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Comparative development and gametophyte morphology of the hart's-tongue fern, *Asplenium scolopendrium* L.

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TESTO, W. L. AND J. E. WATKINS, JR. (Department of Biology, 13 Oak Drive, Colgate University, Hamilton, NY 13346). Comparative development and gametophyte morphology of the hart's-tongue fern, *Asplenium scolopendrium* L. J. Torrey Bot. Soc. 138: 400–408. 2011.—Gametophyte morphology and development of the federally listed American hart's tongue fern *Asplenium scolopendrium* var. *americanum* L. was examined and compared to the old world variety *Asplenium scolopendrium* var. *scolopendrium*. The North American variety is critically threatened and many populations in the Southeastern US have gone extinct while the number of plants in all northeastern populations is plummeting. The ultimate goal of this work was to compare gametophyte biology of these two varieties to understand better potential characters that may help separate plants in North America from those in the old world and generate a better picture of the biology of the North American populations. The two varieties differed significantly in gametophyte ontogeny, morphology, and propensity for sexual and asexual reproduction. Gametophytes of the American variety germinated earlier, grew significantly slower, and produced sporophytes much later (166 d vs. 119 d) than the old world variety. In addition, *A. scolopendrium* var. *americanum* produced copious gametophytic outgrowths that were capable of developing into functional, independent thalli while *A. scolopendrium* var. *scolopendrium* does not. The combined data set aids in understanding the origins of the American variety as its gametophyte biology is similar to several other taxa which now persist in climatically-moderated rockhouse habitats in eastern North America.

Key words: *Asplenium*, conservation, ferns, gametophyte, rarity.

Asplenium scolopendrium L., commonly known as the hart's-tongue fern, is a relatively unusual temperate member of the Aspleniaceae. Unlike most New World Asplenioid taxa, this species produces simple leaves with overlapping linear sori. These characters, in addition to its unusual biogeographic distribution, have resulted in some taxonomic controversy. Some authors place the species in the segregate monotypic genus *Phyllitis* (e.g., Murakami *et al.* 1999) whereas others recognize it as *Asplenium* (e.g., Schneider *et al.* 2004). As currently circumscribed, three varieties of this species are recognized. *Asplenium scolopendrium* L. var. *scolopendrium* is a common fern species found in Europe, Western Asia, and Japan (Bremer 2010). A second variety, of which we know little, *A. scolopendrium* L. var. *lindenii* (Hook.) Viane, Rasbach & Reichst., has been reported from southern Mexico and Hispaniola (Maxon 1900). A third

variety, recognized as *A. scolopendrium* L. var. *americanum* (Fernald) Kartesz & Gandhi, is a rare taxon disjunct in North America, where it is currently treated as a federally-listed threatened species and is known from small populations in New York, Michigan, Alabama, Tennessee, and Canada. The relationship between the varieties remains unclear.

The unusual distribution of these varieties, combined with the relative rarity of the taxon in North America, has produced a great deal of interest from amateur and professional pteridologists alike. Fernald (1935) examined several collections of this species from Europe and North America and concluded that when compared to the European plants, the North American plants exhibit smaller leaves with fewer sori, and scales that are narrower with attenuate apices. Britton (1953) examined the cytology of plants from both continents and determined that *A. scolopendrium* var. *americanum* was a tetraploid. However, these counts come from only two individuals near the town of Durham, Ontario, Canada and no counts have been made on populations from the United States. A single investigation comparing the spores of the two varieties noted some differences in perispore morphology (Arreguin-Sanchez 1986) that support the varietal separation of the European and American plants. The North America variety

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is notably habitat specific, growing in glacial plunge ravines and lime sinks with calcareous substrates (Kelsall *et al.* 2004). These habitats are especially remarkable in the southeastern portion of the taxon's range. The largest southern population occurs in northern Alabama where it grows in a lime sink that is >100 ft deep and >150 ft wide. These southern sites are similar to rockhouse habitats previously described by Farrar (1998) and Watkins and Farrar (2002, 2005).

