# Myxomycete Plasmodia and Fruiting Bodies: Unusual Occurrences and User-friendly Study Techniques

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**Abstract**

Plasmodia, sclerotia, and fruiting bodies are stages in the myxomycete life cycle that are easiest to recognize in the field. These stages can be found on different substrata such as living and dead plants and animals on the forest floor and in the canopy on bark of living trees and vines. This paper describes unusual habitats of myxomycetes on living lizards, mammal skulls, spiders, on other myxomycetes and fungi, and provides additional information needed to collect and identify these fascinating protists. The complete myxomycete life cycle is illustrated in detail, including trophic stages (myxamoebae, swarm cells, and plasmodia), and dormant stages (spores, microcysts, sclerotia, and fruiting bodies). Techniques are described that involve the collection and identification of myxomycetes through the use of moist chamber cultures, harvesting specimens, and slide preparation tips, all of which can be done at home given the proper materials. Studio photographic techniques are described that illustrate larger specimens, for example, the evidence of plasmodial tracks on a dog and deer skull. Moist chamber cultures are miniature "gardens" that may have life forms such as cyanobacteria, lichens, leafy liverworts, mosses, mushrooms, myxomycetes, colorful myxobacteria, nematodes, tardigrades, insects, and arachnids. The culture methods described here were used by school children for research projects, scientific professionals in research laboratories, and amateur hobbyists in their homes.

**Key words**: Daniel Boone National Forest, fruiting bodies, handcrafted tools, Kentucky, mammal skulls, Missouri, moist chamber cultures, myxomycetes, Pertle Springs, photography, plasmodia, plasmodial tracks, sclerotia, slide preparation tips, tree canopy, Warrensburg Middle School

## Introduction

Myxomycetes typically are collected as fruiting bodies and plasmodia on decaying logs or leaf litter during the warmer and wetter months of the year. Generally this occurs during the months of June to September in central and southeastern United States of America (Keller and Braun, 1999).

The myxomycete life cycle is shown in Figure 1 (A–N). Two myxomycete life cycle stages that reach size dimensions large enough to be seen with the unaided eye are the plasmodia (J, L) and fruiting bodies (N). The fruiting body contains the spores (A) and serves as the reproductive unit of the myxomycete life cycle. Spores are a dormant stage, usually visible as a powdery mass, disseminated by wind, and less often by insects, raindrops, or through hygroscopic and drying action of capillitial threads. Individual spores range in size from 5 to 20µm in diameter and are haploid with one set of chromosomes. Spores germinate (B) and produce one of two types of sexual gametes, myxamoebae (C) or swarm cells (D), which mate with other gametes that are sexually compatible.

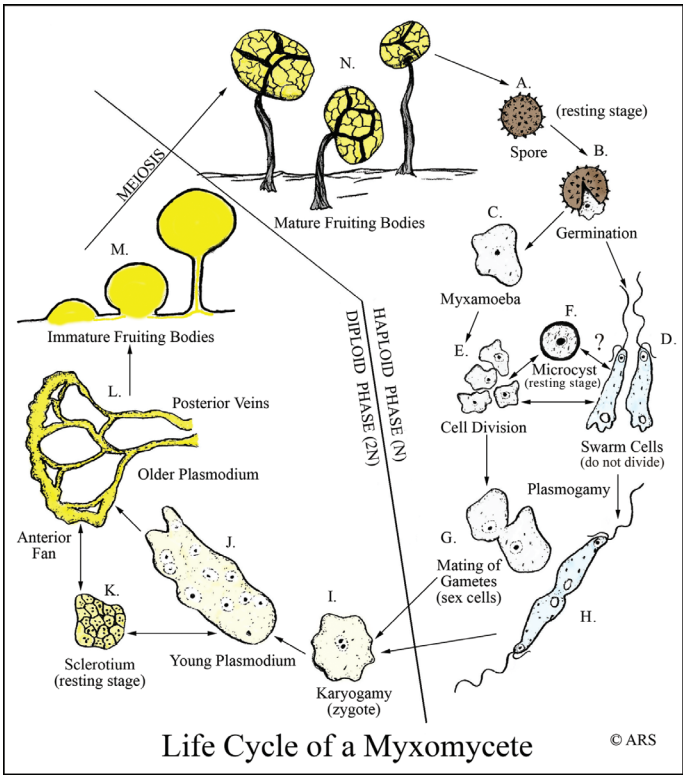


Figure 1. Freehand drawing of *Physarum viride* stalked sporangia. Illustration by Angela R. Scarborough.

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The myxamoeba is amorphous, divides by mitosis (E), and gives rise to a population of cells capable of changing shape as they move across the substratum feeding on bacteria and other microorganisms. Swarm cells do not divide by mitosis and have two unequal, whiplash flagella attached at the anterior end that result in a distinctive corkscrew, rotating, or gyrating swimming movement (D). Swarm cells mate (H) by their sticky posterior ends as their flagella thrash about in a tug of war. This type of gametic fusion in flagellate protists is atypical since in most cases mating occurs at the anterior end when flagella touch tip to tip (Alexopoulos and Koevenig, 1964). Unfavorable environmental conditions, such as desiccation and/or lack of nutrients, often result in the conversion of myxamoebae, and possibly swarm cells, into a dormant stage called a microcyst (F).

Fusion of haploid gametes results in a diploid zygote (I) with two sets of chromosomes that undergo synchronous nuclear divisions without cytoplasmic division, creating a large, multinucleate slime stage called a plasmodium. This amorphous, protoplasmic mass is surrounded by a cell membrane and gives the myxomycetes their common name, the true or plasmodial slime molds. The sclerotium (K), another dormant stage, may form when the plasmodium is exposed to low temperatures, low moisture, overcrowding, lack of food, or accumulation of waste products (Fig. 8f). When favorable conditions return, the hardened mass of spherules becomes hydrated and the plasmodium crawls out, continues feeding, matures, and then produces fruiting bodies (L–N).

The plasmodial stage gradually forms mature fruiting bodies over a 24- to 48-hour period or sometimes longer in the case of larger aethalia (Fig. 2c,d). Immature fruiting bodies often are collected because their conspicuous, bright, glistening colors invite collectors to gather the decaying wood or leaves on which they are developing. Unfortunately these collections result in premature drying into a hardened mass with aborted internal morphology that makes identification impossible.

Myxomycete plasmodia and fruiting bodies sometimes occur in unusual places. Examples are illustrated of plasmodial tracks on mammal skulls such as a dog (Fig. 9a,b) and deer (Fig. 9c,d), and stalked sporangia on a squirrel skull (Fig. 10a-c). Special photographic techniques were used to create digital images of the skulls (Fig. 5). Unusual patterns of plasmodial migration and formation of fruiting bodies on substrata such as rocks, animals, living grasses and plants, mushrooms, and other myxomycetes are recorded. The moist chamber protocol used for culture of mammal skulls as well as for bark from living trees and vines exemplifies user friendly methods used to study myxomycetes in the laboratory. Culture materials are readily available from local stores and are suitable for school children or for amateur hobbyists interested in observing the developing stages of myxomycete plasmodia and fruiting bodies.

