cuticular layer, electron-lucent cuticle proper, and electron-dense cuticle proper. (A) Developmental stage 1 at the middle of the expanding calyptra, showing both the periclinal and anticlinal cell walls with a cuticular peg located at the anticlinal wall. (B–G) Periclinal cell walls. (B) Stage 3 rostrum. (C) Stage 3 inflated base. (D) Stage 6 rostrum. (E) Stage 6 inflated base. (F) Stage 9 rostrum. (G) Stage 9 inflated base. Bars = 500 nm. *Abbreviations:* CL, cuticular layer; CW, cell wall; CWP, cell wall projections of the cuticular layer; eDCP, electron-dense cuticle proper; eLCP, electron-lucent cuticle proper.

surrounded by the epigonium (i.e., tissue that upon detachment from the remainder of the maternal gametophyte is termed the calyptra) to a taller sporophyte covered apically by the calyptra, to finally a sporophyte with an expanded capsule at the apex, as in *F. hygrometrica* (Fig. 1).

All sporophytes and calyptrae used in this study were from a laboratory grown population of the moss *Funaria hygrometrica* (CONN Budke #145), originally collected in Connecticut. Leafy gametophytes were grown from spores on soil in PlantCon tissue culture containers (MP Biomedicals, Solon,

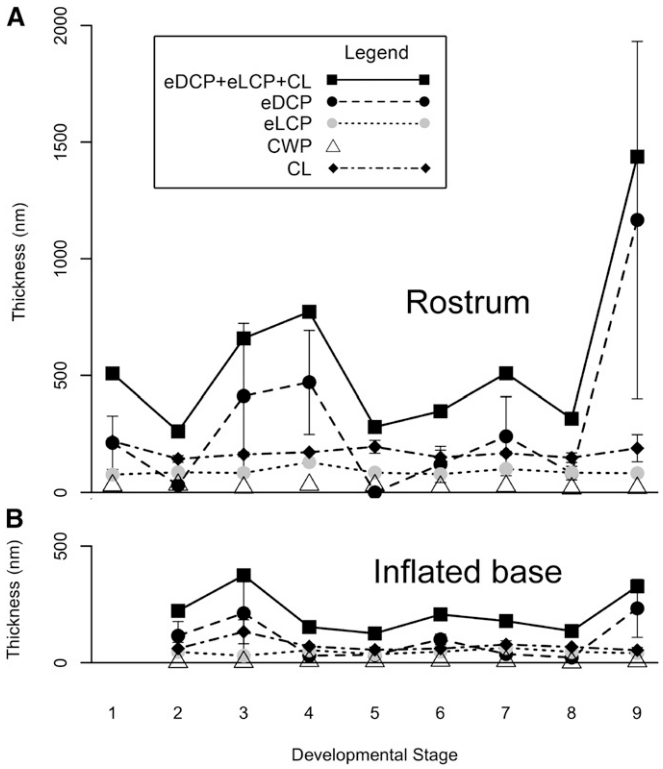

Fig. 3. Thickness of cuticle layers measured from transmission electron micrographs of transverse sections through *Funaria hygrometrica* calyptrae. Mean values are from three cells per individual, one individual per stage, with ±1 standard error bars. (A) Rostrum. (B) Inflated base. *Abbreviations:* CL, cuticular layer (black diamond); CWP, cell wall projections of the cuticular layer (open triangle); eDCP, electron-dense cuticle proper (black circle); eLCP, electron-lucent cuticle proper (gray circle); sum of cuticle layers with the cell wall projections included in the cuticular layer (black square).

Ohio, USA), cold treated to stimulate gametangia production, and postfertilization returned to room temperature conditions to facilitate sporophyte development as detailed in Budke et al. (2011).

***Cuticle and epidermis anatomy*—**All tissues were fixed (1.5% glutaraldehyde, 1.5% formaldehyde in 0.05 mol/L PIPES buffer, pH 7.0), embedded in LR White resin (Pelco, Redding, California, USA), and sectioned as outlined in Budke et al. (2011). For each of the nine developmental stages (Table 1, Fig. 1), an individual sporophyte and its associated calyptra were transversely sectioned to 100 nm thick. At stage 1, both the calyptra and sporophyte were sectioned through the middle at a single location (Fig. 1A). At all other developmental stages, the calyptrae were sectioned at both the mid rostrum and mid inflated base regions (Fig. 1B). At stage 2, these sections also included the sporophyte. Sporophytes at stages 3 and 4 were sectioned through the apex, beneath the rostrum of the calyptra, and through the middle of the seta (Fig. 1C, D). Sporophytes at stages 5 to 9 were sectioned through the apex, middle, and neck (i.e., apophysis) of the developing or fully formed capsules (Fig. 1E–I). All grids were stained in aqueous solutions (w/v) of 1.5% potassium permanganate (5 min), 2% uranyl acetate (5 min), then 2.5% lead citrate (2 min). Sections were examined and photographed using a Tecnai Biotwin (FEI Electron Optics, Eindhoven, Netherlands) transmission electron microscope at 80 kV accelerating voltage.