Unfortunately, the North American variety is critically threatened. Population numbers in New York have dropped by more than half in the 1990s and those in Tennessee have approached local extinction in recent years (Kelsall *et al.* 2004). A recent survey of the one remaining population in Tennessee by the authors (March 2011) revealed only two immature plants of this population that once numbered over 200 plants (McGilliard 1936). Thus, the need to understand the biology of this species is critical. In particular, the gametophyte generation remains poorly understood. A few studies investigating the European variety's gametophytes have focused on the taxon's mating systems and did not include detailed observations of its ontogeny or morphology (Pangua *et al.* 1994, Andersson Kötto 1929). More surprisingly, the gametophyte of the North American variety remains undescribed. The goal of this paper is to describe the gametophyte of the North American *A. scolopendrium* var. *americanum* and to understand better how this stage compares between the European and North American plants.

Materials and Methods. Spore material from *Asplenium scolopendrium* var. *americanum* was obtained from sporophytes collected at Clark Reservation State Park, Onondaga County, New York in the spring of 2010. Material from *Asplenium scolopendrium* var. *scolopendrium* was obtained from sporophytes from a commercial nursery. Multispore cultures were prepared using standard Bold's media (Bold 1957) modified with Nitch's micronutrients (Nitsch 1951). Spores were sown at an approximate density of 25 spores per 100mm × 25mm Fisher Brand Petri dishes. Cultures were exposed to fluorescent growbulbs at approximately 50 μmol m⁻² s⁻¹ PAR on a 12:12 l:d cycle at two temperatures, 20°C and

25°C in two separate walk in Percival (Perry, IA) bench top growth chambers.

Cultures were observed under an Olympus SZX9 stereo-scope every other day until spore germination was observed. After spore germination, cultures were observed twice weekly. To characterize early gametophyte development specimens were removed from culture, mounted in water with Tween 20, and observed under a compound microscope. Large gametophytes were typically observed under the stereoscope, given the difficulty in mounting such large specimens. Images of gametophytes were recorded using Pax-it! microscope imaging software (Midwest Information Systems, Inc., Villa Park, IL). Thallus size was determined by analyzing digital micrographs in the image processing program ImageJ (NIH, Bethesda, MD).

Results. **SPORE GERMINATION.** Spore germination occurred between 11–19 d after sowing in *A. scolopendrium* var. *americanum* and 14–28 d in *A. scolopendrium* var. *scolopendrium* (Table 1). In both varieties, early protonemal growth was consistent with the *Vittaria*-type (Fig. 1) reported for other members of Aspleniaceae by Nayar and Kaur (1971). In most cases, the development of a proximal rhizoid occurred shortly after the formation of the initial cell (Fig. 1 A), though rhizoid formation preceded the formation of a visible cell in several cases. In both varieties, a uniseriate germ filament formed through a series of lateral divisions (Fig. 1 B–D). Early development of the protonemal filament proceeds via anticlinal divisions. Between days 19–26, most protonema of both varieties had reached a length of 5–8 cells. At this stage many filaments begin to broaden via periclinal divisions but the prothalli is still devoid of any pluricellular differentiation. It is also at this stage that many *A. scolopendrium* var. *americanum* gametophytes (~75% of those observed) had developed a single papillate hair on the anterior end of the distal cell (Fig. 1 F–G). This hair type was also observed in *A. scolopendrium* var. *scolopendrium*, but was much less common, occurring in fewer than 25% of the protonema observed. Following hair formation, development of the protonemal filament was replaced by early periclinal divisions which led to the development of the pluricellular meristem (Fig. 1 M–N).

Table 1. Comparison of developmental and morphological characters of the gametophytes of *Asplenium scolopendrium* var. *americanum* and *A. scolopendrium* var. *scolopendrium*.