### Plasmodial Types

The ability to recognize myxomycetes in the field and in culture is facilitated by concentrating on the visible stages of the life cycle. This involves a general understanding of the morphology and ecology of the plasmodium and fruiting bodies.

Free-living plasmodia feed by moving over the surface of the substratum, engulfing yeasts, fungal spores, bacteria, and other microorganisms by phagocytosis. Myxomycetes are thus often considered "microbial predators." There are three distinct plas-

Figure 2 a,b. *Physarum polycephalum* phaneroplasmodia. (a) on decaying log, cans, and leaf litter in Devil's Millhopper Geological State Park (a limestone sink), in Alachua County, near Gainesville, Florida. (b) on tree stump and oyster mushrooms (*Pleurotus ostreatus*). Figure 2 c,d. *Fuligo septica* showing developmental stages of the aethalium fruiting body type. (c) a single, bright, yellow phaneroplasmodium in early stages of forming a sessile, massive aethalium. Note finger ring for scale in Fig. c,d. (d) mature aethalium (shown in Fig. 2 d) approximately 12 hours later. Note the thick calcareous cortex that encloses the mass of black spores.

modial types generally recognized: the protoplasmodium, characteristic of the Echinosteliales; the aphanoplasmodium, characteristic of the Stemonitales; and the phaneroplasmodium, characteristic of the Physarales (Gray and Alexopoulos, 1968; Keller and Braun, 1999). A fourth type intermediate between the aphano- and phaneroplasmodium is termed the trichiaceous plasmodium (Keller, 1971), and is characteristic of the Trichiales. All of these plasmodial types are capable of movement as they feed, migrating different distances, and producing different types and numbers of fruiting bodies under optimal environmental conditions.

The protoplasmodium (Order Echinosteliales) remains microscopic throughout its development and migrates for short distances, usually less than 1mm. It has a plate-like shape that fails to develop advancing fans and trailing vein-like reticulate strands seen in other plasmodial types. Each tiny protoplasmodium gives rise to a single, tiny sporangium (usually less than 1mm), often occurring on the bark surface of living trees and vines. Species of *Echinostelium* and *Clastoderma* develop this type of plasmodium (Keller and Braun, 1999).

The phaneroplasmodium (Order Physarales) is the largest and often most colorful and frequently seen plasmodial type in the field (Fig. 2a,b). Polarity and directional movement at maturity result in an advancing, fan-shaped, anterior, feeding edge and a network of posterior trailing veins. Reversible protoplasmic streaming can be seen in the plasmodial veins with a dissecting stereomicroscope at 50X to 100X magnification. Protoplasmic flow moves toward the anterior end of the plasmodium, slows, then stops, then reverses direction, flowing toward the posterior end. The entire plasmodium has a raised, three-dimensional appearance with definite margins capable of covering and migrating up to 8m to drier sites. The phaneroplasmodial type represented by *Physarum polycephalum* Schwein. (Figs. 2a,b; 8b–e) and by *Diachea arboricola* H. Keller & M. Skrabal (Keller et al., 2004; Fig. 4a–d) may produce either thousands of sporangia, or as in *Fuligo septica* (L.) F. H. Wigg. (Fig. 2c,d), one massive aethalium filled with spores represented by a world record specimen measuring 70 x 54 x 3cm (Keller and Braun, 1999).

Phaneroplasmodia usually grow under drier conditions where free water is absent, traversing habitats both natural and man-made. Natural habitats include decaying leaves and logs, rotting tree stumps, bark surfaces of living trees and vines, and even decaying parts of xerophytic plants growing in desert conditions. Habitats associated with leafy liverworts, mosses, living herbaceous plants, and dung of herbivorous animals also have myxomycetes. Man-made artificial habitats include the dead basal leaves of ornamental flowers in flower beds, on living turf grass, stacked wood or sawdust piles, wood chips, bark or straw mulching, and baled hay or straw stacks weathering in fields (Keller and Braun, 1999; Krug et al., 2004; Spiegel et al., 2004; Figs. 2 a–d; 3a,b; 4 a–d; 8a–f; 9a–d; 10a–c; 11a,b; 12; 13a–d; 14). Unusual man-made habitats include windowsills, eaves of roofs, pressboard in houses, and wooden shingles of roofs (Keller, personal observations).

The aphanoplasmodium (Order Stemonitales) is characterized early in its development by a network of flattened, transparent threads, lack of polarity, and affinity for growing submerged in free water or under wet conditions. Young veins lack a distinct region of ectoplasm and endoplasm that facilitates movement through the interstices of decaying wood suddenly appearing overnight as hundreds of stalked sporangia *in situ* as in species of *Stemonitis* (Keller and Snell, 2002). Mature aphanoplasmodia on decaying leaves exhibit polarity and morphology typical of phaneroplasmodia and may migrate over short distances up to 10cm (Fig. 14). *Stemonitis flavogenita* E. Jahn in agar cultures migrates across the surface as immature stalked sporangia and, when fully mature, sporulates on the sides and lids of plastic Petri dishes (Keller, personal observations).

The trichiaceous plasmodium (Order Trichiales) combines morphological features of both the aphano- and phaneroplasmodial types. *Perichaena depressa* Lib. and *P. quadrata* T. Macbr. are examples of myxomycetes with this kind of plasmodium (Keller, 1971; Keller and Eliasson, 1992). The anterior-posterior polarity of an advancing fan and trailing veins is established early in development. Although the advancing fan is the active growth center for forward movement, the mass of the plasmodium forms fruiting bodies *in situ* without migrating over long distances. Numerous fruiting bodies often develop from a single plasmodium on decaying wood or leaves, but not on living plants. The early stages of trichiaceous plasmodial development are extremely flattened and inconspicuous, and require free water in agar culture, otherwise sclerotization ensues.

**Plasmodial Tracks**

Phaneroplasmodia create evidence of their movements as plasmodial tracks. These tracks show the former location of phaneroplasmodial veins, where plasmodia had fed and migrated over the surface of the substratum. Plasmodial tracks are analogous to footprints and can be recognized by excreted black waste matter along the vein margins, resulting in two distinct black lines separated by a light area in the middle (Keller et al., 2004; Figs. 4b; 9c,d). Outer bark sloughing off from standing dead trees often has plasmodial tracks on the inner, unexposed surfaces. These habitats stay moist for longer periods of time, providing optimal conditions for the plasmodium to grow, migrate, and form fruiting bodies (Keller and Braun, 1999; pp. 20–23).

Plasmodial tracks can be seen in many other places. A recently described new tree canopy myxomycete species, *Diachea arboricola*, represents an example where phaneroplasmodial tracks covered the bark surface and fissures of a living *Quercus alba* L. (white oak) tree, extending vertically approximately 15m (Keller

and Skrabal, 2002; Keller, 2004; Keller et al., 2004; Fig. 4a–d). Samples of this bark placed in moist chamber laboratory cultures yielded large, bright yellow phaneroplasmodia, which migrated over most of the bark surface and filter paper lining the bottom of a plastic Petri dish (Keller et al., 2004; Fig. 4c). Plasmodial tracks may be found on other substrata such as decaying leaf litter, stumps, logs, woody stems, and reproductive structures of plants, for example, on pinecones still attached to living trees.

