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COMPARATIVE ANALYSIS OF THE REPRODUCTIVE ECOLOGY OF *MONOTROPA* AND *MONOTROPSIS*: TWO MYCOHETEROTROPHIC GENERA IN THE MONOTROPOIDEAE (ERICACEAE)[1]

MATTHEW R. KLOOSTER[2,3] AND THERESA M. CULLEY[2]

[2]Department of Biological Sciences University of Cincinnati Cincinnati, Ohio 45221-0006

Studies of mycoheterotrophs, defined as plants that obtain carbon resources from associated mycorrhizal fungi, have fundamentally contributed to our understanding of the importance and complexity of symbiotic ecological interactions. However, to date, the reproductive ecology of these organisms remains empirically understudied, with existing literature presenting hypotheses about traits including a generalist pollination syndrome and autogamous self-pollination. To address this gap in our knowledge of the reproductive ecology of mycoheterotrophic plants, we comparatively analyzed three species of two monotropoid genera, *Monotropa* and *Monotropsis*. During three consecutive years of field observations and manipulations of four populations of *Monotropa uniflora*, seven of *M. hypopitys* (both red and yellow color forms), and two of *Monotropsis odorata*, we investigated flowering phenology, pollination ecology, breeding system, floral herbivory, and reproductive effort and output. Contrary to previous predictions, our results revealed that taxa are largely outcross-pollinated and specialized toward *Bombus* pollinators. Additionally, species differ in breeding system, timing and duration of reproductive development, fluctuations in reproductive effort and output, and fitness impacts of herbivory. This study is the first thorough investigation of the reproductive ecology of mycoheterotrophic species and provides insight into possible limitations in reproductive traits imposed by a mycoheterotrophic life history.

Key words: breeding system; Ericaceae; *Monotropa*; *Monotropsis;* mycoheterotroph; phenology; pollination; reproductive ecology.

Mutualisms and symbioses are some of the most important ecological interactions driving ecosystem function, but these historically have been understudied. Mycoheterotrophy is an important system of symbiotic interactions that has played a key role in our fundamental understanding of highly specialized ecological interactions and the evolutionary ecology of nonphotosynthetic plant biology (Leake, 1994; Bidartondo, 2005). Defined as plant species that obtain carbon resources from obligate mycorrhizal associates, nonphotosynthetic mycoheterotrophs are a phylogenetically, morphologically, and ecologically diverse group of organisms that evolved from photosynthetic ancestors (Wallace, 1975; Leake, 1994; Cullings et al., 1996; Bidartondo, 2005). While they are now free from some of the limitations of “green” plants (e.g., complex vegetative structures and expensive photosynthetic pigmentation [Leake, 1994]), this evolutionary shift in life history to mycoheterotrophy has not come without costs. Consequences of mycoheterotrophy include an obligatory reliance upon mycorrhizal associations for carbon influx (Björkman, 1960) and specialized habitat requirements for survival and propagation (Luoma, 1987; Taylor and Bruns, 1999; Leake et al., 2004; Moola and Vasseur, 2004) that have contributed to rarity, isolation, and divergence among closely related taxa (Kruckeberg and Rabinowitz, 1985; Bidartondo and Bruns, 2001; Taylor et al., 2003). Although much is known about mycorrhizal symbioses (e.g., Bidartondo and Bruns, 2001, 2002) and the many convergent life history traits shared by mycoheterotrophic taxa (e.g., reduced seed size and endosperm, loss of vegetative structures, primary existence as a subterranean root mass [Wallace, 1975; Olson, 1993; Leake, 1994]), the reproductive ecology of these scientifically valuable organisms remains empirically understudied.

In a recent review, Bidartondo (2005) highlighted this gap in our understanding of mycoheterotroph reproduction and suggested that the limitations of the highly specialized life history of mycoheterotrophs may provide direct insight into possible reproductive traits of these plants. Bidartondo suggested that it would be an evolutionarily unstable strategy for a mycoheterotroph already engaged in an obligate symbiotic interaction to assume additional compulsory associations, including those related to reproduction. Subsequently, such taxa would be expected to possess a reproductive ecology free from further specialization, as evident by traits such as a generalist pollination syndrome and/or autogamous self-pollination. To date, we are not aware of any other study that has experimentally addressed Bidartondo’s ideas; existing information consists largely of anecdotal reports of “pollinator” visitations, informal assessments of breeding systems, and hypothetical commentary on possible reproductive traits (e.g., Wallace, 1977; Leake,

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[3] Author for correspondence (e-mail: mklooster@oeb.harvard.edu); present address: Department of Organismic and Evolutionary Biology, Harvard University Herbaria, 22 Divinity Ave., Cambridge, Massachusetts 02138 USA

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1994; Imamura and Ushimaru, 2007; Ushimaru and Imamura, 2002). To empirically address this fundamental gap in our understanding of mycoheterotroph reproductive traits, we targeted the Monotropoideae (Ericaceae) as an ideal study system.

The Monotropoideae is an entirely nonphotosynthetic subfamily with many species having narrow geographic ranges and endemism (Kruckeberg and Rabinowitz, 1985) that likely correlate with the limited distribution of their specialized mycorrhizal associates (Bidartondo and Bruns, 2001). Most genera are morphologically distinct in traits such as color and size of reproductive stems, flower number per stem, floral fragrance, pollen morphology, seed shape, and fruit type (e.g., Copeland, 1935, 1937, 1938, 1939; Bakshi, 1959; Wallace, 1975, 1977; Leake, 1994). Additionally, the Monotropoideae has been used as a model system in pioneering studies of plant–fungal symbioses (Björkman, 1960) and for reconciling other mycoheterotrophic life history traits including the complexity of plant–mycorrhizal associations (Bidartondo and Bruns, 2001, 2002; Yang and Pfister, 2006), developmental biology (Olson, 1990, 1991, 1993), and life history chronology (Leake et al., 2004; Bidartondo and Bruns, 2005).

The goal of this study was to thoroughly investigate and compare the reproductive strategies of related mycoheterotrophic species in the Monotropoideae, providing a foundation and facilitating a broader understanding of nonphotosynthetic, mycoheterotrophic plant reproductive traits. Specifically, we assessed similarities and differences in reproductive traits across species by comparing the pollination ecology, breeding system, flowering phenology, reproductive effort and output, and frequency and fitness impacts of floral herbivory of two closely related mycoheterotrophic genera, *Monotropa* and *Monotropsis*. Ultimately, we aim to discuss our findings in light of Bidartondo's (2005) theory, offering insight into possible evolutionary limitations in reproduction in organisms with an obligate mycoheterotrophic life history.

For this study, we developed and tested four hypotheses. First, taxa should have high pollinator diversity and abundance consistent with a generalist pollination syndrome, as indicated by Bidartondo's theory. Second, these taxa have breeding systems that ensure reproductive success such as self-compatibility, autogamy, and geitonogamy, which is also consistent with Bidartondo's ideas. Third and more generally, taxa should have unique flowering phenologies with distinct fluctuations in yearly reproductive effort and fecundity, as indicated in numerous anecdotal and published reports (e.g., Wolf, 1922; Luoma, 1987; Wallace, 1975, 1977; Leake, 1994). Finally, in conjunction with yearly fluctuations in reproductive effort, taxa likely vary in the frequency, type, and fitness impacts of floral herbivory.