Within each region, three epidermal cells equally spaced around the circumference were measured for thicknesses of the cuticle layers (cuticular layer, cell wall projections of the cuticular layer, electron-lucent cuticle proper, electron-dense cuticle proper), at the region of the periclinal wall, as defined in Budke et al. (2011). Also the cell wall thicknesses and lumen sizes for each of these cells were measured. All measurements were taken from digital images using the program ImageJ (http://rsb.info.nih.gov/ij/). Measurements from each of these sets of three cells were averaged to calculate a mean value for each region of each developmental stage. All data were analyzed using the program R 2.11.0 (R Development Core Team, 2010).

***Morphology*—**Nine sporophytes, with their associated calyptrae, were randomly selected, and the calyptrae lengths were measured once a week for 6 wk to determine whether they grow across development. The first measurement was made with 1.0 mm of the seta visible below the detached calyptra, immediately following developmental stage 2 (Table 1). At the first measurement, calyptrae ranged from 3.0–3.75 mm long.

RESULTS

The calyptra cuticle develops precociously, reaching maturity early relative to the sporophyte cuticle. Calyptrae are covered by a mature, multilayered cuticle at all nine developmental stages, whereas sporophyte cuticle maturation is delayed until late sporangium formation. Sporophyte cuticle development includes both the addition and thickening of cuticle layers, which occurs in an acropetal wave.

***Calyptrae*—**Following nine individual calyptrae during sporophyte development (Fig. 1C–I; Table 1, stages 3–9) reveals that their lengths do not increase (lengths not shown). Additionally, the cells of the rostrum and inflated base of the calyptra do not undergo an increase in either lumen size or a thickening of any of the epidermal cell walls (including outer periclinal, inner periclinal, anticlinal), and the number of cells in circumference remains constant (data not shown).

Four components of the cuticle, the cuticular layer, cell wall projections of the cuticular layer, electron-lucent cuticle proper, and electron-dense cuticle proper (CP), are present on both regions of the calyptrae at all developmental stages (Figs. 2, 3). The thickness of the cuticular layer, cell wall projections, and electron-lucent CP are relatively constant across development (Fig. 3). The electron-dense CP does not follow a similar pattern; the thickness of this layer is highly variable across development and within an individual, ranging from completely absent in stage 5 to a mean thickness of over 1 μm at stage 9 (Fig. 3). Within a single individual, the thickness of the electron-dense CP varied by two orders of magnitude, indicated by the larger standard error bars (Fig. 3A, stages 3, 9). Thickenings of the cuticular layer at the anticlinal cell walls (cuticular pegs) were present only on the calyptra rostrum and were observed on all nine developmental stages (Fig. 2A).

***Sporophytes*—**The sporophyte grows in height and changes shape from a spear to a stalk with an expanded capsule during development (Fig. 1). Concurrently, the epidermal cells change in both size and shape, as observed in transverse section. Cells of the sporophyte apex increase in both lumen width and depth, whereas cells of the mid capsule increase in lumen width and decrease in depth, thus undergoing a change in shape, but not in size (transverse lumen area), whereas cells of the apophysis are relatively similar across development (Fig. 4). The epidermal cell lumens of the seta do not change in size or shape (data not shown). The epidermal cell walls, most significantly the outer periclinal cell walls, thicken across development for all three regions of the sporophyte capsule (apex, mid capsule, apophysis; Fig. 4). During early sporophyte development (stages 1–4), the average number of epidermal cells in circumference is similar between the apex (N = 4, 61.3 cells) and seta (N = 3, 62.7 cells). Late in development (stages 5–9), the average number of