### Fruiting Bodies

The mature fruiting body is the life cycle stage used to identify a myxomycete to species (Fig. 1N). It is primarily the combination of morphological characters inside the fruiting body such as the columella, capillitium, and spores (ornamentation, size, and color in mass), which are important in myxomycete classification and used in keys to different taxa (Martin and Alexopoulos, 1969). Fruiting bodies are represented by sporangia (Fig. 3a; 4d), plasmodiocarps (Fig. 3b), aethalia (Fig. 2d), and pseudoaethalia with both generic and detailed species descriptions based in part on these four different morphological types. In addition, the basic morphological type of a fruiting body is based on the presence or absence of a peridium, capillitium, pseudocapillitium, columella, stalk, and hypothallus.

The peridium is an acellular wall that encloses the spore mass and may exist in a variety of shapes and colors, depending on the fruiting body type and species of myxomycete. Some peridia are brilliantly colored in hues of orange, yellow, violet, and red, while others are iridescent or infused with calcium carbonate ($CaCO_3$) as in the Order Physarales, hence the designation "the biological jewels of nature" (Keller, 2004).

The capillitium is a network of threadlike strands sometimes attached to the columella (if present) that intermingles with the spores but are not connected to them. In some cases, the capillitial threads may actively disseminate spores as in some members of the Trichiales. In the genus *Trichia* the capillitial threads are hygroscopic, and when wetted and dried, twist and expand, actively expelling spores over a longer period of time. Pseudocapillitia occur in pseudoaethalia and aethalia as in the genus *Fuligo* and in the genus *Lycogala*. Pseudocapillitium is unlike true capillitium in that the strands are usually irregular in diameter. The columella is often an extension of the stalk within the peridium. Capillitial threads may be attached to the columella or to the peridium itself or to both. One may envision the columella as a "backbone" and the capillitial threads as the "limbs." The columella is not present in any members of the Orders Trichiales and Liceales. Spores are the reproductive units formed within the peridium. Spore shape, color, size, and ornamentation vary among species and are valuable in myxomycete identification.

A stalk may or may not be present as part of the sporangium. The primary function of the stalk is to support and elevate the spores above the substratum, protecting the sporangium from excessive moisture, and also aiding in the dissemination of the dry, powdery spore mass. The stalk may be useful in identifying myxomycetes such as in the Order Physarales where the stalk contains $CaCO_3$ and in the Order Stemonitales where the stalk is either hollow or filled with fibrous strands. The hypothallus is found at the base of the stalk and may be transparent or opaque (Gray and Alexopoulos, 1968; Martin and Alexopoulos, 1969; Stephenson and Stempen 1994; Keller and Braun, 1999).

### Field Collections, Photography, and Laboratory Cultures

Bark samples taken from living trees and vines, decaying leaves from forest litter, decaying bark from decomposing logs on ground sites, and other organic materials can be cultured using the moist chamber technique as described by Keller and Braun (1999, pages 24-25; Snell and Keller, 2003).

*Collection of substrata*.—A canine (dog) skull was discovered in the Daniel Boone National Forest, Redbird District, Clay County, Kentucky near the Redbird District Office on June 16, 2006. This area was a bottomland riparian habitat community of *Carya glabra* (Mill.) Sweet (pignut hickory) and *Platanus occidentalis* L. (sycamore), with steep slopes on both sides. Scattered sycamore trees provided most of the leaf litter where the dog skull was found next to a dry shallow stream bed about 3m wide. The canine skull was partially buried in leaf litter with 6cm of leaves covering the upper jaw and side of the head. The lower jaw was missing and no other skeletal remains were present. A few leaves adhered to the upper palate and leaf litter debris was in the nasal

Figure 3. Examples of substrata that support myxomycete diversity. (a) *Physarum rubiginosum* (sessile sporangia) fruiting on moss. (b) *Physarum cinereum* fruiting bodies (sessile, spherical sporangia and elongated plasmodiocarps)) on a blade of living, Saint Augustine grass.

Figure 4. *Diachea arboricola,* a new tree canopy myxomycete species discovered in the Cades Cove area of Great Smoky Mountains National Park. (a) University of Central Missouri undergraduate student Melissa Skrabal, using the double rope climbing method to sample bark with plasmodial tracks and sporangia from a living white oak tree #88 (*Quercus alba*)**.** (b) phaneroplasmodial tracks on white oak bark collected in the field. (c) three different yellow phaneroplasmodia migrating on filter paper in moist chamber culture. Note the darker veins that leave plasmodial tracks on the bark surface seen in Fig. b. (d) stalked sporangium with iridescent, glittering silvery peridium with a basal ring of rainbow-like colors in contrast to the pink-orange stalk (total height 1.2mm).

chamber. Plasmodial tracks recognized by students were on portions of the skull (ARS, CMK, and SEE; Fig. 9a,b).

A female deer skull was found June, 2005 at Larkin Fork in Jackson County, Alabama. The field site was located atop a large bluff leading down to Opossum Hollow. The skull was partially buried in oak leaves not yet softened due to decay. The skull was prostrate, teeth against the ground, completely devoid of skin and well weathered. No other skeletal remains were present. One author (CMK), who was not familiar with myxomycetes at the time of collection, kept the skull wrapped in tissue paper on a shelf at home. Not until collection of the dog skull the next summer did she realize the deer skull might also have plasmodial tracks. Indeed, conspicuous plasmodial tracks were on the underside of the deer skull (Fig. 9c,d). Skull specimens were brought to the laboratory to be photographed and cultured (Fig. 5; 6).

*Macro-photography of specimens.*—Photographs were taken of the plasmodial tracks before placing the skulls in moist chamber culture. Photographic equipment included a Canon EOS 10D and Nikon D2X digital single lens reflex cameras. Lenses used were a Canon MP-E 65mm lens, Tamron 28-300 mm lens, and a micro Nikkor 105mm lens. Other related equipment included a tripod, laptop computer, daylight balanced compact fluorescent bulbs, gooseneck desk lamps, background material, background holder, turntable (lazy susan), glass pie dish, and salt (Fig. 5)**.**

Cameras were operated to capture the sharpest images possible with remote capture software (DSLR Remote Pro by Breeze Systems and Nikon Capture 4) and images were saved directly to the laptop. Capture software connected the camera to the computer via a USB cable. Aperture and shutter speed was set through the computer and triggered via remote control, reducing vibration caused by depressing the shutter manually. The image was sent directly to