Character	<i>A. scolopendrium</i> var. <i>americanum</i>	<i>A. scolopendrium</i> var. <i>scolopendrium</i>
Germination	11–19d	14–28d
Plate Formation	26–38d	27–34d
Antheridia	44–62d	41–88d
Archegonia	67–90d	63–94d
Hair Formation	34–52d	63–98d
Days to formation of first sporophyte	166d	119d
Proliferation type	Filamentous or cordiform, viable when excised	None present
Thallus width at 150 days	0.8–1.4cm	1.1–1.8cm
General morphology	Cordiform to reniform, margin ragged due to copious proliferation	Cordiform, margin smooth to wavy
Hair density	Profuse along margin, dense on thallus	Sparse along margin

THALLUS DEVELOPMENT. In both varieties, the development of the prothallial plate generally followed the *Aspidium* pattern reported by Nayar and Kaur (1971) beginning with periclinal divisions at the anterior end of the germ filament and eventually pluricellular differentiation (Fig. 1 M–N, Fig. 2 A–D). This initial division occurred between 26–38 d in *A. scolopendrium* var. *americanum* and 27–34 d in *A. scolopendrium* var. *scolopendrium* (Table 1). In *A. scolopendrium* var. *americanum*, it was typically the penultimate cell which divided, though the terminal cell did actively divide in many cases. When this occurred, the anterior hair was borne on one of the resulting cells, and that cell did not contribute to plate development.

During the broadening of the prothallial plate, differences between the two varieties became more apparent. In the *A. scolopendrium* var. *americanum*, unicellular papillate hairs became profuse on the margins of the young prothalli between 34–52 d (Fig. 3), with most marginal cells bearing hairs prior to the differentiation of a pluricellular meristem. In *A. scolopendrium* var. *scolopendrium*, hair density was much lower, and hair development did not occur until 63–98 d, generally after the gametophyte had reached its mature cordiform morphology. Between 29–67 d, large finger-like outgrowths began to develop along the margins of many *A. scolopendrium* var. *americanum* gametophytes (Fig. 1 M–N, Fig. 4). In many cases, these outgrowths became so prolific that they significantly altered the appearance of the gametophyte, resulting in an unusually ragged margin

(Fig. 2 A–D, compared to E–H and Fig. 5 A–D, compared to E–F). These outgrowths were also observed in *A. scolopendrium* var. *americanum* gametophytes collected from the field. When such outgrowths were detached and placed on Bold's media in the lab, they developed into large cordiform thalli capable of producing additional thalli and sporophytes. These outgrowths clearly have potential for both asexual and sexual reproduction. No such outgrowths were observed in *A. scolopendrium* var. *scolopendrium* gametophytes. In *A. scolopendrium* var. *americanum* and other Aspleniaceae, severing of proliferations appears to be the product of the passive process of decay, rather than active release as occurs in the Hymenophyllaceae, Vittariads and Grammitids. This type of proliferation has been described by Chiou and Farrar (1997, 1998) for members of the Polypodiaceae and Elaphoglossaceae and appears to be incorporated into normal maturation of the thallus, resulting in a gametophyte stage with a strong tendency toward clonal formation and ecological advantages associated with that growth form.

There were two distinct developmental pathways that gave rise to these outgrowths. In some instances, these outgrowths started as broad flattened marginal projections that rapidly developed a pluricellular meristem to develop a cordiform thallus (Fig. 4 A). Yet in other cases, these outgrowths started as linear filaments that developed by a series of anticlinal divisions and formed broader spatulate appendages (Fig. 4 B). These filaments were typically 1–2 cells wide except at the anterior end, which were typically 5–8 cells wide.