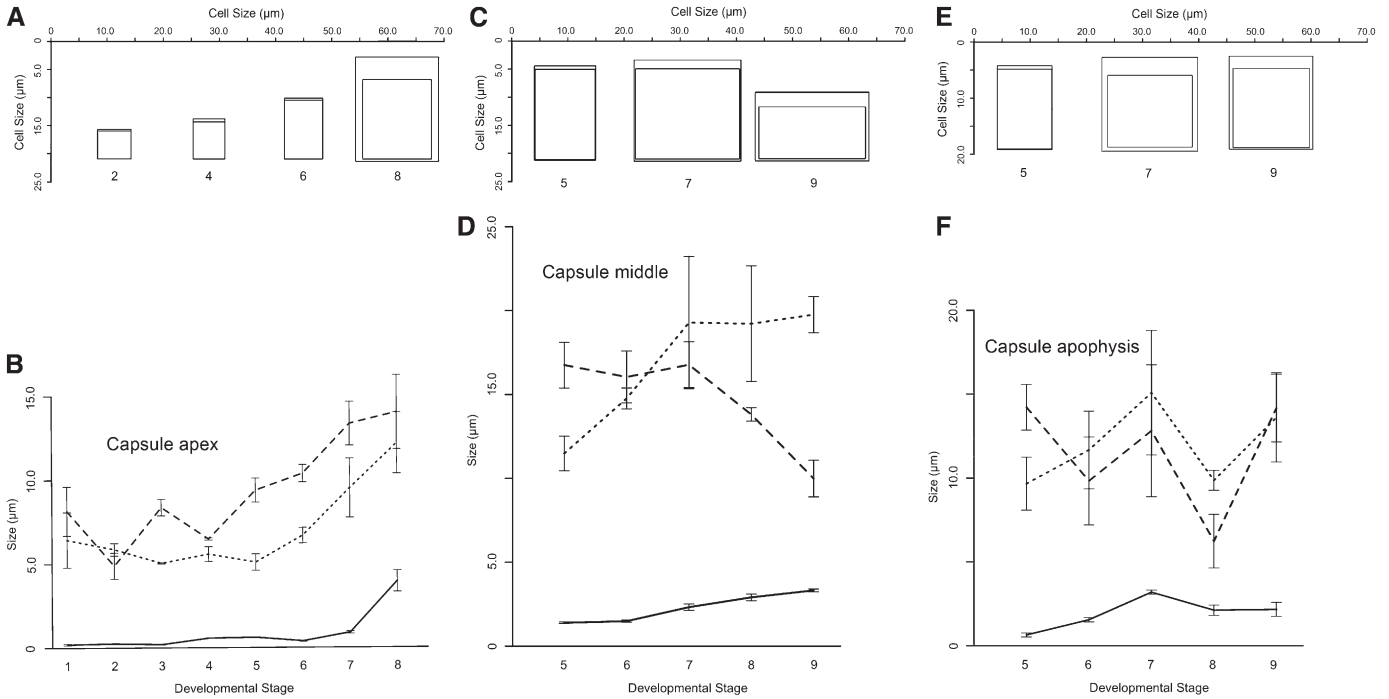

Fig. 4. Cell lumen sizes and cell wall thicknesses measured from sporophytes of *Funaria hygrometrica* averaged from three cells per region per individual. (A, B) Apex. (C, D) Capsule middle. (E, F) Apophysis. (A, C, E) Cell outlines illustrate mean lumen width and depth and mean cell wall thickness (inner and outer periclinal and two anticlinal walls) at representative developmental stages. (B, D, F) Line graphs display lumen width (short dashed lines), lumen depth (long dashed lines), and thickness of outer periclinal wall of the epidermis (solid lines) across all developmental stages examined (means ±1 standard error).

epidermal cells for the sporophyte capsule increases for the apex ($N = 4$, 134.0 cells), mid capsule ($N = 5$, 160.4 cells), and apophysis ($N = 4$, 120.3 cells).

Early in development the cuticle is present as only a single layer (cuticular layer or procuticle) on the embryonic sporophyte that is completely surrounded by gametophyte tissue (Figs. 1A, 5A, 6A). The sporophyte apex continues to be covered only by this single layer through stage 3 (Figs. 1C, 5A). Below the apex, the seta has a cuticle that progresses from two-layered when covered by the calyptra (Figs. 1B, 5B stage 2, 6B, 7) to a four-layered cuticle when exposed (Figs. 1C, D, 5B stages 3 and 4, 6D, 7). At stage 4, the sporophyte apex is covered by a thicker cuticle that consists of both a cuticular layer and an electron-dense CP (Figs. 1D, 5A, 6C, 7). Subsequently, the sporophyte apex undergoes a significant developmental transition from an undifferentiated apical region to an actively differentiating and expanding capsule (Fig. 1E). All three capsule regions (apex, middle, apophysis) are covered by the calyptra through stage 6 (Figs. 1F, 7). The lower region, the apophysis, has a three-layered cuticle, whereas the upper two regions are covered by only a two-layered cuticle (Figs. 5C–E stages 5 and 6, 6E–G, 7). The apophysis is no longer covered by the calyptra at stage 7 and beyond (Fig. 1G–I). The cuticle of the apophysis also has cell wall projections of the cuticular layer on stages 7 and 9 (Fig. 5E). At stage 7, the mid capsule is partially covered by the bottom edge of the calyptra and has a three-layered cuticle (Fig. 5D). When the mid capsule is no longer covered by the calyptra at stages 8 and 9, the cuticle is composed of all four layers (Figs. 5D, 6I–J). The sporophyte apex maintains a cuticle that consists of only two layers through stage 8, while covered by the calyptra. Observations of the cuticle for the sporophyte apex at stage 9 are missing. In addition to the increase in number of cuticle layers, the cuticle thickness increases during development for all regions of the sporophyte (Figs. 5, 8).

DISCUSSION

As the first comprehensive analysis of moss sporophyte cuticle development, this study broadens our perspectives on cuticle development and evolution across embryophytes. Plant cuticles are widely known to function in desiccation protection, and the moss calyptra provides a unique cross-generational example that may have been critical for moss sporophyte evolution. We previously demonstrated the occurrence of a thick, multilayered calyptra cuticle at maturity (Budke et al., 2011). We report here that the calyptra cuticle develops precociously with the potential to protect the sporophyte from desiccation in early development, whereas the sporophyte has a thin cuticle during early development that thickens late during sporangium formation. These observations support the hypothesized role of the calyptra in desiccation prevention of the immature sporophyte apex.