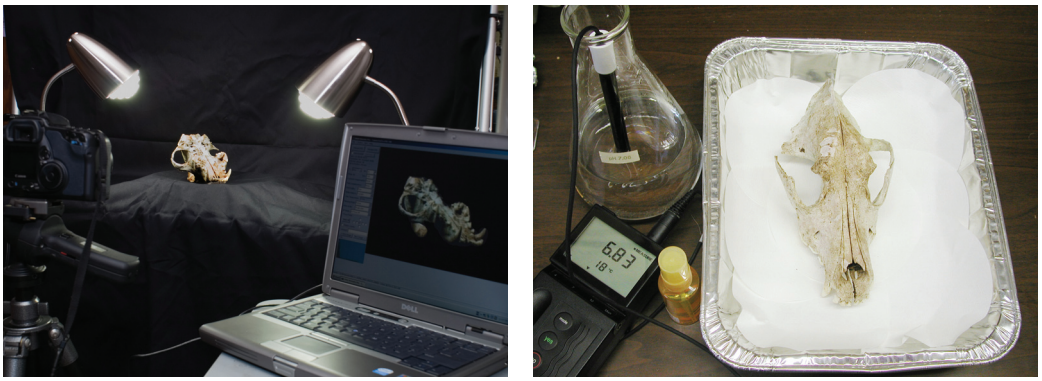

Figure 5 (left)**.** Macro-photography studio arrangement of the dog skull. Figure 6 (right). Moist-chamber culture of dog skull. Note pH meter with flat probe and mist spray bottle.

the computer to allow for visual inspection of the image at full screen. Focal adjustments, exposure, and lighting were made without having to remove the digital media, since the tiniest movement of the camera can significantly affect focus. The camera was set to mirror lockup so there was no vibration from the mirror during shutter release. The images were captured in raw format to allow for the greatest adjustments to exposure and white balance.

When the whole specimen was photographed, the area was staged with simple black fabric for the background. Daylight balanced bulbs were installed in desk lamps and positioned on either side of the specimen. Background lighting depended on the specimen and how much working distance there was between the lens and the specimen. The daylight balanced fluorescent bulbs emitted little heat, so they were ideal for macro work to adequately light the field of view when the light source needed to be close to the lens. A shallow glass container was filled with salt to hold the specimen in place and to allow for proper rotation and angle to achieve maximum depth of field. The salt conformed to the shape of the skull and held the skull in position when tilted or rotated. The glass container was placed on a turntable to aid in positioning of the specimen. Any other room lighting was turned off to prevent color shift from mixed lighting sources. Natural daylight did not interfere with color (Fig. 5).

*Various laboratory moist chamber culture techniques*.—Moist chamber cultures were first used by Gilbert and Martin (1933) who wanted to show their botany classes the algae that grew on the bark surface of living trees. The surprise discovery of two undescribed myxomycete species has resulted in the popular use of moist chamber cultures today. These cultures have been used to obtain myxomycete fruiting bodies from dormant stages (spores, microcysts, sclerotia) on different substrata gathered from different habitats. Various laboratory protocols and materials for culture chambers are available depending on the laboratory conditions and purpose of the moist chamber technique. Research protocols (technical-professional) use supplies, equipment, and laboratory conditions that may not be available for use in the classroom or at home (Snell and Keller, 2003). General information about moist chamber cultures and harvesting fruiting bodies is available in books (Stephenson and Stempen, 1994; Keller and Braun, 1999). More details about moist chamber cultures (Braun and Keller, 1977; Snell and Keller, 2003) and useful tools (Sundberg and Keller, 1996) can be found in journal papers.

*Technical-professional*.—Moist chamber culture techniques used for research projects by undergraduate and graduate students at the University of Central Missouri followed a specific protocol. Petri plates (sterile, plastic, disposable, and 150 X 25mm) were each fitted with a single, sterilized sheet of P8-creped filter paper that covered the bottom. Bark was placed face up on the paper in a single, non-overlapping layer that covered the bottom of the Petri dish. Approximately 35ml of de-ionized, distilled water sterilized in an autoclave was adjusted to pH 7 manually using potassium hydroxide and/or hydrochloric acid. Distilled water is often acidic (pH of 5-6). Non-buffered water (pH 7) was used so any change in pH would be influenced solely by the tree bark. Three pH measurements were taken for each moist chamber culture at 24 hours from the wet date. All pH measurements were taken using an ORION model 610 flat probe pH meter, which was calibrated prior to each use (Fig. 6). The moist chamber culture dishes were incubated at room temperature (23-25°C) under normal indirect sunlight (Snell and Keller, 2003).

Cursory scanning of cultures was conducted for each plate to check for existing fruiting bodies. Each plate was examined 48 hours after wetting and thereafter on day 4, 8, 16 and 32. Cultures were examined at 50X to 150X magnifications using a dissecting stereomicroscope with a low heat, fiber optic light source (Fig. 7a,b). The entire plate of bark samples was scanned meticulously to detect myxomycete plasmodia and sporangia, and all observations were recorded. Attentive scanning of the moist chamber cultures at higher magnifications detected most myxomycete species, but some fruiting bodies were difficult to see. For example, some Echinostelia are only 50 to 100µm in total height. Mature fruiting bodies were removed from the bark as small pieces to reduce disturbance to the sample. A final scanning was performed after plates dried, to search for any remaining fruiting bodies on the surface and on the undersides of the bark. Moist chamber culture incubation was limited to 32 days in order to reduce the probability of non-corticolous species that would not naturally grow on the tree bark.

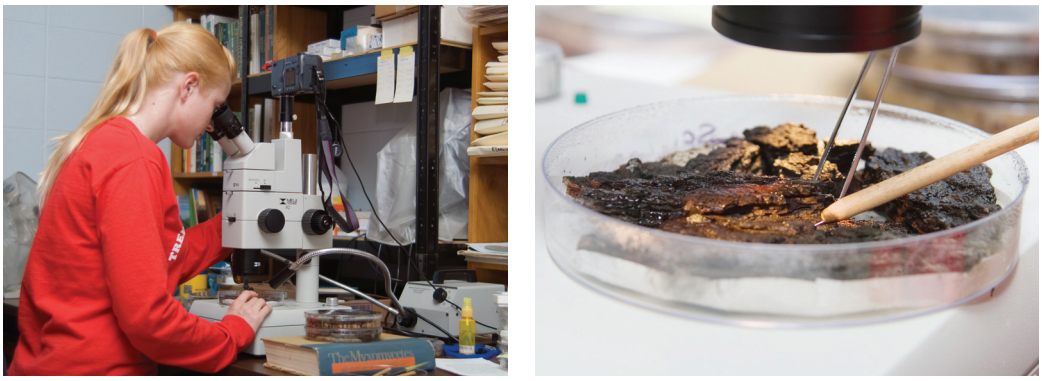

Figure 7. (a) Courtney Kilgore scanning moist chamber bark cultures. (b) plastic Petri dish with lid removed to show moist bark.

Dried bark samples with myxomycete fruiting bodies were harvested from the moist chamber cultures and carefully glued into the bottom or top of a small slide box (7.62cm x 5.08cm x 3.18cm). The collection boxes served as voucher specimens. Each box was labeled with the myxomycete species name, the location and habitat, the tree species, tree number, height of sample, plate number, wet date, harvest date, the collector's initials, and the collection number (e.g. *Comatricha laxa* Rostaf., found on the bark of living *Juniperus virginiana* L. (eastern red cedar), Pertle Springs, Johnson County, Missouri, tree #279, 3m. Plate 1, wetted April 10, 2005, harvested April 2, 2005, collector: HWK #). The side and bottom of the box was also labeled with the collector's initials and the collection number in order to prevent switching the lid with the label and the bottom containing the specimen (Keller and Braun, 1999). Myxomycetes were identified to species using dichotomous keys (Martin and Alexopoulos, 1969; Keller and Braun, 1999).