MATERIALS AND METHODS

Study system—Much of the highly influential literature in the field of mycorrhizal biology and mycoheterotrophy has focused on the genus *Monotropa* (e.g., Björkman, 1960; Olson, 1990, 1993; Bidartondo and Bruns, 2001, 2002, 2005; Leake et al., 2004; Bidartondo, 2005). *Monotropa* consists of two species, *M. uniflora* (indian pipe) and *M. hypopitys* (pine sap), which both have a circumboreal distribution (Wallace, 1975; Leake, 1994). As in other monotropoid genera, multiple reproductive stems may be produced per plant, each stem containing one to many flowers (Wallace, 1975). In this genus, reproductive stems and flowers are not noticeably fragrant but are conspicuously colored, ranging from solitary white flowers (*M. uniflora*) to yellow or red inflorescences (*M. hypopitys*). Each species is capable of producing sizable reproductive stems that stand 10–30 cm tall. Flowers emerge from the ground in a nodding position and, upon fertilization, assume an erect orientation (Wallace, 1975). Fruits are dry capsules that contain seeds with wing-like integuments for wind dispersal (Olson, 1980). Within the species *M. hypopitys* are two distinct color forms, yellow and red, causing much confusion among taxonomists. Once classified as separate species, these color forms are currently thought to simply represent natural color variation within the species without any distinct ecological function (Wallace, 1975, 1977). Because the red and yellow forms have unique flowering phenologies (Neyland, 2004), rarely occur in sympatry, and are ecologically understudied, we treated each form as a separate experimental unit and separate taxa in this study.

Another related taxon with unique reproductive traits is the monotypic genus *Monotropsis*. *Monotropsis odorata* (sweet pine sap or pygme pipes) is a rare endemic of the Appalachian Mountains and southeastern United States and is presently listed as threatened or endangered by four states of its limited range (USDA NRCS, 2009). Found almost exclusively growing in upland, mixed oak–pine forests (Jones, 2005), these plants possess coralline roots, which produce one to many reproductive stems that stand 5–10 cm tall (Wolf, 1922; Copeland, 1939). Known for their highly fragrant flowers (similar to baking cloves), these plants are notoriously difficult to visually locate due to their small stature and the seemingly cryptic, brownish bracts and sepals covering the reproductive stems and encircling each flower (M. Klooster, D. Clark [Alma College], and T. Culley, unpublished manuscript). Flowers are deep pink to lavender, typically with a white tip, and after fertilization, form indehiscent, fleshy fruits with seeds that are animal dispersed (Wallace, 1975; Leake, 1994).

Site description and sampling—Populations of each taxon were identified in Ohio, Indiana, Kentucky, and Tennessee (eastern United States) in 2004 and early 2005 (Table 1). All populations were located in mixed deciduous, old growth forest remnants, and plants were typically observed to grow in close proximity to *Fagus grandifolia* and *Quercus* species. In two populations (Cowan Lake State Park, Highland Co., Ohio and Nature Conservancy Land, Adams Co., Ohio), multiple taxa were observed growing in sympatry, and each was treated independently of all other co-occurring taxa. *Monotropa uniflora* and *M. hypopitys* (yellow form) were observed to grow across a broader geographic distribution than populations of the red color form of *M. hypopitys*, which was rare and only observed to grow in forests on the edge of the Appalachian Mountains in southern Ohio. *Monotropsis odorata* was also restricted to a southern portion of the Appalachian Mountains and primarily occurred in secondary growth, oak–Virginia pine (*Pinus virginiana*) stands.

Individual genets of these mycoheterotrophs could not be identified without exhuming roots and severing essential mycorrhizal connections. Therefore, individual plants of each species were identified as isolated clumps of reproductive stems that were located more than 1 m from the next neighboring clump and were marked with semipermanent flagging ($N \approx 20$–200 plants per population). Consequently, the total number of plants identified in a population using this method is likely an underestimate of the actual population size.

Pollinator diversity and visitation rates—Preliminary research in the summer and fall of 2004 showed nectar production was highest in the late morning and early afternoon, indicating that pollinators may likely visit during the day (M. Klooster, unpublished data). Additionally, observations conducted by Wallace (1977) on a number of monotropoid genera revealed that *Bombus* spp. may play significant roles as floral visitors. Subsequently, pollinator observations were conducted within the largest population of each taxon between the daylight hours of 0900 and 1900 hours EST. Pollinator activity was monitored for each taxon over two consecutive years (30 h per year per taxon) from 2005 to 2006 with the exception of the yellow form of *M. hypopitys*, which was examined in 2007 only. Pollinators were defined as pollen dispersal agents that contacted stamens and pistils, whereas floral visitors failed to make contact with male and/or female sex parts. At peak bloom in a given year, pollinators were videotaped each day for three consecutive days (10 h per day) and video footage was reviewed at a later time to determine the average number of flowers, stems, and plants visited during each complete pollinator foraging event. Additionally, the total duration of each complete pollination event and the average time spent by each pollinator visiting each plant was also determined from video obtained during the blooming period. Complete foraging events were defined as the activity of a given pollinator that was observed from initial entry into the population and commencement of floral visitations until the pollinator exited the study area. Any visitation event that was not observed and recorded in its entirety was excluded from further analyses. Because pollinator observations for the yellow form of *M. hypopitys* were conducted over a single year and few complete visitation events were observed, videorecordings of pollination in this taxon were excluded from detailed analyses. Voucher specimens of pollinators and floral

Table 1. Site locations of taxa used to conduct this study. Included are site identifications (site ID) used when referencing individual populations in the text, the approximate population sizes (*N*), and latitude and longitude coordinates for each location.

Taxon	Study site location,county, state	Site ID	*N*	Latitude (N), longitude (W)
Monotropsis odorata	Daniel Boone National Forest, Powell Co., Kentucky	PT	~30	37°48.469, −83°39.150
	Cherokee National Forest, Monroe Co., Tennessee **	CNF	233	35°19.778, −84°16.975
Monotropa uniflora	Nature Conservancy Land, Adams Co., Ohio **	BCI	>225	38°48.005, −83°24.082
	Cowan Lake State Park, Highland Co., Ohio	CLI	24	39°22.899, −83°54.658
	Lockwood Property (Private), Hamilton Co., Ohio	LP	17	39°13.920, −84°39.950
	Wood Creek (Private), Hamilton Co., Ohio *	WC	~19	39°12.353, −83°15.365
Monotropa hypopitys (yellow form)	Germantown Metropark, Montgomery Co., Ohio	GT	41	39°38.444, −84°25.135
	Cowan Lake State Park, Highland Co., Ohio	CL	60	37°22.899, −83°54.658
	Flat Woods (Private), Jefferson Co., Indiana	FW	65	38°50.221, −85°26.388
	Stonelick State Park, Clermont Co., Ohio **	SL	>250	39°12.451, −84°04.234
	Nature Conservancy Land, Adams Co., Ohio *	BCY	~12	38°48.005, −83°24.082
Monotropa hypopitys (red form)	Nature Conservancy Land, Adams Co., Ohio **	BC	211	38°48.005, −83°24.082
	Shawnee State Forest, Scioto Co., Ohio *	SSF	~15	38°41.876, −83°10.250

Notes: *Used for reproductive phenology study only, **used for pollination studies

visitors were collected during observations to visually assess pollen load; insects were identified to family or genus when possible. These voucher specimens were used in conjunction with video footage to determine which floral visitors were primary pollen dispersal agents (i.e., pollinators) and to ascertain if nonpollinating floral visitors forage for pollen and nectar in this system. Furthermore, pollinator foraging behavior was observed both in the field and by video to reinforce and better resolve various components of the plant breeding system (i.e., geitonogamy, self-compatibility, obligate outcross fertilization, and buzz-pollination).