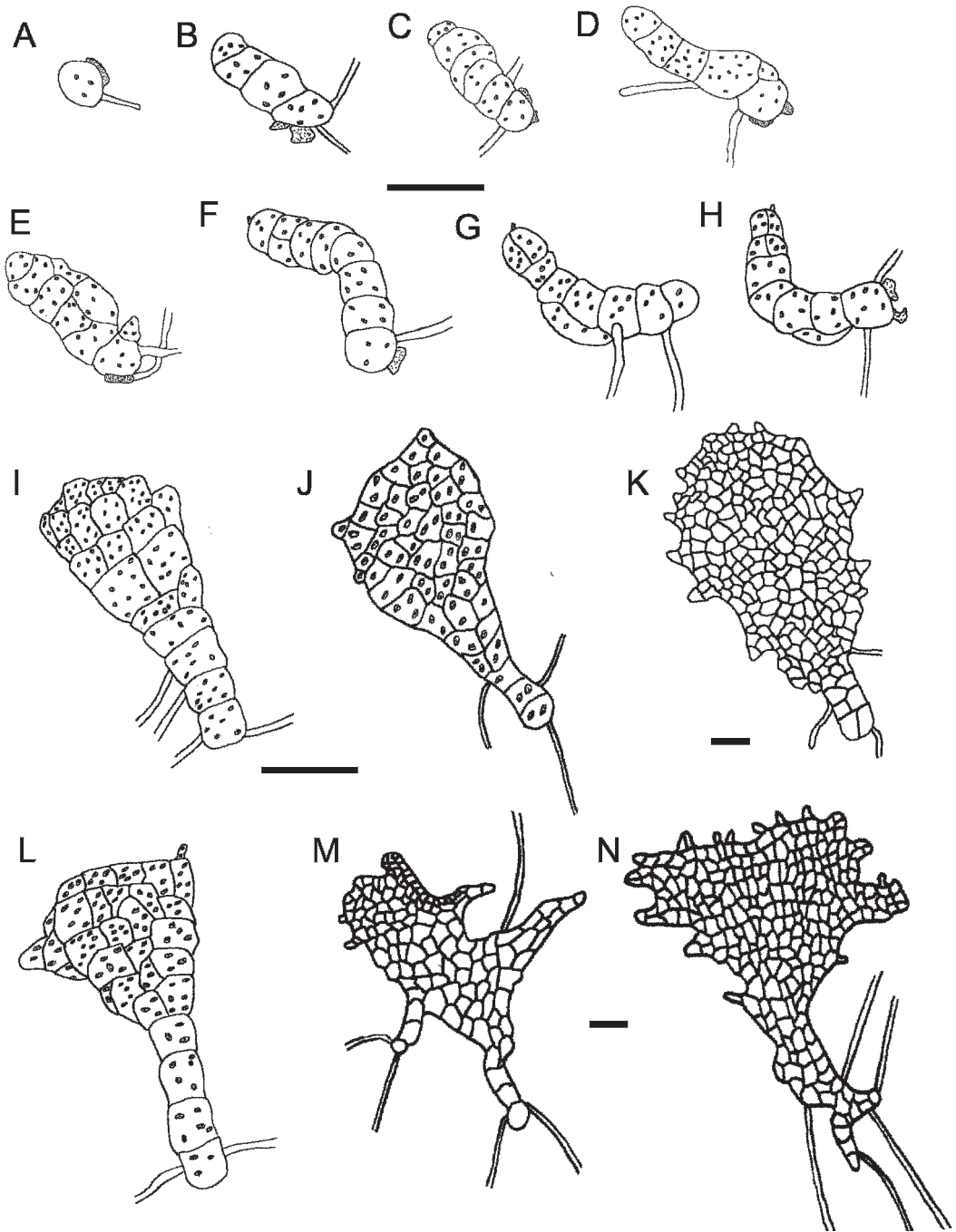


FIG. 1. General developmental pathway and comparison of prothallial plate formation in *Asplenium scolopendrium* var. *americanum* and *A. scolopendrium* var. *scolopendrium*. (A) Initial cell with proximal rhizoid, 15d. (B-E) Uniseriate germ filament, 19-33d. (F-G) Prothallial filament displaying first longitudinal cell division at anterior or penultimate cells, 27-36d. Note single papillate hair on terminal cell. (I-K) Plate formation in *A. scolopendrium* var. *scolopendrium*, showing typical spatulate morphology. 27-38d. (L-N) Plate formation in *A. scolopendrium* var. *americanum*, showing outgrowth formation and asymmetric development pattern. 29-35d. Scale bars = 100 μ m.