Myxomycete fruiting bodies harvested from moist chamber cultures often had insects, especially collembola, that were unwanted in specimen boxes. One way to kill insects and avoid the use of toxic chemicals is to store the specimen box overnight in the freezer compartment of a refrigerator.

*Classroom-educational.*—Warrensburg Middle School 7th-grade life-science students used a slightly modified version of the moist chamber culture described above. A pH meter may not be available, so narrow-range paper pH indicator strips are used to measure the approximate pH. Scanning timetables of moist chamber cultures coincide with classroom meetings. Student stereomicroscopes are used at 20X to 40X magnifications.

Students learn about our tree canopy biodiversity project in the Great Smoky Mountains National Park (GSMNP) and at Pertle Springs (Univ.y of Central Missouri outdoor land laboratory), Warrensburg, Missouri through three phases: the Adventure Phase, the Laboratory Phase, and the Publication Phase. The Web site "Exploring Life in the Forest Canopy" (http://warrensburg.k12.mo.us/ iadventure/GSMNPiadventure), mirrors the three phases and enables students to realize a virtual real world experience and learn about the All Taxa Biodiversity Inventory in GSMNP (Keller, 2005).

A second tier of this same web site represents a problem solving activity in which students determine the direction and outcome of a parallel research study, using resources available on the Internet, particularly resources providing real-world data and primary documents. This activity is designed to help students discover how to access and use unlimited data and information from the Internet to conduct research, solve problems, and make choices. This web site also includes a detailed teacher's guide. Teachers are encouraged to access the teacher side of the web site to develop and conduct parallel field-based research in their locality.

*Do-it-yourself for beginners.*—Moist chamber cultures can be prepared with easy-to-obtain materials not mentioned in the above publications. Instead of sterile plastic Petri dishes a round pie tin

Figure 8. *Physarum polycephalum* yellow phaneroplasmodium associated with the mushroom *Lentinus tigrinus* in the field (b, c) and in moist chamber culture (d–f). (a) habit of mushroom in the field, caps 1-3 cm across. (b) phaneroplasmodium mostly on five different mushrooms. (c) close-up view of phaneroplasmodium on cap and stalk of mushroom. (d) phaneroplasmodium migrating onto the plastic wrapping that covered the moist chamber culture. Note the "blob" now has an advancing, anterior, fan-shaped, feeding edge and a network of trailing veins. (e) phaneroplasmodium four days after wetting. Note the thick veins of the phaneroplasmodium covering the decayed branch. (f) yellow sclerotium on filter paper in pie tin cultures.

substitute can be used and is available in different sizes, preferably 22cm wide and 3cm deep. The surface of the pie tin should be wiped with 70 percent rubbing alcohol and the bottom lined with absorbent paper toweling (Fig. 8f). This toweling does not have to be sterile but can be microwaved to decrease mold contaminants. De-ionized water is preferable to tap water with chlorine but both will work. Water can be sterilized by boiling it then letting it cool to room temperature before use. Place the bark or leaves (without overlapping pieces) on the paper toweling lining the bottom of the pie tin. Add 2.5 tablespoons of water around the bark to ensure that the bark stays moist. Do not submerge the bark in water because excess water on the bark surface interferes with development of some myxomycete species. Place white, waterproof adhesive tape on the inside rim of the pie tin and use a lead pencil to record the collection data and wet date. Cover the surface of the pie tin with transparent PVC Cling Film Wrap stretched tightly and sealed under the lip of the pan. This actually works better than Petri dish lids because less water condenses on the PVC. Place the pie tins on a flat shelf surface near a window that provides indirect sunlight. These cultures may produce plasmodia and fruiting bodies that can be seen with the unaided eye or with a 10X hand lens or by scanning with a dissecting stereomicroscope. Myxomycetes often develop fruiting bodies at night, so if you are a "night owl," you will see the developmental stages as the plasmodium changes color and shape to form the fruiting bodies. Moist chamber cultures are miniature "gardens" that may have life forms such as cyanobacteria (formerly called blue green algae), green algae, lichens, leafy liverworts, mosses (Fig. 3a), mushrooms, colorful myxobacteria, myxomycetes, nematodes, tardigrades, insects, and arachnids (Fig.11a,b; Keller, 2005).

Instructions for making handcrafted fine dissecting needles, flattened dissecting and lifting tools, dissecting micro-knives, a hand-held small air stream blower (microblower) used in preparing myxomycete fruiting bodies for examination are given in Sundberg and Keller (1996). For example, the microblower aids in the removal of spores to show the internal structures such as the capillitial system and attachments to the columella and peridium often used in species descriptions and identification keys.

*Microscopic Slide Preparation*.— Microscope glass slides, 25 X 75 X 1mm, preferably with rounded corners and with a frosted end, are used with a compound microscope to observe fruiting body parts (spores and capillitium) at higher magnifications (400X high power objective lens or 1,000X oil immersion objective lens). Species descriptions and illustrations of spore ornamentation in Martin and Alexopoulos (1969) were based on spores measured at 400X total magnification with an ocular micrometer. More accurate spore descriptions and spore diameter measurements recorded in optical section from tip of ornamentation to tip are possible using an oil immersion objective lens at 1000X. Tap water may be used for temporary slides and clear lactophenol without cotton blue for permanent mounts. One drop of either liquid is all that is needed for the slide.

Slide preparation involves removing the myxomycete fruiting body from the moist chamber culture using tweezers or a needle and placing the specimen centered in the liquid. Water is the best general purpose mounting medium to show spore and capillitial details in calcareous and non-calcareous fruiting bodies. Water mounts should be made by dropping a cover glass (No. 1 ½ 22mm sq) on the water droplet with myxomycete fruiting bodies to create isolated air bubbles with spores and capillitial threads trapped inside. These bubbles produce a "lens effect," increasing magnification above that of the optical system of the compound microscope, so spore and capillitial thread ornamentation can be seen in greater detail (Sundberg and Keller, 1996). Fruiting bodies (dried and matured) immersed directly in a water droplet often float on the surface due to surface tension of the water. In these cases a small droplet of 70% alcohol can be added next to the water droplet and the fruiting body submerged in the alcohol (this lowers the surface tension) then intermixed in the water droplet. Slides and cover glasses should be cleaned with soap and water prior to use. Cover glasses often stick together, preventing use of the oil immersion lens due to the extra thickness. Thin edges of the cover glass are sharp so caution and a forceps should be used in making slides.

Preparation of slides using clear lactophenol as a mounting medium should not be used for the calcareous bearing myxomycete species. Lactic acid dissolves calcium carbonate in the capillitium of *Badhamia* or *Physarum* species, resulting in the release of bubbles. Species in the Orders Liceales, Stemonitales, and Trichiales, can be mounted in clear lactophenol by gently placing a cover slip against the liquid allowing the surface tension to break. Once the liquid is attracted to the cover slip, using a forceps slowly lower the cover slip onto the slide to prevent formation of air bubbles. Air bubbles result in gradual drying of the lactophenol over time after being sealed. Lactophenol mounts should be sealed with resin (Zut–slide ringing compound) or clear nail polish to make a permanent slide (Keller, personal observations). A sharpened No. 2 lead pencil can be used to inscribe the label on the frosted end of the slide then covered with transparent scotch tape to prevent smudging. Slides should be stored in a horizontal position in a slide box to prevent the specimen from drifting to the edge of the cover slip over time. Some lactophenol slide mounts were still in good condition after 35 years (Keller, personal observations).