Breeding system—Self-compatibility and autogamy (including prior- and delayed-selfing mechanisms) and their reliance upon floral visitors for successful reproduction were examined in these taxa using ca. 18–61 nylon pollinator exclusion tents constructed in the largest population of each taxon. These tents were placed over newly emerged reproductive stems of randomly selected individual plants ($N \geq 4$ stems/tent) before anthesis. Tents were constructed using very fine mesh and in such a way as to exclude flying and crawling insects while allowing proper light, moisture, and airflow to the plants. Flowers on two stems within each tent were self-pollinated by hand to determine self-compatibility. All remaining flowers on the other stems within each tent were left unmanipulated to test for autogamy (including prior- or delayed-selfing). Because of population size constraints for each species, we could not balance this experimental design with an outcross-pollinated, untented treatment that, when compared to a control group, would be useful in measuring pollinator limitation (Kearns and Inouye, 1993). All tented treatments were performed across multiple populations or years and data were pooled, with the exception of a single yearly treatment of the yellow form of *M. hypopitys*.

At the end of flowering, senesced reproductive stems containing the intact fruits and flowers in each tented treatment were harvested. Differences in viable seed set between self-compatibility and autogamy treatment groups were determined by studying fruits under a dissecting microscope and observing seed development. Flowers were placed in one of two categories: mature fruit or aborted fruit/ovary. Relative self-compatibility and autogamy were then determined based on the presence or absence of mature fruits in each treatment group.

To determine whether herkogamy (spatial separation of male and female sexual parts) exists in these taxa, samples of 30 reproductive stems at anthesis were randomly selected from the largest population of each taxon. Flowers on each stem were dissected, and the relative position of anthers to the receptive stigmatic region was assessed using a dissecting scope at 2–4× magnification, with distinct physical separation classified as herkogamy. A population was determined to have a mixed breeding system when floral morphs consistently varied in the separation of anthers and stigmas and this trait resulted in at least some self-pollination and production of mature fruits in the autogamy tented treatment. Additionally, observations of pre- and postanthesis flowers were used to determine if temporal separation in the reproductive maturation of stamens and pistils (dichogamy) exists in this system.

Flowering phenology and reproductive effort—Populations were monitored throughout three years (2005–2007) to determine when plants became reproductive and to assess the duration of flowering phenology for each species. Reproductive effort, defined in this study as the average number of reproductive stems produced per plant, was assessed in each population across the three years of study. Reproductive stems were used as a measure of reproductive effort instead of flower number to standardize this measurement across taxa because each species typically produces a variable number of flowers per stem, with the exception of *M. uniflora*. All stem data for each population were log transformed to enhance normality. Repeated measures ANOVAs were conducted across years for each taxon to test the null hypothesis that plants do not differ in reproductive effort, using population and year as independent variables and numbers of stems produced per plant as the dependent variable. The red form of *M. hypopitys* was excluded from this analysis because of the lack of multiple populations for comparison. Because there was a significant drought in the eastern United States during the summer and fall of 2007, year was treated as a fixed factor.

Fecundity and herbivory—Yearly fecundity was analyzed to determine the potential reproductive capacity of populations of each species. To assess fecundity, reproductive stems from a subset of flagged plants (N = 20–60 plants; 1–8 stems per plant) in each population were randomly selected prior to anthesis and flower numbers were recorded. At the end of blooming, these stems were collected, and their flowers and fruits were analyzed with a dissecting microscope to place them into one of three categories: mature, aborted, or damaged via herbivory. Ratios were then calculated from this data by dividing the number of flowers or fruits in each category by the total number of flowers per stem. Data from all stems of a given plant were then averaged to yield mean mature, aborted, and herbivory ratios, and these were used in further population and species level analyses. Plant ratios were used as a means of standardizing data, as flower number produced per plant varied greatly across the different taxa.

Because it was extremely difficult to count or germinate the dust-like seeds or to rear offspring in controlled settings, mean mature fruit ratio per plant was used instead as a measure of reproductive output of each population across years. Mature fruit ratios and herbivory ratios were arcsine-square root transformed to enhance normality prior to analysis. A three-way nested ANOVA was used to determine significant differences in the reproductive output of taxa, with taxon, year, and population nested within taxon as independent variables and mature fruit ratio as the dependent variable. An additional three-way nested ANOVA was performed using ratios of only flowers and fruits that had signs of herbivory as the dependent variable to determine whether there were significant differences in herbivory rates of taxa and populations nested within taxa across all years of study. All factors in these statistical models were treated as fixed because of the nonrandom event of a drought in 2007. Least-squares means were used for post hoc comparisons of species level effects on differences in mature fruit ratios and herbivory ratios. Finally, herbivory data were combined across populations and further classified under one of the following four categories to determine which herbivory type most commonly affected each taxon: floral herbivory (consumption of flower sex parts), stem herbivory (consumption of part of the stem or severing the stem from its roots), nectar robbing (small chew holes at the base of petals, adjacent to nectaries), or seed predation (burrowing into a fruit to consume developing or mature seeds). The herbivory category of seed predation was not applied to berries of *Monotropsis odorata* because we could not accurately quantify damage and separate fruits attacked by seed herbivores from those consumed by animal seed-dispersal agents.

RESULTS

Pollinator diversity and visitation rates—Over 210 hours of observations across three years implicated only a few insect taxa as floral visitors of *Monotropsis* and *Monotropa*. *Monotropsis odorata* attracted the greatest abundance and diversity of floral visitors across both years, with minimal diversity observed in *Monotropa* species (Table 2). Most insect floral visitors consumed nectar from flowers with a few insects possibly functioning as minor pollen vectors from infrequent contact with plant sex parts (i.e., Aprocrita, *Erynnis* spp., *Epargyreus clarus*, Halictidae, Vespidae). Those floral visitors with short mouthparts (i.e., Tachinidae and Syrphidae) were often observed foraging on the sticky exudates excreted from the stigma and did not function as nectar robbers or pollinators.

Bumblebees (*Bombus* spp.) were the most abundant floral visitors across all years of observations, harvesting both pollen and nectar. Additionally, *Bombus* spp. were the primary pollen dispersal agents across all years in populations of *Monotropa* and *Monotropsis*, demonstrating relatively high fidelity while foraging on flowers of each species (Table 3). *Monotropa hypopitys* (yellow form) received the fewest number of observed pollinator visitations with only six in 2007, and *Monotropsis odorata* elicited the highest frequency of pollinator visitations each year with 154 and 77 in 2005 and 2006, respectively. Video footage and field observations revealed that "buzz pollination" played a substantial role in all pollination events for *Monotropsis* and was observed less frequently in *Monotropa*. Additionally, *Bombus* spp. were observed to commonly visit on average ≥2 flowers per stem and ≥2–4 stems per plant, supporting the likelihood of geitonogamous pollination in self-compatible taxa. *Monotropa uniflora* elicited the overall shortest duration (median of 2:07 and 2:34 min in 2005 and 2006, respectively) of pollinator visitations by *Bombus* spp., which visited the fewest stems and plants per complete visitation. Alternatively, *Bombus* spp. spent the longest duration of time per complete visitation event (median of 4:18 and 4:08 min in 2005 and 2006, respectively) foraging on *Monotropa hypopitys* (red form) and on average, visited the most flowers, stems, and plants of this taxon.