The calyptra of *Funaria hygrometrica* undergoes precocious development relative to the sporophyte, achieving its mature shape and size prior to detaching from the remainder of the maternal gametophyte (Fig. 1B), with no subsequent increases in cell size or wall thickness. A multilayered cuticle is present at the earliest stage and persists throughout development. The thickness of these layers is quite stable for all but the electron-dense CP on the rostrum (Fig. 3A). As the outermost layer of the cuticle, the electron-dense CP may be the most susceptible

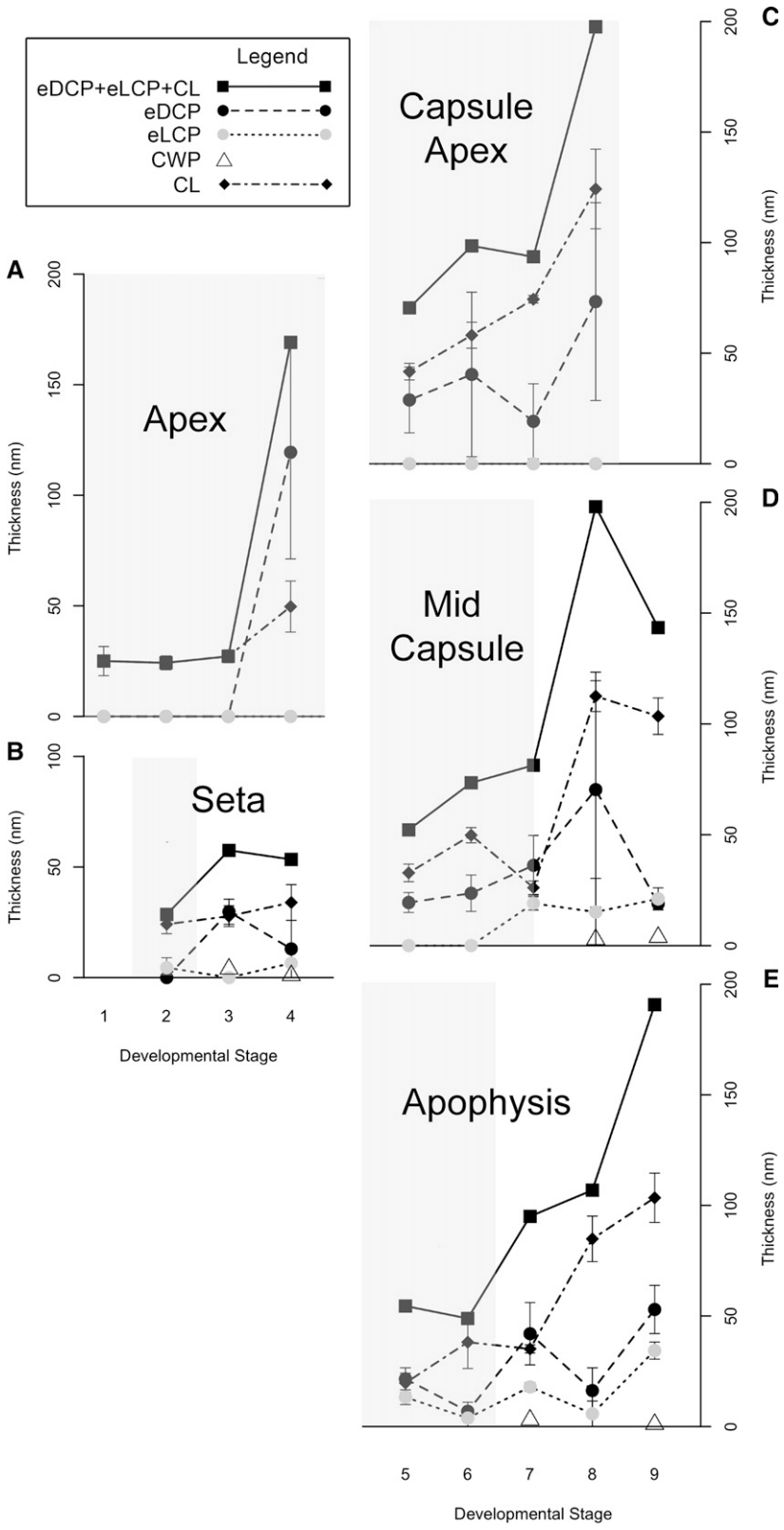

Fig. 5. Thickness of cuticle layers measured from transmission electron micrographs of transverse sections through *Funaria hygrometrica* sporophytes. Mean values from three cells per individual with ±1 standard error bars. Shaded portions indicate regions where the sporophyte is covered by the calyptra. (A) Sporophyte apex of the spear-shaped sporophyte. (B) Middle of seta. (C) Capsule apex. (D) Capsule middle. (E) Apophysis. *Abbreviations:* CL, cuticular layer (black diamond); CWP, cell wall projections of the cuticular layer (open triangle); eDCP, electron-dense cuticle proper (black circle); eLCP, electron-lucent cuticle proper (gray circle); sum of cuticle layers with the cell wall projections included in the cuticular layer (black square).