*Special modifications for oversized specimens*.—The large size of the skulls (both were approximately 24cm in length, 16.5cm width, and 8.9cm deep) required a special container. An aluminum-roasting pan 5cm deep, 30.5cm in length, and 25cm wide was wiped clean with 70% rubbing alcohol and the bottom covered with several layers of sterile filter paper. Sterile, de-ionized,

distilled water adjusted to a pH of 7 was poured into the bottom of the pan and the surfaces of the skulls were sprayed with a squirt bottle. Transparent PVC Cling Film Wrap was used to enclose the skull within the pan to prevent drying. The skulls were observed and wetted with a misty spray every 48 hours during a 32-day period starting January 20, 2007 (Fig. 6).

### Unusual Sites for Myxomycete Plasmodial Tracks and Fruiting Body Development

Bright yellow phaneroplasmodia of *Physarum polycephalum* are often associated with *Pleurotus ostreatus* Fr. fruiting bodies (oyster mushroom; Fig. 2b) on standing dead or dying trees or on decaying logs or stumps (Fig. 2a,b). Plasmodia may migrate on to living ground cover such as ferns, poison ivy, shrubs, and herbaceous plants to form stalked sporangia (Keller and Braun, 1999). *Physarum polycephalum* was found as both phaneroplasmodia and stalked sporangia, during the months of September and October at River Legacy Parks near the Living Science Center in North Arlington, Texas. This Texas site was where the plasmodium had developed the largest continuous mass observed (8m) on *P. ostreatus*, growing along the side of a dead, lodged tree trunk at about 2 meters off the ground. Portions of the mushroom had fallen on ground leaf litter where thousands of sporangia had formed (Keller, personal observations). Unfortunately no photograph was taken. I did consider taking my shoes and socks off (HWK) to see if the plasmodium would crawl onto my feet, but I did not have the patience to wait overnight. An interesting contrast is the recent first report of *Physarum pusillum* (Berk. & Curtis) G. Lister sporangia fruiting on the head, body, and legs of a living, cryptic lizard *Corytophanes cristatus* (Merrem.). This represents an unusual situation where the living phaneroplasmodium had enough time to grow, then migrate and fruit on the skin surface of a motionless animal (Townsend et al., 2005; *Mycologia* front cover image).

A bright yellow plasmodium was found near Warrensburg, Missouri, Johnson County, at the Ralph and Martha Perry Memorial Conservation Area on March 30, 2007. Temperatures were approximately 27° C during an early spring warm spell. The decorticated tree branch was near a small creek and the ground litter was well saturated from previous spring rains. The phaneroplasmodium was confined to the fruiting bodies of *Lentinus tigrinus* (Bull.) Fr., formerly called *Panus tigrinus*, and on the wood surface (Fig. 8b–e). The decaying branch was placed in a pie tin as previously described. Sterilized, old fashioned oat flakes were scattered near the plasmodium and in several days the mushroom began to decompose as the phaneroplasmodium increased in size, migrating over the surface of the wood, the mushroom, the sides of the pie tin, and on to the plastic wrapping (Fig. 8d,e). Unfortunately, the plasmodium did not form sporangia but instead produced a yellow, sheet-like, flattened sclerotium (Fig. 8f).

The sclerotial stage when encountered in nature is a source for starting agar cultures or moist chamber cultures. Sclerotia often form in protected areas of a decaying log next to the ground or under the sloughing bark. Removal of the sclerotium with a forceps and rewetting in a moist chamber culture will trigger the formation of a plasmodium to begin actively feeding (Keller, personal observations). Collectors should mark the location of logs with the myxomycete sclerotial stage and revisit them over time as a future source of fresh plasmodia in culture.

A second attempt to culture the sclerotium from the decaying branch was successful. The bright yellow plasmodium covered the branch and filter paper eventually producing fruiting bodies typical of *Physarum polycephalum* on the filter paper and sides of the pie tin.

*Fuligo septica* is one of the most common myxomycetes and

Figure 9. Phaneroplasmodial tracks on dog and deer skull. (a) dog skull. (b) c lose-up of phaneroplasmodial tracks on upper palate of dog skull. (c) deer skull. (d) close-up of phaneroplasmodial tracks on left molar and cheek bone of deer skull.

can be found fruiting just about anywhere, on bark mulching in parking lots, campuses, and parks, on thatch in lawns, decaying piles of grass or leaves, on decaying logs, near the base of living tree trunks, and on tree stumps (Kilgore, personal observations; Fig. 2c,d). The common name for this species is the "dog vomit" myxomycete because that is what the mature aethalium resembles. The edibility of the plasmodial stage of myxomycetes has been shrouded largely in myth. However, the large, yellow phaneroplasmodium of *Fuligo septica* is sought by the Indians in the vicinity of Veracruz, Mexico as a food source. Supposedly, the plasmodium is fried with onions and peppers and is eaten on a tortilla. It is referred to as "caca de luna" or "poop of the moon" (Lopez et al., 1982; Keller and Braun, 1999).

*Physarum cinereum* (Batsch) Pers. is frequently found covering extensive areas several meters across in grassy lawns (Fig. 3b). Immature stages appear blackish and give the grass an oily appearance that causes urban homeowners to express concern that their grass has a turf disease. In a few hours the fruiting bodies dry out and give an ashen-gray appearance due to the formation of calcium carbonate in the peridium. This myxomycete has been implicated in killing grass, but usually mowing or hosing down the area with water will remove the cause of concern. However, resting stages survive in the soil since rainy conditions will produce *P. cinereum* in the same grassy location year after year, often in a circular pattern much like mushroom fairy rings (Keller, personal observations).

This is the first known report of plasmodial tracks discovered on a dog skull from Kentucky and a deer skull from Alabama. No myxomycete stages developed on either skull in moist chamber culture. The plasmodial tracks were the phaneroplasmodial type and therefore were made by a taxon in the Order Physarales. One of the most common genera of myxomycetes found in leaf litter was *Didymium*, and it is possible that the plasmodial tracks belonged to a species in that genus.

The phaneroplasmodial tracks on the dog skull covered a 40mm area on the molars along the right side of the jaw, 22mm on the posterior part of the roof of the mouth, 45mm length and 21mm width on the roof of the mouth (Fig. 9a,b). Two distinct areas of phaneroplasmodial tracks were present on the deer skull. The largest plasmodial track extended along the entire length of the upper jaw, even appearing on the teeth, and measured 130mm in length from the back molar to the tip of the nose, a 36mm width extending to the cheek bone, and upwards to the lower bone of the eye socket. On the underside of the skull the plasmodial tracks were present on the back of the head, measuring 37mm in length, 6mm in width. Tracks also occurred where the spinal column connects to the brain case, measuring 8mm in length, 14mm width on the left side and 15 mm length, 10mm width on the right side (Fig. 9c, d).