TABLE 2. The identity of insect taxa and total number of floral visitations on flowers of four mycoheterotrophic taxa. Each year represents 30 h of independent observations conducted at peak bloom in the largest population of each taxon from 2005 to 2007.

Taxon	Year	Floral visitor	Visitations
Monotropsis odorata	2005	*Bombus* spp.	154
		Solitary wasp (Apriocrita)	31
		Erynnis spp.	17
		Tachinid fly (Tachinidae)	8
		Epargyreus spp.	1
	2006	*Bombus* spp.	77
		Solitary wasp (Apriocrita)	12
		Erynnis spp.	1
Monotropa uniflora	2005	*Bombus* spp.	91
		Syrphid fly (Syrphidae)	35
		Halictid bee (Halictidae)	2
	2006	*Bombus* spp.	26
		Syrphid fly (Syrphidae)	23
		Halictid bee (Halictidae)	11
M. hypopitys (yellow form)	2007	*Bombus* spp.	6
		Syrphid fly (Syrphidae)	1
M. hypopitys (red form)	2005	*Bombus* spp.	44
	2006	*Bombus* spp.	42
		Vespid wasp (Vespidae)	1

Breeding system—Tented treatments, designed to exclude insect floral visitors and pollinators, revealed striking differences in levels of self-compatibility and autogamy among taxa of *Monotropsis* and *Monotropa* (Table 4). Plants in both populations of *Monotropsis odorata* had negligible self-compatibility with approximately 1% fruit set in self-pollinated treatments and were not autogamous (0% fruit set), while *Monotropa* spp. had moderate to high levels of self-compatibility ranging from 20% in *M. uniflora* to 72% in *M. hypopitys* (yellow form). *Monotropa* spp. differed substantially in levels of autogamous self-pollination with tented, unmanipulated stems of *M. uniflora* and *M. hypopitys* (red form) having <11% fruit set and plants of the yellow form of *M. hypopitys* producing approximately 68% autogamous fruits.

The morphology of flowers collected from the largest population of each taxon exhibited approach herkogamy with orientation of the stigma above the level of the anthers, with the notable exception of the yellow form of *M. hypopitys* (Fig. 1). The flowers sampled from the SL population of this taxon were consistent with a mixed breeding system with one or both whorls of anthers in direct contact with the receptive stigmatic region in 91% of flowers sampled at reproductive maturity, and the remaining 9% of flowers exhibited approach herkogamy. Additionally, there was no clear evidence for dichogamy in *Monotropsis* or *Monotropa* because there was no discernible temporal separation in pistil and stamen maturation.

Flowering phenology and reproductive effort—Each taxon exhibited unique timing and duration of reproductive phenology (Fig. 2). Additionally, each population of plants showed high fidelity for the timing of reproductive phenology among years with no population varying in the timing of anthesis beyond 7–10 d across all three years. *Monotropsis odorata* took the longest time (32–34 wk) for reproductive stems to develop, floral receptivity to subside, and fruits to mature. Taxa in the genus *Monotropa* had substantially shorter flowering periods than *Monotropsis*. *Monotropa uniflora* had the most variable reproductive phenology across populations ranging from late June (i.e., BCI population) into early October (i.e., LP and WC populations). This variation in *M. uniflora* phenology among populations did not appear to be clearly attributable to a latitudinal gradient, geographic distance, or habitat type, although this was not empirically tested. The red and yellow forms of *M. hypopitys* differed distinctly in the timing of their reproductive phenologies, with the five populations of the yellow form consistently reproducing during midsummer (from 11 July to 14 August), whereas populations of the red form were strictly fall blooming (15 September to 5 October).

Reproductive effort was highly variable for all taxa in the three consecutive years of observations. According to the repeated measures ANOVAs used to compare reproductive effort, there was a significant interaction of year and population ($P < 0.001$) in each taxon with the exception of *Monotropsis odorata*, which had no significant interaction ($P = 0.64$; Table 5). Additionally, populations of each taxon significantly differed in reproductive effort across years ($P < 0.0001$), reflecting inconsistent production of reproductive stems by individuals within populations. A small percentage of plants across populations reproduced consistently in all years (ranging from 8–20%), with an average of 87% of plants having at least one year of

TABLE 3. Visitation duration and frequency of *Bombus* spp. pollinators, foraging in the largest population of three mycoheterotrophic taxa. A total of 60 h of observations were conducted per taxon across 2005 and 2006. Data collected from complete visitation events include median and range of duration of all visitation events as well as the mean number of flowers, reproductive stems, and plants visited by *Bombus* pollinators during each foraging bout.

Taxon	Year	Median duration (min:s) of visitation (range)	Mean no. of flowers	Mean no. of stems	Mean no. of plants
Monotropsis odorata	2005	3:32 (1:11–20:04)	24	12	3
	2006	3:13 (1:11–16:37)	27	13	3
Monotropa uniflora	2005	2:07 (0:31–8:36)	10	10	4
	2006	2:34 (1:06–11:45)	15	15	6
Monotropa hypopitys (red form)	2005	4:18 (1:20–10:32)	29	13	4
	2006	4:08 (1:04–27:30)	30	15	7

dormancy in the three years of observations. Additionally, there was no clear synchronicity in the timing of reproductive years or dormancy among individual plants within populations. A few individuals in each population had substantially higher reproductive effort (>50 stems per plant) in a given year than other individuals, although these reproductive "bursts" were often preceded and followed by years of dormancy or minimal reproductive effort.

Fecundity and herbivory—Fecundity was highly variable across populations of each taxon and years of study (Fig. 3). A three-way nested ANOVA was initially used to quantify differences in the reproductive output among populations and taxa across the three years of study. Results from this model demonstrated a significant interaction of taxon and year ($P < 0.0001$), with significant differences in the overall reproductive output among taxa ($P = 0.014$) and across years ($P < 0.031$, Table 6). Additionally, populations nested within species differed significantly in reproductive output ($P < 0.0001$), and these results are consistent with broad fluctuations in fecundity among individuals within populations. Post hoc comparisons revealed that *Monotropa uniflora* had significantly lower mature fruit ratios per plant than either *M. hypopitys* (yellow form; $P = 0.0012$) or *Monotropsis odorata* ($P = 0.046$), with all other comparisons among taxa yielding nonsignificant differences. *Monotropsis odorata* had the highest and most consistent overall reproductive effort and relative fruit set between populations across all three years of study. The BC population of the red form of *Monotropa hypopitys* had the largest overall decrease in relative fruit set across years, from 45% in 2005 and 56% in 2006 to 0% in 2007. Overall, 2005 was the most fecund year for all taxa, after which fecundity either remained consistent or steadily declined in the subsequent two years. A drought in the summer and fall of 2007 likely contributed to the overall decline in reproductive effort and output of taxa that bloomed later in the year, with the fall-blooming LP population of *M. uniflora* and the BC population of *M. hypopitys* (red form) declining substantially in reproductive effort and 0% reproductive output for that year. The 2007 drought and other ecological factors contributed to rates of floral abortion that varied strongly among taxa and across years, ranging from 29% and 13% in 2005 for the LP and BC populations of *M. uniflora* and *M. hypopitys* (red form), respectively, to 100% floral abortion in 2007 for these same populations.