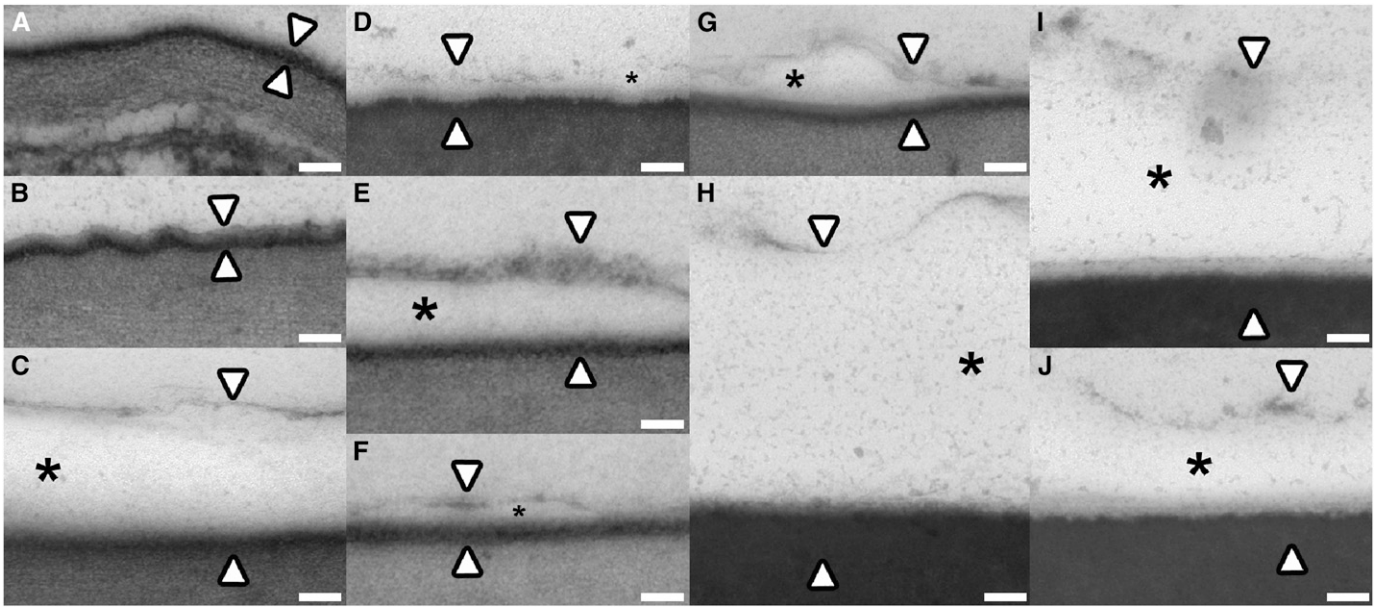

Fig. 6. Transmission electron micrographs of transverse sections through *Funaria hygrometrica* sporophytes across developmental stages. Inner and outer edges of cuticle are indicated by arrowheads; the asterisk (*) indicates artifactual separation of the layers. (A) Stage 2 apex of spear-shaped sporophyte. (B) Stage 2 seta. (C) Stage 4 apex of spear-shaped sporophyte. (D) Stage 4 seta. (E–J) Regions of the expanding sporophyte capsule. (E) Stage 6 apex. (F) Stage 6 middle. (G) Stage 6 apophysis. (H) Stage 8 apex. (I) Stage 8 middle. (J) Stage 8 apophysis. Bars = 100 nm.

to loss during specimen processing (Viougeas et al., 1995), which may obscure an accurate assessment of this layer's thickness across development (absent at stage 5, thickest at stage 9) and within an individual section (see Fig. 4A in Budke et al., 2011). We cannot rule out the possibility that the electron-dense CP of the rostrum thickens across development culminating in a thick layer at stage 9; alternatively the electron-dense CP may be thick at all nine developmental stages, but is removed during specimen processing. Destructive sampling for transmission electron microscopy does not allow for a single calyptra to be followed across development; the examination of separate calyptrae may also account for the variation observed in our data (Fig. 8). All other aspects of the calyptra, including cell size, cell wall thickness, and cuticle, indicate that the calyptra is a static structure that does not change after detachment from the leafy gametophyte.

Unlike the calyptra, the sporophyte of *Funaria hygrometrica*, undergoes significant modifications to its morphology, anatomy, and cuticle across development. The similar changes in exterior morphology observed here in *F. hygrometrica* (Fig. 1) occur in all mosses (Crum, 2001). The cuticle, in the form of a single cuticular layer (procuticle), is present on the embryo of *F. hygrometrica* (Fig. 6A). This mirrors the findings in vascular plants that the cuticle develops early during plant embryogenesis, while completely surrounded by maternal tissues (Bruck and Walker, 1985; Rodkiewicz et al., 1994). As a defining feature of embryophytes, the cuticle is an important innovation for protection of the plant body from the desiccative effects of life on land (Kenrick and Crane, 1997). However, the simple, single-layered procuticle and the protection that it offers may be minor compared to that of a multilayered cuticle. In *F. hygrometrica*, cuticle development proceeds from single to multilayered during ontogeny and in a wave from proximal to distal regions of the sporophyte (see Fig. 7 for summary). The cuticle layering starts with the cuticular layer alone and ends with all four layers. However, the sequential addition of these layers is not consistent across sporophyte regions (i.e., comparing the seta and apex, Fig. 5A, B respectively). Cuticle maturation does not reach the sporophyte apex by stage 8 (Figs. 5C, 6H); however, without observations at stage 9 a four-layered cuticle on this region cannot be ruled out. These transitions in cuticle development are mirrored by changes to the epidermis including increases in periclinal wall thickness and cell lumen size (Fig. 4). These observations indicate that both the cuticle and epidermis of the sporophyte transition from immature at early developmental stages to mature at later stages, thus representing a dynamic structure that undergoes significant modifications across development.