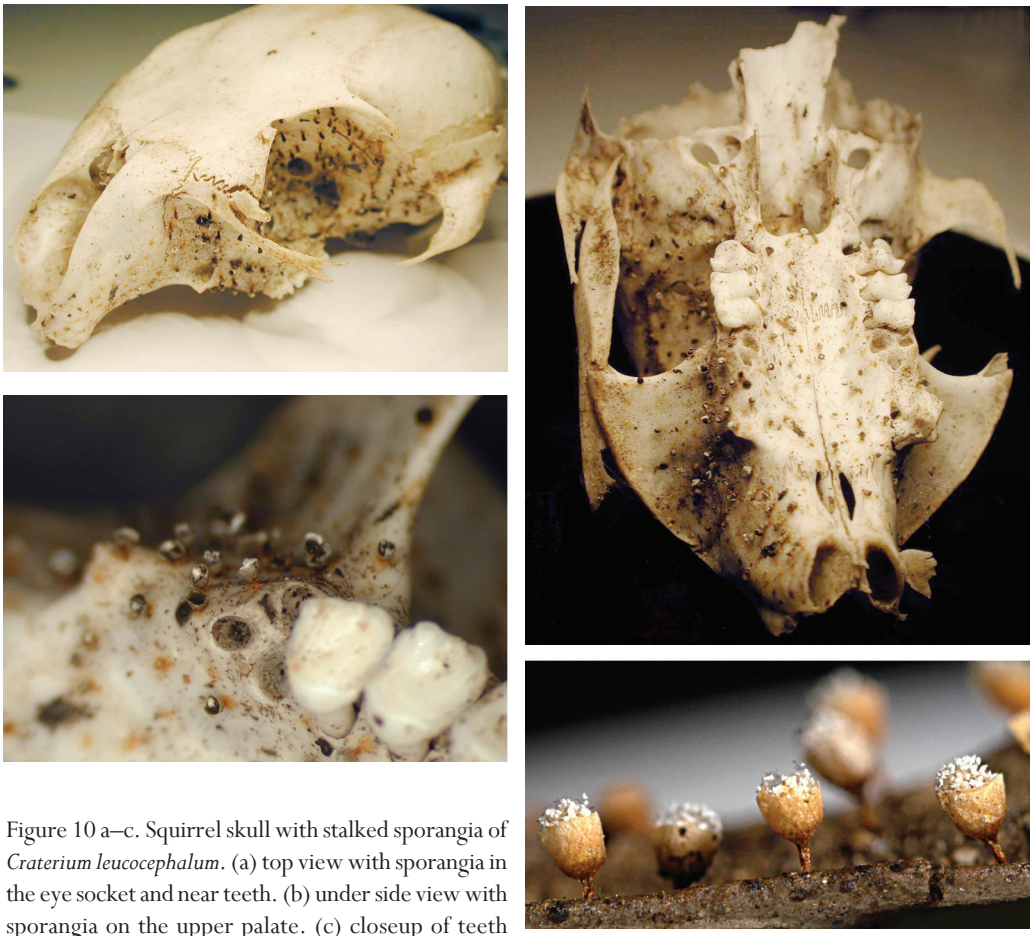

Figure 10 a–c. Squirrel skull with stalked sporangia of *Craterium leucocephalum*. (a) top view with sporangia in the eye socket and near teeth. (b) under side view with sporangia on the upper palate. (c) closeup of teeth showing sporangia nearby on upper palate. (d) close-up of the vase-shaped, stalked sporangia typical of this genus (0.5-0.7 mm in diameter and 1–1.5 mm in total height) easily visible to the naked eye.

*Craterium leucocephalum* (Pers.) Ditmar is an example of a myxomycete species that forms a phaneroplasmodium capable of migrating on leaves, twigs, and in this case a squirrel skull. Numerous stalked sporangia appear in the orbit of the eye and the roof of the mouth (Fig. 10a-c). El-Hacène Seraoui collected this specimen in the area of La Chaise-Dieu, Haute-Loire, France, in November, 1997. The ground litter was derived mainly from coniferous forest. Skulls may serve as a hardened surface much like other debris such as twigs, wood fragments, and decaying

Figure 11. *Licea poculiformis* stalked sporangium on a spider leg. (a) general habit (total height 0.2) at low magnification. (b) enlargement of sporangium shown in Fig. 11a.

logs and leaves where myxomycete plasmodia and fruiting bodies typically are found (Fig. 10d).

*Licea poculiformis* Ukkola was collected by Kayoko Fujioka (KF 1217) August 2, 2005, Japan, Yakage-cho, Odagun, Okayama Prefecture, on the leg of a dead spider on a living *Pinus* tree (Fig. 11a,b). The spider was near a green moss where the protoplasmodium of this species sometimes sporulates. The tiny protoplasmodium forms a single, tiny, stalked, goblet-shaped sporangium, separating into two halves marked by a line of dehiscence clearly visible in Figure 11b. This corticolous myxomycete is rare, known from the type locality in Africa, Tanzania, Tanga Province, Lushoto District, East Usambara Mountains on mosses of bark from living *Cupressus* sp. in moist chamber cultures and also from Mexico, Quintana Roo, El Eden, on bark of living *Hematoxylon compechianum* L. in moist chamber culture. This is the first published record of a myxomycete sporangium photographed on an arachnid.

*Physarum albescens* Ellis is a common snowline species that forms short-stalked, obovoid sporangia covering extensive areas. Bright yellow phaneroplasmodia migrate under the melting snowbanks then migrate to higher, drier substrata such as rocks shown herein (Fig. 12). This specimen was collected in Colorado, Archuleta County, in the Upper Blanco Basin, altitude 2530 meters, April 17, 2004.

Figure 12. *Physarum albescens* sporangia on a rock

*Diachea arboricola* H. W. Keller & M. Skrabal is a myxomycete species that only occurs in the canopy of living trees (Keller and Skrabal, 2002; Keller, 2004; Keller et al., 2004; Fig. 4a–d). Members of the genus *Diachea,* especially *Diachea leucopodia* (Bull.) Rost. on leaf litter, often migrate several meters in distance to form stalked fruiting bodies at higher and drier sites. The phrase "biological jewels of nature" aptly describes the stalked, iridescent spore case (glittering gold, silver, and a ring of rainbow colors at the base) of *D. arboricola*. These beautiful peridial colors contrast with the pink to reddish-orange stalk (Fig. 4d).

One stalked sporangium of *Physarum pusillum* (Berk. & Curt.) G. Lister (total height 1.5mm) formed from a watery white phaneroplasmodium that typically produces many sporangia (Eliasson et al., 1988). This sporangium developed in moist chamber culture on a sessile plasmodiocarp of *Perichaena vermicularis* (Schw.) Rost. In this case the phaneroplasmodium separated into a small protoplasmic unit that migrated some distance on top of another myxomycete to form a typical stalked sporangium (Fig. 13a).