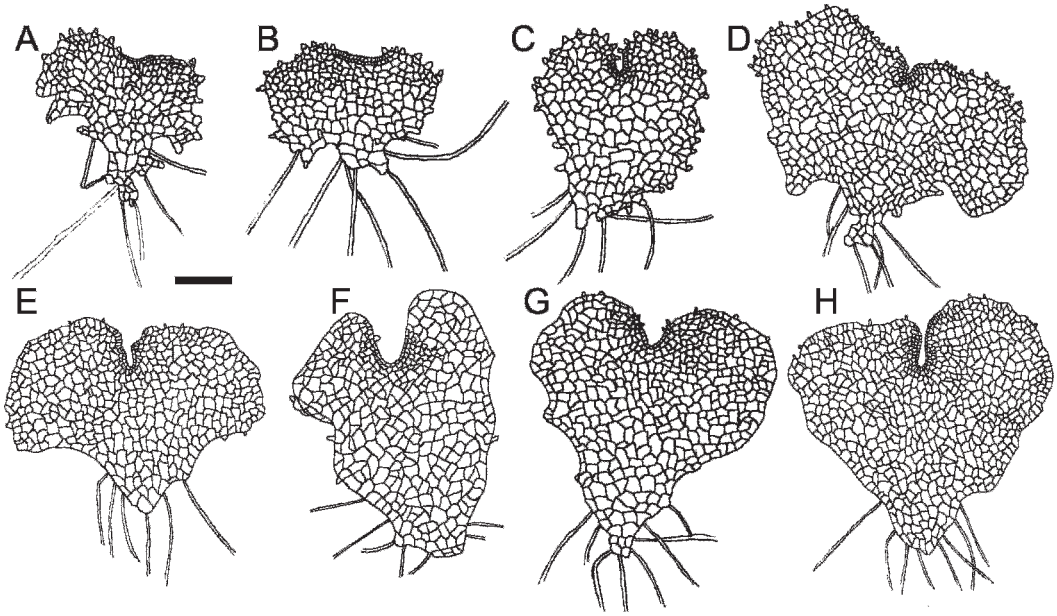


FIG. 2. Mature gametophytes of *Asplenium scolopendrium* var. *americanum* and *A. scolopendrium* var. *scolopendrium* showing variation in morphology within and between varieties. (A–D) 84–90d *A. scolopendrium* var. *americanum* gametophytes, ranging from cordiform to roughly reniform morphology. (E–H) 82–92d *A. scolopendrium* var. *scolopendrium* gametophytes, each with cordiform morphology. Note irregularity in form in *A. scolopendrium* var. *americanum* relative to *A. scolopendrium* var. *scolopendrium* and differences in hair density between varieties. Scale bar = 100 μ m.

GAMETANGIA. In *A. scolopendrium* var. *americanum*, antheridia formation occurred between 44–62 d, compared to 43–88 d in *A. scolopendrium* var. *scolopendrium* (Table 1). Archegonia formation was also observed within a similar range for both varieties, between 67–90 d for *A. scolopendrium* var. *americanum* and 63–94 d for *A. scolopendrium* var. *scolopendrium* (Table 1). Though not quantified, a clear difference was noted in the sexual composition of populations of the two varieties, with male gametophytes occurring with much greater frequency in *A. scolopendrium* var. *scolopendrium* than *A. scolopendrium* var. *americanum*. Additionally, no bisexual gametophytes were observed in populations of *A. scolopendrium* var. *americanum*, while bisexual gametophytes composed a large proportion (~33%) of mature *A. scolopendrium* var. *scolopendrium* gametophytes (Table 1), which is consistent with the findings of Pangua *et al.* (1994).

MATURE GAMETOPHYTE MORPHOLOGY. Mature *A. scolopendrium* var. *americanum* gametophytes displayed a wide variety of appearances,

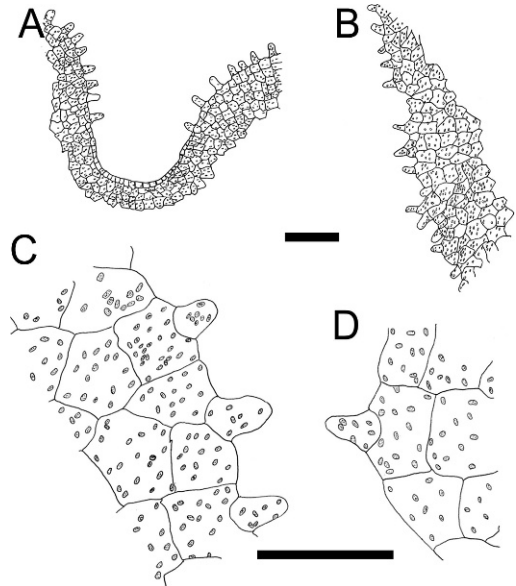


FIG. 3. Detail of thallus margin of *Asplenium scolopendrium* var. *americanum* showing hair density and morphology. Scale bars = 100 μ m (A, B) and 50 μ m (C, D).