Cuticle developmental studies of bryophytes are limited to the stomatal guard cells in *Funaria hygrometrica* (Sack and Paolillo, 1983) and to protonemata of *Sphagnum fimbriatum* Wilson (Cook and Graham, 1998). In the former study, developmental comparisons are confounded by the cuticle developing concurrently with the physical separation of the cell walls that result in pore formation (Sack and Paolillo, 1983). In the latter, the initial developmental stages resemble those observed here in the sporophyte of *F. hygrometrica* with the presence of a single procuticle layer (Cook and Graham, 1998). Later in development the *Sphagnum* cuticle becomes multilayered, including an "amorphous osmophilic" layer resembling the cuticular layer (CL) overlain by a "loosely attached lamellate surface layer" that we interpret as a CP (Cook and Graham, 1998). Cuticle developmental patterns observed in angiosperms have been thoroughly summarized by Jeffree (2006) and begin with a procuticle that transitions to a multilayered cuticle. Cuticle development of the *F. hygrometrica* sporophyte does not achieve the complexity observed in angiosperms. The initial stages of cuticle development are similar transitioning from a procuticle to a distinct CL and CP (Jeffree, 2006, fig. 2.22a–e); however, the CL of *F. hygrometrica* cannot be separated into an interior and exterior CL or a multilamellate CP.

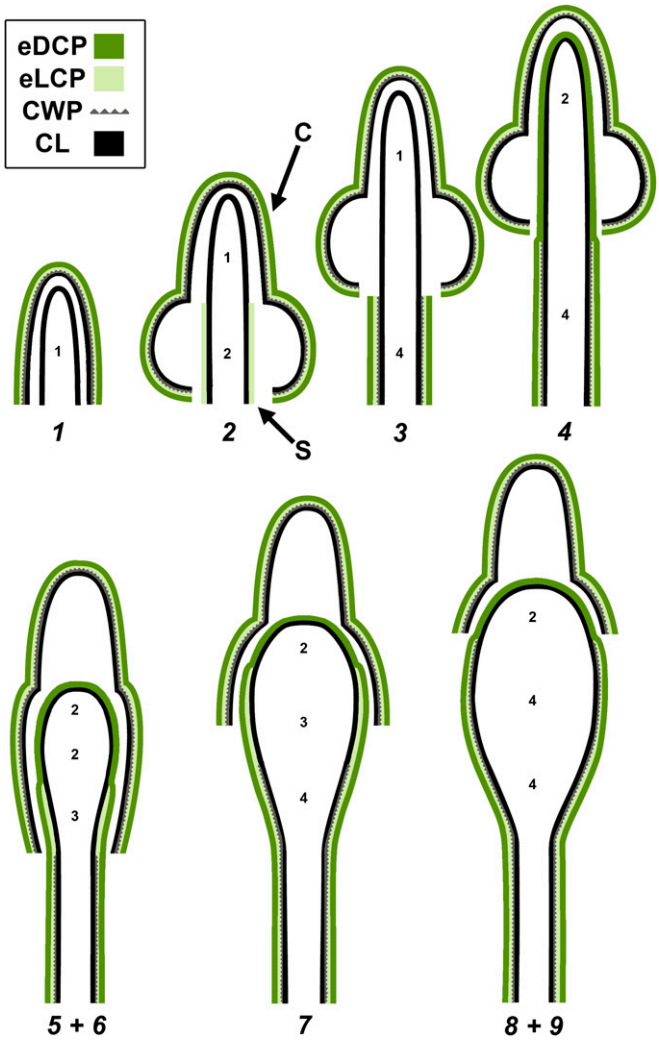

Fig. 7. Diagram illustrating cuticle layers on the calyptra and sporophyte for stages 1 to 9 (Table 1), with numbers of the developmental stages identified beneath each diagram. The calyptra is covered by a multilayered cuticle at all nine developmental stages. Numbers inside the sporophytes indicate the different number of cuticle layers present at each region of each developmental stage. *Abbreviations:* C, calyptra; CL, cuticular layer; CWP, cell wall projections of the cuticular layer; eDCP, electron-dense cuticle proper; eLCP, electron-lucent cuticle proper; S, sporophyte.