A tiny, stalked sporangium of *Barbeyella minutissima* Meyl. (0.5mm in total height) has sporulated on the columella of *Lepidoderma tigrinum* (Schard.) Rost. (Fig. 13b). *Barbeyella* has not been grown from spore-to-spore in culture to observe the plasmodial stage. The small droplets associated with leafy liverworts suggest the protoplasmodial type that forms a single sporangium. This specimen was gathered by Marianne Meyer as collection #9176, November 8, 1996, in France, 73-Savoie, Rognaix, elevation 864m, on a well-decayed spruce log covered with moss.

*Diachea subsessilis* Peck is an example of a stalked myxomycete sporangium (total height of 1.2mm) that sporulated on top of a fungus (Fig. 13c). This is the only species of *Diachea* that has reticulate spore ornamentation (Gaither and Keller, 2004). The calcareous, white stalk and iridescent blue peridium are the hallmark characters of the external morphology. A small portion of the phaneroplasmodium migrated on top of the fungus and formed a single sporangium instead of many sporangia, as is usu-

ally the case. El-Hacène Seraoui collected this distinctive species August, 2004 in Pompaples, Vaud, Switzerland, elevation 600m, in a wetland area dominated by *Alnus*.

*Clastoderma debaryanum* Blytt has tiny, stalked sporangia (1-1.3mm in total height) and a spore case (0.2mm in diameter) that develops from a tiny protoplasmodium. It is generally found on decaying logs and wood fragments on ground sites, on bracket fungi attached to dead standing trees, and on the bark of living trees. The single sporangium shown here (Fig. 13d) is the result of the protoplasmodium that migrated on to the peridial surface of *Diderma chondrioderma* (de Bary and Rost.) G. Lister (another myxomycete) in moist chamber culture. The source of these specimens was a living tulip poplar (*Liriodendron tulipifera* L.), 24m high in the canopy. Bark samples were collected May 19, 2007 from Rock Creek Park, Washington, D.C. during a Bioblitz sponsored by the National Geographic Society and the National Park Service.

Stalked sporangia of a *Stemonitis* sp. sporulated on the white stem of the achlorophyllous, vascular plant, *Monotropa uniflora* L. (indian pipe; Fig. 14). This unusual discovery was observed on a forested trail near the Appalachian Highlands Science Learning Center at Purchase Knob on August 5, 2006, (approximately 1524m elevation) in Haywood County, on the NC side of Great Smoky Mountains National Park. Fortunately, this specimen was photographed in the field by CMK, because afterwards it was placed in the refrigerator and lost.

Another example not shown here is a stalked sporangium of *Didymium ovoideum* Nann-Bremek. that sporulated on top of *Didymium minus* (Lister) Morgan as part of an intermixed field collection (Eliasson, et al., 1988; see Fig. 2, p. 377). *Didymium minus* was represented by a prematurely developed sporangium and a small part of a *D. ovoideum* phaneroplasmodium that migrated to the top of one sporangium and sporulated.

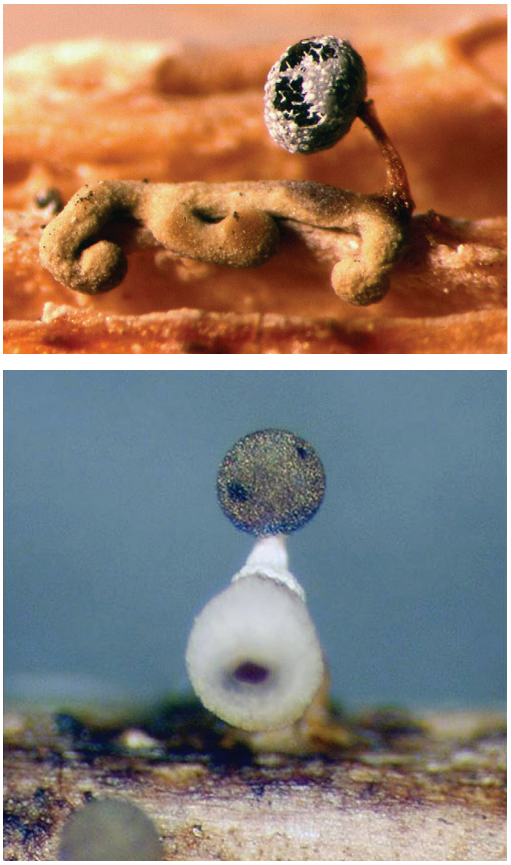

Figure 13. Stalked myxomycete sporangia sporulating in unusual places. (a) *Physarum pusillum* on top of plasmodiocarp of *Perichaena vermicularis*. (b) *Barbeyella minutissima* on columella of *Lepidoderma tigrinum*. (c) *Diachea subsessilis* on a fungus. (d) *Clastoderma debaryanum* on *Diderma chondrioderma*.

**Conclusions**

Myxomycetes form plasmodia and fruiting bodies wherever there is adequate moisture, moderate temperatures, and organic matter. These conditions may result in the plasmodial stage migrating onto the surface of rocks, living animals, dead animal remains (skulls), living plants, fungi, and other myxomycete fruiting bodies. These unusual occurrences in most cases are accidental and are the result of the plasmodium migrating and sporulating on a higher and drier site. However, the plasmodium of *Physarum polycephalum* is so frequently associated with fleshy mushrooms that this association cannot be ascribed to chance. Perhaps a combination of food organisms and nutrients available from the decomposing mushroom fruiting bodies accounts, in part, for this association.

Phaneroplasmodia migrate the greatest distances, produce the greatest number of individual sporangia, and also develop into the largest myxomycete fruiting body, as a single aethalium. A small portion of the phaneroplasmodium (with a large surface to volume ratio) may develop into a single, stalked sporangium such as in *Diachea subsessilis*. In this case, a tiny portion separated from a large phaneroplasmodium and migrated to the top of a fungus to form a perfect stalked spo-

Figure 14. Stalked sporangia of *Stemonitis* sp. on stem of *Monotropa uniflora* (indian pipe).

rangium. This pattern of development has not been documented in previous publications.

Protoplasmodia are usually microscopic and form a tiny, single sporangium. Examples such as a stalked sporangium of *Licea poculiformis* on a spider leg and *Clastoderma debaryanum* A. Blytt on another myxomycete represent the first observed reports of this unusual occurrence.

The tools, culture methods, and specimen and slide preparation described here can be used by school children for research projects, scientific professionals in research laboratories, and amateur hobbyists in their homes.


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Photo credits: Henry C. Aldrich=Figs. 2a,c,d; Samuel S. Ristich=Fig. 2b; Alain Michaud=Figs. 3a, 10d, 13b; Ray Simons=Fig. 3b; Harold W. Keller=Figs. 4a; 6; James Murray=Fig. 4b,c; Sydney E. Everhart=Fig. 4d; Glenda Carmack=Figs. 5, 7a,b, 8d,e,f, 9a–d; Christopher D. Crabtree=Fig. 8a–c; El-Hacène Seraoui=Fig. 10a–c, 13c; Michel Poulain=Fig. 11a,b; 13b; Theodore Stampfer=Fig. 12; Uno Eliasson=Fig. 13a. This color image was published as a black and white photograph in Mycological Research by Eliasson, 1991, page 264, Figure 17 and is used here with permission of the British Mycological Society. Courtney M. Kilgore=Fig. 13d, 14. Lisa Schmidt assisted with the preparation and design of the color plates.

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