TABLE 4. Results from breeding system experiments using pollinator exclusion tents to determine percentages of fruit set in self-compatibility and autogamy treatment groups for four mycoheterotrophic taxa. Plants examined in this study (*N*) possessed at least two stems per treatment group. Flowers on stems in the self-compatibility treatment were self-pollinated by hand, whereas stems in the autogamy group were left tented but unmanipulated.

	Autogamy		Self-compatibility	
Taxon	*N*	Fruit set (%)	*N*	Fruit set (%)
Monotropsis odorata	61	0	56	0.6
Monotropa uniflora	35	1.4	27	20.4
M. hypopitys (yellow form)	18	67.6	13	71.8
M. hypopitys (red form)	48	10.9	49	69.5

Herbivory rates and the type of herbivory also differed between *Monotropsis* and *Monotropa*. Herbivory rates significantly differed across taxa ($P = 0.0028$) and among populations nested within taxa ($P < 0.0001$; Table 6). However, there was no significant effect of year on herbivory rates ($P = 0.11$), although there was a significant interaction of taxon and year ($P < 0.0001$). Post hoc comparisons demonstrated that plants of *Monotropa uniflora* had significantly higher ratios of flower and fruit loss to herbivory than all other taxa ($P < 0.01$), with no significant differences detected among the other multiflowered taxa ($P > 0.05$). *Monotropsis odorata* plants had the lowest ratio of flower loss to herbivory with an average of 10.8% per year. Additionally, the reproductive stems and flowers of *M. odorata* were typically damaged by predation of viable floral and stem tissue (63% and 36% of total herbivory, respectively), whereas the reproductive stems and flowers of *Monotropa* taxa were more likely to suffer from seed predation once flowers were fertilized (Table 7). All forms of herbivory for *Monotropsis odorata* peaked in frequency in early spring with 55–85% of damage occurring prior to reproductive maturity and an average of 23% of herbivory observed after anthesis. In contrast, approximately 9–16% stem herbivory and 7–20% floral herbivory were observed while *Monotropa* plants were developing or reproductively active. However, once viable tissue began to dry and seed development was near completion, stem-boring lepidopteran larvae were observed consuming seed within mature fruits, accounting for 71–81% of the total herbivory observed in *Monotropa*.

DISCUSSION

Nonphotosynthetic, mycoheterotrophic plants, specifically those within the Monotropoideae (Ericaceae), have played a substantial role in enhancing our understanding of the dynamics and complexity of plant–fungal symbiotic interactions. However, despite the continued use of the Monotropoideae as a model system for investigating mycoheterotrophic plant biology, there remains a substantial void in our understanding of the reproductive ecology of these intriguing organisms (e.g.,

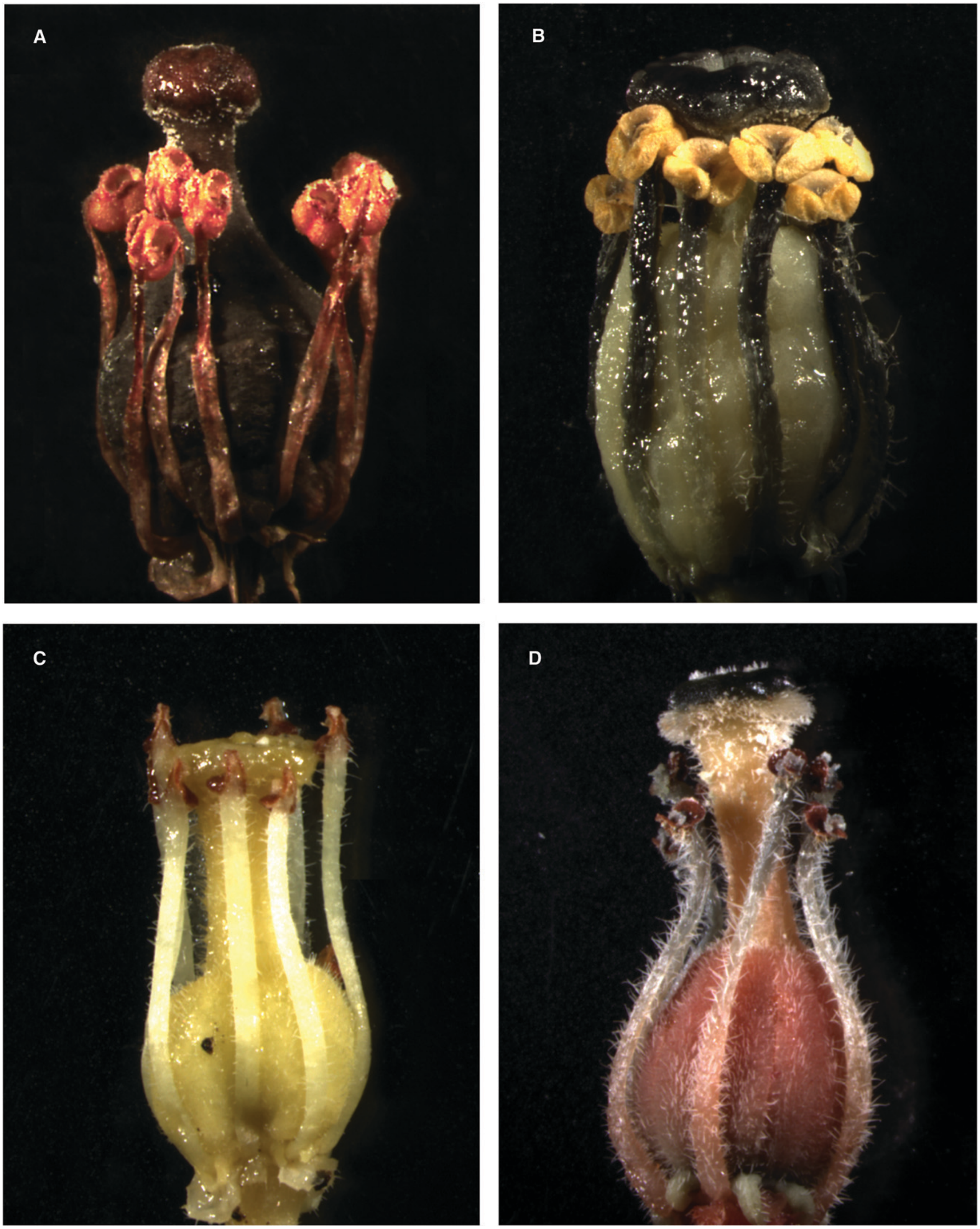

Fig. 1. The most frequently observed floral morphology of four mycoheterotrophic taxa. Thirty stems were sampled from individual plants of the largest population of each taxon and were assessed for relative anther to stigma position. (A) *Monotropsis odorata* and (B) *Monotropa uniflora* consistently displayed approach herkogamy, while (C) *M. hypopitys* (yellow form) had a mixed breeding system (>90% of flowers showed no anther to stigma separation; pictured here) and (D) *M. hypopitys* (red form) displayed approach herkogamy.

Leake, 1994; Bidartondo, 2005). Results from this analysis empirically document a broad suit of reproductive traits for three species in the Monotropoideae and further demonstrate that each taxon possesses complex reproductive strategies, many of which markedly differ among species. Ultimately, our findings fail to support Bidartondo's (2005) theory, which postulates the likelihood of convergence among mycoheterotrophic taxa on a generalist pollination syndrome and autogamous self-pollination.