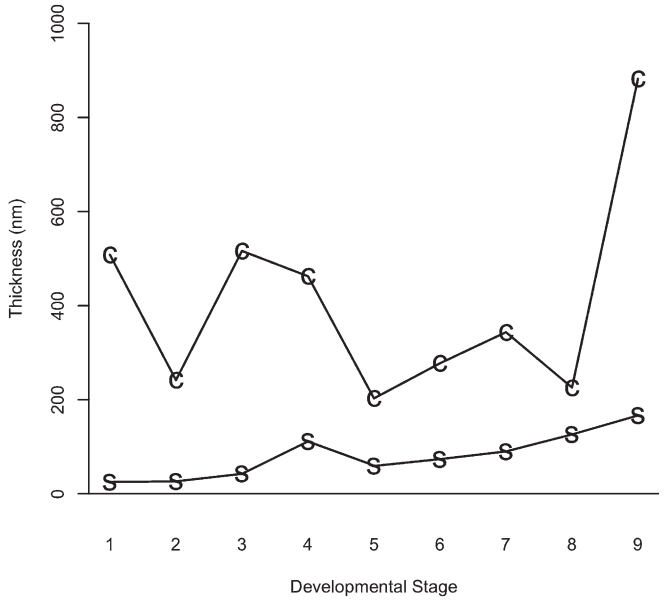

Fig. 8. Average thickness of cuticle layers from transmission electron micrographs of transverse sections through *Funaria hygrometrica* calyptrae and sporophytes. For each developmental stage, the average thickness was calculated from the sum of cuticle layers for each region (black squares on Figs. 3, 5). Calyptra average of rostrum and inflated base. Sporophyte stages 2 to 4 average of apex and seta, stages 5 to 9 average of apex, middle, and apophysis of capsule. *Abbreviations:* c, calyptra; s, sporophyte.

Cuticle development should be considered in relation to the morphological expansion and cell wall thickening of the underlying tissues, including the epidermis. In angiosperm leaves, these are two distinct developmental stages. During stages of expansion, both cuticle layers are added and cuticle thickness increases; in later stages when expansion has stopped and cell wall thickening occurs, the cuticle continues to increase in thickness, but additional cuticle layers are not added (e.g., Reiderer and Schonherr, 1988; Viougeas et al., 1995). Capsule expansion in *Funaria hygrometrica* (stages 5–9) is similarly characterized by the addition of cuticle layers and thickening of the cuticle. However, thickening of the exterior periclinal cell walls of the capsule epidermis occurs concurrently with its morphological expansion (Fig. 4). By stage 9, meiosis has occurred, and subsequent stages involve senescence and drying of the capsule for spore release. The longer lifespan of an angiosperm leaf allows for an extended period of cuticle thickening after all cuticle layers have been established. In moss sporophytes, the thin mature cuticle may be due to the relatively rapid capsule expansion and ephemeral nature of the moss sporophyte.

The sporophyte of *Funaria hygrometrica* becomes acropetally exposed to the surrounding air from beneath the calyptra. All regions of the sporophyte that have a cuticle with two layers or fewer are covered by the calyptra. Two cuticle layers may be insufficient to protect the sporophyte from the desiccative effects of the surrounding air. When calyptrae are removed, wilting of the apex occurs more severely for sporophytes of developmental stages 1 to 5 compared to stages 6 to 9, as measured by a decrease in width of the sporophyte apex (J. M. Budke, personal observations). The mid capsule and apophysis have a cuticle that includes the electron-dense CP, electron-lucent CP, and CL at the time of, or prior to, emergence from beneath the calyptra, whereas the cell wall projections of the cuticular layer were only observed on exposed portions of the sporophyte. The cuticle transitions beyond two layers prior to emergence from beneath the calyptra, which may indicate a preparation for exposure to the exterior environment (Fig. 7 stages 2, 5–7). Air exposure has been found to trigger the deposition of epicuticular waxes on the leaves of barley when prematurely exposed from the leaf sheath (Richardson et al., 2005). Whether the developmental progression of the moss sporophyte cuticle is triggered by an environmental cue remains unexplored.

Cuticle development on the sporophyte of *Funaria hygrometrica* is clearly delayed compared to the calyptra and indicates that early in development the sporophyte is ill prepared to prevent desiccation. On the other hand, the calyptra cuticle develops precociously and is both multilayered and thick (Fig. 8), with all of its protective abilities established at the earliest developmental stage examined herein. These observations of

cuticle development are best explained in the context of a waterproofing role for the calyptra in early moss sporophyte development. A broader survey of moss taxa is necessary to determine whether these observations of cuticle development apply across a wide range of calyptra and sporophyte morphologies. The calyptra is an organ that has been retained in all of the ~12 500 moss taxa. The calyptra is not simply an example of a vestigial structure, but rather the role of the calyptra in desiccation prevention offers a functional explanation for calyptra retention during the 450 Myr of moss evolution and points toward a critical role of the calyptra in the evolution of moss sporophyte development.

LITERATURE CITED

BOPP, M. 1954. Untersuchungen über Wachstum und Kapselenwicklung normaler und isolierter Laubmoossporogone. *Zeitschrift für Botanik* 42: 331–352.

BOPP, M., AND E. STEHLE. 1957. Zur Frage der Wasserleitung im Gametophyten und Sporophyten der Laubmoose. *Zeitschrift für Botanik* 45: 161–174.

BRUCK, D. K., AND D. B. WALKER. 1985. Cell determination during embryogenesis in *Citrus jambhiri*. II. Epidermal differentiation as a one-time event. *American Journal of Botany* 72: 1602–1609.

BUDKE, J. M., B. GOFFINET, AND C. S. JONES. 2011. A hundred-year-old question: Is the moss calyptra covered by a cuticle? A case study of *Funaria hygrometrica*. *Annals of Botany* 107: 1279–1286.

COOK, M. E., AND L. E. GRAHAM. 1998. Structural similarities between surface layers of selected Charophycean algae and bryophytes and the cuticles of vascular plants. *International Journal of Plant Sciences* 159: 780–787.