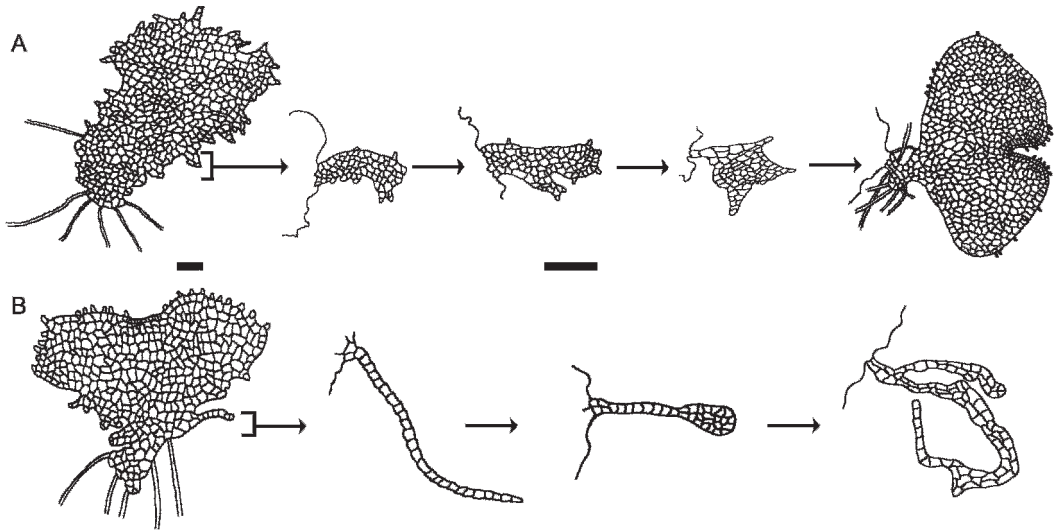


FIG. 4. Two pathways of marginal outgrowth formation in *Asplenium scolopendrium* var. *americanum*. (A) Development of cordate proliferation from outgrowth on margin of gametophyte. Stages of development shown occurred between 55 and 78 days after germination. Note hairs, defined meristematic region and basal rhizoids on fully-developed proliferation. (B) Development of filamentous proliferation with eventual broadening from outgrowth on margin of gametophyte. Stages of development shown occurred between 88 and 113 days after germination. Scale bars = 250 μ m.

ranging from a cordiform-thalloid morphology with a deep apical notch, to a roughly oblong shape with a broad, flattened meristematic region (Figs. 2 A–D, 5 A–D). Hair density along the margin remained very high throughout gametophyte development and unicellular (rarely two-celled) hairs were also observed on the body of the thallus. In all *A. scolopendrium* var. *americanum* gametophytes observed, the margin was irregular in appearance due to proliferation of the outgrowths mentioned previously. After approximately 120 d, these outgrowths developed pluricellular meristems, gametangia and basal rhizoids and when detached the outgrowths could live independently. At 150 d, gametophytes measured between 0.8–1.7 cm across, and the first incidence of sporophyte formation was observed at 166 d. In older (>180 d) gametophytes, these proliferations became so common that the gametophyte entirely lost its cordate-thalloid form and grew to 3–5 cm across (Table 1). At this stage of development, hundreds of cordiform proliferations existed on a single gametophyte, which resulted in an unusual three-dimensional appearance (Fig. 5 I). No proliferations were observed in *A. scolopendrium* var. *scolopendrium* gametophytes, regardless of age (Fig. 5 J).