Due to Bidartondo's theory and because these mycoheterotrophs were previously reported to reproduce unreliably across years with unique reproductive phenologies (Wallace, 1975, 1977; Leake, 1994), we expected taxa to be generalist-pollinated by a locally abundant and diverse group of insects (Waser et al., 1996). However, *Bombus* spp. appeared to function as the most reliable floral visitors and were the primary pollen dispersal agents for these taxa, indicating that populations of *Monotropa* and *Monotropsis* analyzed had low pollinator diversity, failing to support our first hypothesis. The low pollinator diversity observed in this system is indicative of specialization on *Bombus* spp., which have a generalist preference for many plant taxa and are abundant and reliable pollinators in the endemic ranges of these mycoheterotrophs. Although taxa shared a common pollinator, *Bombus* behavior differed while foraging on flowers of *Monotropa* and *Monotropsis*, likely resulting from differences in anther morphology. *Monotropa* produces anthers that dehisce along a slit, spilling pollen onto the inner walls of the corolla, which results in pollen collecting on the head and thorax of *Bombus* spp. as they probe flowers for nectar. In *Monotropsis*, each anther possesses two pores that dehisce but do not readily release pollen from the anther sac. Instead, *Bombus* spp. induce the release of pollen from anther sacs through high frequency buzzing upon probing each flower (e.g., Hermann and Palser, 2000). Despite the higher number and frequency of insect taxa observed visiting flowers of *M. odorata*, *Bombus* spp. appear to be the primary pollen dispersal agents because "buzz pollination" is necessary for pollen release. Additionally, the number and duration of pollinator visitations varied among *Monotropa* spp., which may be suggestive of differential preferences by *Bombus* spp. for particular taxa. Alternatively, variation in visitation rates may correspond to differences in the blooming periods of *Monotropa* spp., resulting in differential competition for pollinator fidelity with discrete groups of co-occurring plant species that share a blooming period.

The high frequency and abundance of *Bombus* visitations, as well as the production of copious nectar and pollen, suggests reliance by plant taxa upon pollinators for successful fertilization. Tented treatments further demonstrated this dependency upon pollinators because low to negligible autogamous self-pollination was detected in most taxa, which failed to support our second hypothesis and Bidartondo's prediction that autogamy would be favored in this system. *Monotropsis odorata* was determined to be self-incompatible, which stood in stark contrast to the highly self-compatible and autogamously self-pollinating yellow form of *Monotropa hypopitys*. *Monotropa uniflora* had low self-compatibility and negligible autogamy, whereas the red form of *M. hypopitys* had a high level of self-compatibility similar to that of the yellow form of *M. hypopitys* but had low autogamous fruit set. Additionally, nearly all taxa exhibited approach herkogamy, consistent with outcross-pollinated and animal-pollinated species (Barrett, 2003). Although the vast majority of flowers sampled for the yellow form of *M. hypopitys* had no spatial separation between anthers and stigmas, it remains unclear to what degree and in what proportions this breeding system persisted in other populations. Results indicate that these taxa have unique breeding systems, and most taxa distinctly rely on pollinators for successful pollen dispersal and fertilization. Therefore, pollinator abundance and fidelity are vital for successful reproduction in all taxa but substantially less so for the autogamous, yellow form of *M. hypopitys*.

Ultimately, these results demonstrated a specialist reproductive syndrome and obligate outcross pollination in most taxa, which failed to support assertions of Bidartondo's (2005) theory, namely, a generalist pollination syndrome and autogamous self-pollination. However, in a broader evolutionary–ecology context, it remains unclear whether there are inherent tradeoffs of plants engaged in obligate mycorrhizal and pollinator interactions. We encourage the use of two possible alternative approaches to address questions of trade-offs associated with specialization in multiple life-history traits. A comparative phylogenetic approach (e.g., Harvey and Pagel, 1991) would compare the reproductive biology of mycorrhizal generalist (e.g., *Arctostaphylos*, *Pyrola*), mycorrhizal specialist (e.g., *M. hypopitys*, *M. uniflora*), and extreme mycorrhizal specialist (e.g., *Allotropa*, *Pterospora*) ericaceous species. This methodology is suited for cases where specialization is a fixed trait within species. A comparative population approach, championed by Thompson (2005), would examine both mycorrhizas and reproductive traits for multiple populations of a single mycoheterotrophic species. This approach is suited to cases where there is intraspecific variation in specialization. Our study of reproduction in a limited number of populations, and the available mycorrhizal data for numerous populations, indicate that a population approach will be most enlightening and specifically useful for investigations of *M. uniflora*, which possesses variable reproductive traits and is differentially specialized on *Russula* fungi across its geographic range. Future research on this and like systems will further elucidate any ecological and evolutionary trade-offs in the degree of specialization of plants with obligate mycorrhizal and pollinator associations.

Additional field observations and analyses aimed to resolve anecdotal reports of monotropoid reproductive traits revealed that taxa of *Monotropa* and *Monotropsis* had distinctly different timing and duration of various stages of reproductive development and phenology, supporting our third hypothesis and previous observations (e.g., Wallace, 1975, 1977; Leake, 1994). Specifically, reproductive development and phenology of *Monotropsis* lasted up to 36 weeks, which was nearly four times the duration of *Monotropa* spp. Additionally, each population of *M. uniflora* differed in the timing of flowering phenology, ranging from early summer to late fall, whereas populations of all other taxa consistently reproduced within a discrete period each year. Interestingly, populations of the two color forms of *M. hypopitys* had different blooming periods, supporting temporal reproductive isolation in these taxa. Even when both color forms occurred in sympatry (as was the case for the BC and BCY populations), flowering phenologies were separated by 6–8 wk, which may have implications for genetic divergence and possibly speciation in these morphs. Genetic divergence between color forms has further been examined with molecular markers in a related study (Klooster et al., 2009; M. Klooster and T. Culley, unpublished manuscript).

The variation in reproductive effort and fecundity observed in most of these taxa may also be attributable to the interaction of a number of factors, including pollinator behavior, breeding