CRUM, H. 2001. Structural diversity of bryophytes. University of Michigan Herbarium, Ann Arbor, Michigan, USA.

CROSBY, M. R., R. E. MAGILL, B. ALLEN, AND S. HE. 1999. A checklist of the mosses. Missouri Botanical Garden, St. Louis, Missouri, USA.

FRENCH, J. C., AND D. J. PAOLILLO JR. 1975a. Intercalary meristematic activity in the sporophyte of *Funaria* (Musci). *American Journal of Botany* 62: 86–96.

FRENCH, J. C., AND D. J. PAOLILLO JR. 1975b. On the role of the calyptra in permitting expansion of capsules in the moss *Funaria*. *The Bryologist* 78: 438–446.

GARNER, D., AND D. J. PAOLILLO JR. 1973. A time-course of sporophyte development in *Funaria hygrometrica* Hedw. *The Bryologist* 76: 356–360.

GOEBEL, K. 1905. Organography of plants especially of the archegoniate and spermatophyta, part II, Special organography, authorized English edition by Isaac Bayley Balfour. Clarendon Press, Oxford, UK.

HEIDE-JØRGENSEN, H. S. 1991. Cuticle development and ultrastructure: Evidence for a procuticle of high osmium affinity. *Planta* 183: 511–519.

HEINRICHS, J., H. ANTON, S. R. GRADSTEIN, R. MUES, AND I. HOLZ. 2000. Surface wax, a new taxonomic feature in Plagiochilaceae. *Plant Systematics and Evolution* 225: 225–233.

JEFFREE, C. E. 2006. The fine structure of the plant cuticle. In: M. Riederer and C. Muller [eds.], Biology of the plant cuticle, 11–125. Blackwell, Oxford, UK.

JANZEN, P. 1917. Die Haube der Laubmoose. *Hedwigia* 58: 158–280.

KENRICK, P., AND P. R. CRANE. 1997. The origin and early diversification of land plants: A cladistic study. Smithsonian Institution Press, Washington, D.C., USA.

KERSTIENS, G. 1996. Signalling across the divide: A wider perspective of cuticular structure–function relationships. *Trends in Plant Science* 1: 125–129.

KOCH, K., J.-P. FRAHM, AND R. POLLAWATN. 2009. The cuticle of the *Buxbaumia viridis* sporophyte. *Flora* 204: 34–39.

LIU, Y., J. M. BUDKE, AND B. GOFFINET. In press. Phylogenetic inference rejects sporophyte based classification of the Funariaceae (Bryophyta): Rapid radiation suggests rampant homoplasy in sporophyte evolution. *Molecular Phylogenetics and Evolution*. doi:10.1016/j.ympev.2011.09.010

MILLER, D. H., AND H. A. MILLER. 2007. 12.3 *Funaria*. *In* Flora of North America Editorial Committee [eds.], Flora of North America, vol. 27, Bryophyta, part 1, 118–194. Oxford University Press, New York, New York, USA.

NEINHUIS, C., AND R. JETTER. 1995. Ultrastructure and chemistry of epicuticular wax crystals in Polytrichales sporophytes. *Journal of Bryology* 18: 399–406.

PRESSEL, S., K. M. Y. P'NG, AND J. G. DUCKETT. 2010. A cryo-scanning electron microscope study of the water relations of the remarkable cell wall in the moss *Rhacocarpus purpurascens* (Rhacocarpaceae, Bryophyta). *Nova Hedwigia* 91: 289–299.

PRESSEL, S., K. M. Y. P'NG, AND J. G. DUCKETT. 2011. An ultrastructual study of the liverwort *Mizutania riccardioides* Furuki et Iwaksuki: New insights into its systematic affinities and unique surface ornamentation. *The Bryologist* 114: 38–51.

PROCTOR, M. C. F. 1979. Surface wax on the leaves of some mosses. *Journal of Bryology* 10: 531–538.

R DEVELOPMENT CORE TEAM. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website http://www.R-project.org.

RICHARDSON, A., R. FRANKE, G. KERSTIENS, M. JARVIS, L. SCHREIBER, AND W. FRICKE. 2005. Cuticular wax deposition in growing barley (*Hordeum vulgare*) leaves commences in relation to the point of emergence of epidermal cells from the sheaths of older leaves. *Planta* 222: 472–483.

RIEDERER, M., AND J. SCHONHERR. 1988. Development of plant cuticles: Fine structure and cutin composition of *Clivia miniata* Reg. leaves. *Planta* 174: 127–138.

RODKIEWICZ, B., B. FYK, AND E. SZCZUKA. 1994. Chlorophyll and cutin in early embryogenesis in *Capsella*, *Arabidopsis*, and *Stellaria* investigated by fluorescence microscopy. *Sexual Plant Reproduction* 7: 287–289.

SACK, F. D., AND D. J. PAOLILLO JR. 1983. Stomatal pore and cuticle formation in *Funaria*. *Protoplasma* 116: 1–13.

VIOUGEAS, M. A., R. ROHR, AND A. CHAMEL. 1995. Structural changes and permeability of ivy (*Hedera helix* L.) leaf cuticles in relation to leaf development and after selective chemical treatments. *New Phytologist* 130: 337–348.