Discussion. With its unusual taxonomy, distribution, and rarity, *Asplenium scolopendrium* var. *americanum* has generated a significant amount of interest from amateur and professional botanists alike. The data presented in this paper suggest that *A. scolopendrium* var. *americanum* and *A. scolopendrium* var. *scolopendrium* differ significantly in gametophyte ontogeny, morphology, and propensity for sexual and asexual reproduction. As such, they strongly support the current separation of these taxa at the varietal level. One particularly interesting difference is the length of time to first sporophyte production in *A. scolopendrium* var. *americanum*. While this variety produces significantly larger gametophytes relative to *A. scolopendrium* var. *scolopendrium*, it was much delayed in sporophyte production, a character that likely influences the long term survival of the taxon.

The ability of *A. scolopendrium* var. *americanum* to produce outgrowths from the gametophyte that are capable of developing into functional, independent thalli (while *A. scolopendrium* var. *scolopendrium* does not) is noteworthy, as these outgrowths act as asexual propagules that produce new thalli which often produce sporophytes in lab culture. This may produce more robust gametophyte capa-

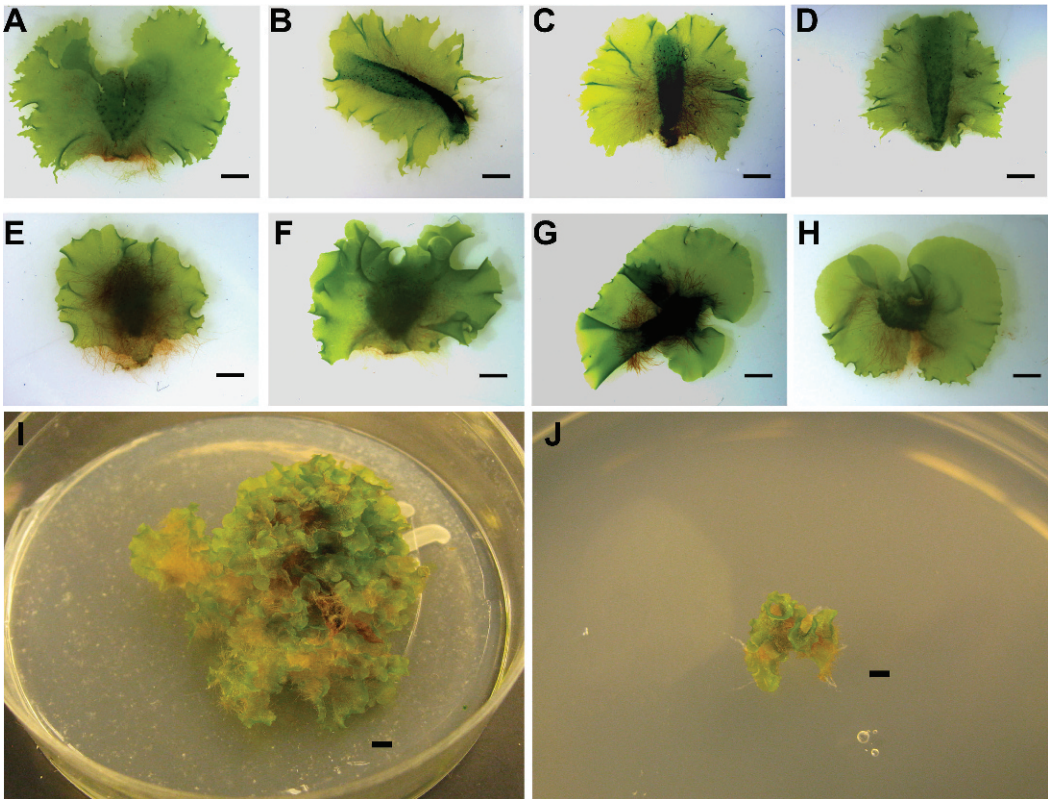


FIG. 5. Mature gametophytes of *Asplenium scolopendrium* var. *americanum* (A–D) and *A. scolopendrium* var. *scolopendrium* (E–H). At 150d, *A. scolopendrium* var. *americanum* gametophytes produced ragged thallus margins due to formation of marginal proliferations (A–D) which were not observed in gametophytes of *A. scolopendrium* var. *scolopendrium* of the same age (E–H). Extreme proliferation of these marginal outgrowths resulted in *A. scolopendrium* var. *americanum* (I) thalli that were significantly larger than those of *A. scolopendrium* var. *scolopendrium* (J) (Both images taken at 360d). Scale bars = 2.5mm.

ble of persisting during periods of physiological stress (Watkins *et al.* 2007).