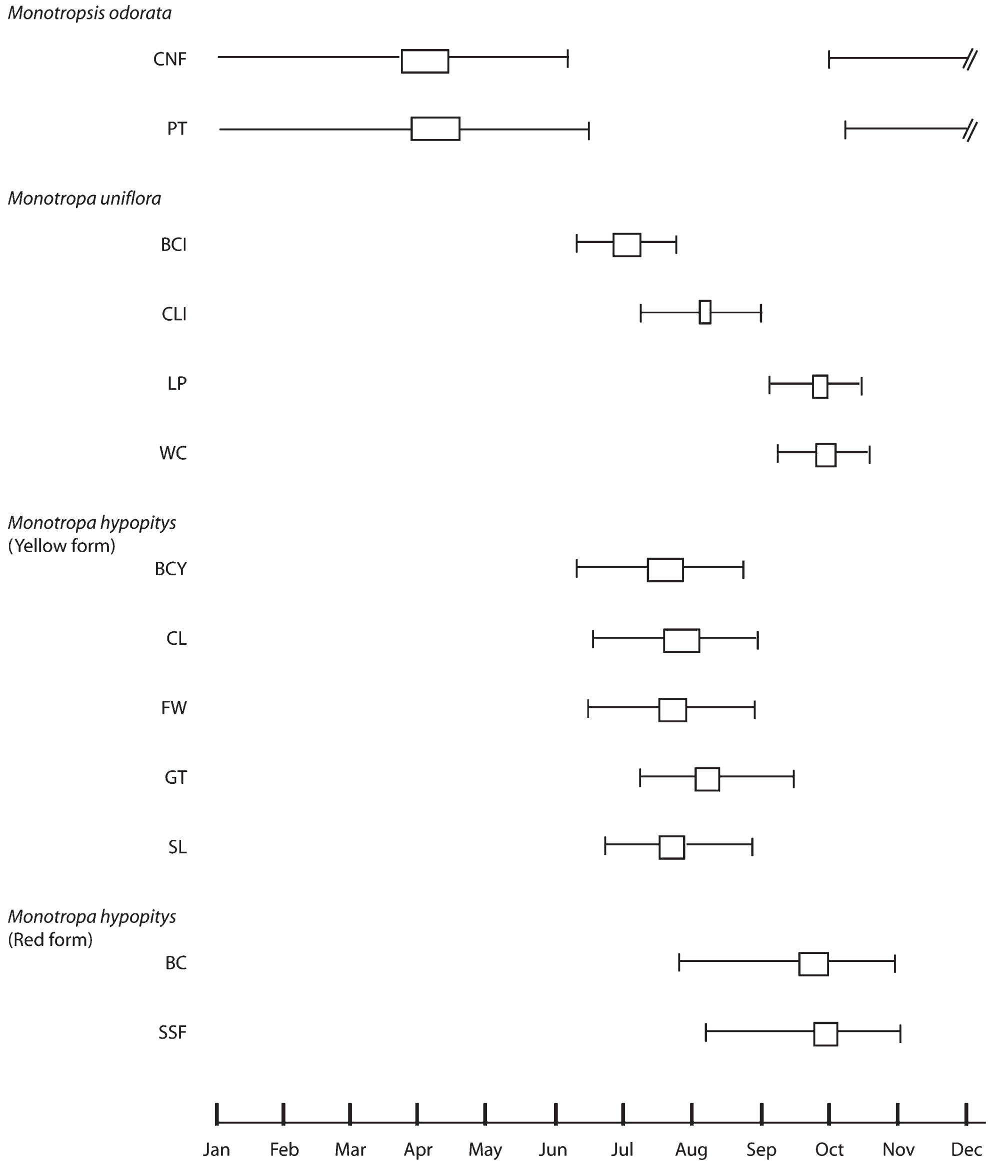

Fig. 2. Reproductive phenologies of multiple populations of four mycoheterotophic taxa monitored from 2005 to 2007. Shown for each population are the mean duration and timing of flowering observed for the 3-yr period. Lines to the left of the boxes represent reproductive stem and floral development from initial emergence to reproductive maturity. Boxes indicate reproductive maturity from anthesis to fertilization. Lines extending to the right of boxes represent the duration of fruit and seed development from fertilization to fruit set.

systems, and the environment. Although *Bombus* spp. did show relatively high fidelity when foraging on flowers of these taxa, the frequency of visitations uniformly declined from 2005 to 2006, possibly reflecting yearly fluctuations in the number of reproductive plants and variation in the reproductive effort of those plants that reproduced. Additionally, it is unclear if pollinator limitation exists in this system and to what degree, possibly reducing pollen dissemination and subsequent fertilization in years of low pollinator abundance. Differences in plant morphological traits and breeding systems also appear to elicit differential visitation rates by *Bombus* spp. For instance, the small, highly fragrant, and self-incompatible *Monotropsis odorata* attracted the highest frequency of *Bombus* visitations across both years of observations, whereas the robust, conspicuous, autogamous yellow form of *Monotropa hypopitys* attracted the fewest pollinator visitations in a given year. Furthermore, the production of numerous reproductive stems per plant and pollinator foraging behavior favored geitonogamous self-pollination, which could be a potential waste of nectar and pollen resources for self-incompatible taxa and may potentially promote inbreeding and reduce plant fitness in the self-compatible *Monotropa* spp. Finally, yearly fluctuations in moisture and temperature appeared to play a substantial role in both reproductive effort and output. In the summer and late fall of 2007, a drought struck the eastern United States. Associated with the lack of precipitation came a notable decline in the reproductive effort of plants observed within the majority of summer and fall blooming populations of *M. uniflora* and *M. hypopitys* (both color forms). This overall reduction in reproductive stem and flower production was accompanied by increased floral abortion and a substantial

TABLE 5. Results of repeated measures ANOVAs for the reproductive effort of multiple populations of three mycoheterotrophic taxa surveyed over three consecutive years.

Taxon	Sources	df	MS	*F*	*P*
Monotropsis odorata	Year	2	2.67	13.44	<0.0001
	Year × Pop.	2	0.077	0.39	0.6394
	Error(Year)	524	0.19		
Monotropa uniflora	Year	2	7.43	102.04	<0.0001
	Year × Pop.	4	0.36	4.99	0.0006
	Error(Year)	516	0.072		
Monotropa hypopitys (yellow form)	Year	2	3.59	48.25	<0.0001
	Year × Pop.	4	0.42	5.75	0.0002
	Error(Year)	292	0.074		

decrease in fecundity with some populations of *Monotropa* spp. failing to produce any mature fruits that year. Although specific correlations between particular environmental factors and plant reproduction were not examined here, the change in levels of precipitation from 2005 to 2007 and the observed decline in reproductive effort and output indicate that these mycoheterotrophic taxa are sensitive to environmental fluctuations.

Furthermore, because mycoheterotrophs rely solely upon mycorrhizal associates for water and nutrients and indirectly upon autotrophic plants for carbohydrates, it can be assumed that this dependency upon other organisms as part of a web of ecological associations may potentially limit resource availability, affecting the growth and yearly reproductive effort of mycoheterotrophs (Bidartondo, 2005; Selosse et al., 2006). Consequently, it is possible that slow or inconsistent mycorrhizal resource contributions may explain differences in the resource pool among taxa and thus the duration of reproductive stem development and flowering phenologies. Furthermore, temporal variation in the reproductive effort of plants of a given taxon (such as in populations of *M. uniflora* or the color forms of *M. hypopitys*) may result from association with unique mycorrhizal taxa at the population level and could lead to speciation (Taylor and Bruns, 1999; Bidartondo and Bruns, 2002; Taylor et al., 2003). Plants for which successful fertilization takes place must further rely upon this web of interactions for resources necessary for seed and fruit production. Fluctuations in or early termination of resource flow may result in the random or selective abortion of some fruits and seeds so that others can develop (e.g., Marshall and Ellstrand, 1988; Martin and Lee, 1993; Burd, 1998), possibly explaining at least some of the variation in reproductive output observed across taxa. Further research concerning the provisioning of resources by fungal associates may explain, at least in part, the differences in the

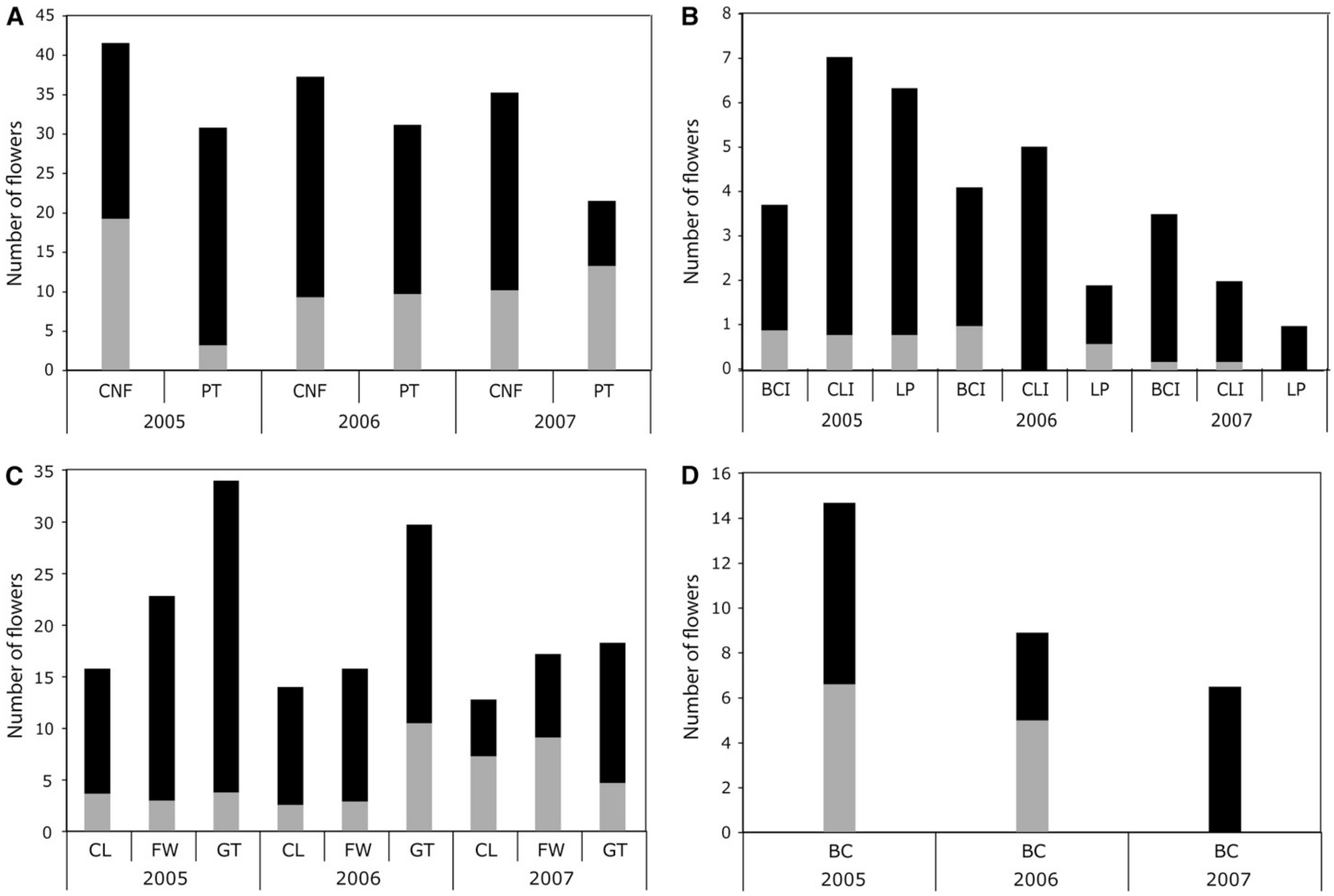

Fig. 3. Mean frequency of flowers produced by plants in populations of four mycoheterotrophic taxa surveyed from 2005 to 2007. Each bar represents the mean number of flowers produced by plants in the respective population in each year of study (see Table 1 for population abbreviations). Regions of each bar shaded in gray represent the proportion of flowers that successfully developed into mature fruits. (A) *Monotropsis odorata*, (B) *Monotropa uniflora*, (C) *M. hypopitys* (yellow form), (D) *M. hypopitys* (red form).

TABLE 6. Results of three-way nested ANOVAs for reproductive output and ratios of combined floral and fruit herbivory of four mycoheterotrophic taxa across three years of study.

	Reproductive output				Floral and fruit herbivory			
Sources	df	MS	*F*	*P*	df	MS	*F*	*P*
Taxon	3	3150.50	3.60	0.0141	3	2986.90	4.73	0.0028
Year	2	3056.40	3.50	0.0319	2	1417.60	2.24	0.1067
Population(Taxon)	5	7018.62	8.03	<0.0001	5	4921.42	7.79	<0.0001
Taxon*Year	6	3385.46	3.87	<0.0001	6	2874.06	4.55	<0.0001

relative productivity of mycoheterotrophs and the reproductive timing of populations.

Another factor shown to strongly influence the reproductive ecology of these mycoheterotrophs was herbivory, which significantly differed among taxa and populations, with a significant interaction of taxon and year. Additionally, the type of herbivory differed between *Monotropa* and *Monotropsis,* likely indicating that different guilds of animal herbivores target members of each genus. These results support our final hypothesis that these plant taxa vary in the frequency, type, and fitness impacts of floral herbivory, possibly owing to specialization by herbivores in the unique ranges of distribution and possibly owing to endemism. Results also suggest that distinctive plant defense strategies may have evolved across taxa. For instance, taxa within the genus *Monotropa* produce large reproductive stems with conspicuous coloration ranging from white to yellow and red, yet reproductive stems and floral tissue of these taxa have a relatively lower proportion of herbivory, possibly owing to the production of systemic herbivore defense compounds (Kunze, 1878; Orton, 1922) and possibly aposematic coloration (Rubino and McCarthy, 2004). In contrast, the small reproductive stems and flowers of *Monotropsis odorata* are covered with brown, scarious bracts that cover colorful petal and stem tissue. When the bracts are removed and the colorful floral and stem tissue of *M. odorata* becomes exposed, flowers and stems are significantly more likely to suffer from herbivory than when they remain concealed by bracts (M. Klooster, D. Clark [Alma College], T. Culley, unpublished manuscript), supporting the possibility of cryptic coloration (camouflage) useful in herbivore defense (Wiens, 1978). Overall, the selective pressures imposed by floral, stem, and seed herbivores in each taxon have likely played a substantial role in the evolution of defense mechanisms, which may account for at least some fundamental trait differences observed among genera (i.e., floral fragrance, coloration, size, and morphology).

Conclusion—This study addresses a fundamental gap in our knowledge of reproductive traits of mycoheterotrophs, using members of the Monotropoideae as a model system. Results from these analyses demonstrate that mycoheterotrophic plants have evolved a variety of reproductive strategies (e.g., differences in developmental and reproductive phenologies, pollinator interactions, and breeding systems) for dealing with a concert of intrinsic and extrinsic ecological factors to ensure successful propagation. Also, broad fluctuations in the reproductive output and fecundity of taxa, especially in lieu of ecological fluctuations, demonstrate possible reproductive limitations associated with a mycoheterotrophic life history. This study is the first thorough empirical exploration and comparative analysis of mycoheterotroph reproductive ecology and sets a methodological and theoretical foundation for future investigations of the evolutionary ecology of nonphotosynthetic plants.

TABLE 7. The relative proportion of flowers and fruits damaged by various types of herbivory of four mycoheterotrophic taxa. Included are the number (*N*) of predated flowers and fruits and the proportion of flowers and fruits with the following herbivory types: floral herbivory (FH), stem herbivory (SH), nectar robbing (NR), seed predation (SP). Seed predation was not measured in *Monotropsis odorata* because it was not possible to differentiate between fruits predated by herbivores and those consumed by seed dispersal agents.

Taxon	*N*	FH	SH	NR	SP
Monotropsis odorata	119	63.0	36.1	0.9	–
Monotropa uniflora	126	7.1	10.3	1.6	81.0
M. hypopitys (yellow form)	245	9.4	15.9	0	74.7
M. hypopitys (red form)	112	19.6	8.9	0	71.5

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