The biology of this taxon fits well into our conceptual understanding of the origins of several other taxa which now persist in climatically-moderated rockhouse habitats in eastern North America. Farrar (1998) has proposed a rockhouse refugia hypothesis to explain the occurrence and persistence of certain fern and bryophyte species in North America. Some authors (Farrar 1967, 1978, 1990, 1998; Watkins and Farrar 2002, 2005) have argued that species of tropical and subtropical affinities may have passed the Pleistocene glacial period tucked away in the recesses of these moderated habitats. Data from Sato (1982) and Watkins *et al.* (2007) have shown that gametophytes of temperate and tropical ferns are more stress tolerant relative to the sporophytes, suggesting that

gametophytes are capable of overwintering and/or surviving in fairly extreme habitats where the sporophyte can not. Additional and ongoing work of stress tolerance in fern gametophytes suggests that gametophytes are markedly tolerant to desiccation relative to sporophytes (Watkins *et al.* 2007). Thus, gametophytes seem better equipped to handle periods of extreme stress compared to sporophytes.

One critical element that has been repeatedly shown in such rockhouse species is the capacity for gametophytic proliferation. In order to survive for long periods in these suboptimal conditions in the absence of the relatively-sensitive sporophyte generation, these plants would have needed to reproduce in the gametophyte stage. All tropical fern species limited to these moderated habitats exhibit this capability, including notable ex-

amples such as *Vittaria appalachiana* Farrar and Mickel (Farrar and Mickel 1991), *Trichomanes intricatum* Farrar (Farrar 1992), *Thelypteris burksiorum* Watkins and Farrar (Watkins and Farrar 2002, 2005) and *Asplenium monanthes* L. (Shaw 2003). As with other rockhouse pteridophytes, *A. scolopendrium* var. *americanum* exhibits prolific asexual reproduction in the gametophyte stage, something that was not observed in the European gametophytes.

Additionally, compared to related populations in the tropics and elsewhere, rockhouse endemics generally exhibit the absence of (*V. appalachiana*, *T. intricatum*) or reduction in (*T. burksiorum*, *Hymenophyllum tayloriae*) the sporophyte generation, possibly due to long-term stress experienced by these taxa during the Pleistocene (Farrar 1998). Fernald (1935) and others have noted that fronds of *A. scolopendrium* var. *americanum* are reduced compared to those of *A. scolopendrium* var. *scolopendrium*. Although this reduction is limited relative to the previously mentioned taxa, it is noteworthy within the context of rockhouse pteridophyte flora.

Finally, the differences in gametophyte development between American and European populations are notable. Compared to the European variety, the American plants develop more slowly and exhibit a complete absence of bisexual gametophytes, and produce few antheridiate gametophytes. Such aberrant mating systems are common amongst rockhouse taxa, including several bryophytes which are known only by a single sex (Schuster 1983). These differences in developmental rates and reproductive strategies may help explain the rarity of the American variety and the relative commonness of the European one.

If the rockhouse refugia hypothesis is correct, why then does the European variety fail to exhibit the same characters as the American plants? These differences may be due to the fact that Pleistocene glaciation events in Europe were less extensive than those experienced in North America (Tribsch and Schönswetter 2003, Brochmann *et al.* 2003), allowing for the persistence of suitable habitat throughout a significant portion of the current range of *A. scolopendrium* var. *scolopendrium*. As such, the European plants were never restricted to refugia and were able to maintain a normal life cycle throughout the Pleistocene and colonize their current range in the Holocene.

Clearly, a great deal of work is needed in order to develop a full understand of the biogeography and taxonomy of *Asplenium scolopendrium*. In particular, further studies including the populations in the southeastern United States are necessary. Unfortunately, these populations are significantly threatened and many are now extinct. In order to develop an understanding of modes of origin and the relationships between European, North American, and Mexican taxa, we are currently utilizing molecular techniques relying on herbarium specimens from threatened and extinct populations.

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