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The Effect of Insects on Seed Set of
Ozark Chinquapin, *Castanea ozarkensis*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Entomology

by

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Missouri State University
Bachelor of Science in Biology, 2014

May 2017
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This thesis is approved for recommendation to the Graduate Council.

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Abstract

Ozark chinquapin (*Castanea ozarkensis*), once found throughout the Interior Highlands of the United States, has been decimated across much of its range due to accidental introduction of chestnut blight, *Cryphonectria parasitica*. Efforts have been made to conserve and restore *C. ozarkensis*, but success requires thorough knowledge of the reproductive biology of the species. Other *Castanea* species are reported to have characteristics of both wind and insect pollination, but pollination strategies of Ozark chinquapin are unknown. Experiments were conducted to determine the influence of insects on successful pollination of *C. ozarkensis*, as measured by production of burs and seeds. Exclosure treatments were applied to *C. ozarkensis* inflorescences during flowering, including exclusion of both insects and wind, exclusion of insects but allowing wind, and a control that excluded neither insects nor wind. Exposure to both wind and insects (no exclusion) resulted in a greater proportion of successfully pollinated flowers, greater numbers of burs and a significantly greater proportion of burs that produced seeds than both exclosure treatments. There were no differences between the treatments that excluded insects or both insects and wind. Pollination occurred in the absence of insects, demonstrating wind alone could pollinate flowers, but at levels far less than when flowers were exposed to both wind and insects. Limited surveys of nocturnal and diurnal insects present on Ozark chinquapin inflorescences yielded 122 species in 6 orders. The number of insect species recorded was much greater than the numbers reported in studies of other *Castanea* species. Insects were present on male flowers, foraging for nectar and pollen, and on female flowers. The combination of the presence of insects on flowers and the reduced pollination in the absence of insects indicate Ozark chinquapin to be an amphiphilic species. This better understanding of pollination

strategies suggest that increasing pollinator presence could increase seed production, thus contributing to restoration practices for this functionally extinct species.

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Steven Bost, Cheyne Matzenbacher, and Tim Smith of the Ozark Chinquapin Foundation introduced me to the story of the Ozark chinquapin. They started me down the path to better understanding reproductive strategies of the tree. In 2014, I got to accompany Cheyne on trips to artificially cross pollinate some of the largest remaining Ozark chinquapin trees and to observe and collect insects off the flowers. The Foundation provided funding and contacts for potential study sites. Steve is the greatest advocate of the Ozark chinquapin and has been a tremendous guide on this project.

Finally, I would like to thank my parents for their continued support of my interests. I am grateful that in our childhood, they convinced my brother and me to play outside, and took us camping, biking, fishing, and hunting.

Dedication

I dedicate this thesis to the late Kelley Williamson. Most knew Mr. Williamson as a storm chaser from the Weather Channel TV show, "Storm Wranglers." I knew him as the landowner of my study site. Mr. Williamson and his family provided me with access to their wonderful property and made sure I was able to complete my work safely and effectively. For this, I owe him a debt of gratitude.

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I. Introduction

Will the blight end the chestnut?
The farmers rather guess not.
It keeps smoldering at the roots
And sending up new shoots
Till another parasite
Shall come to end the blight.
"Evil Tendencies Cancel," Robert Frost 1932

The Genus *Castanea*

The plant family Fagaceae contains three well-known genera: oaks (*Quercus*), beeches (*Fagus*), and chestnuts (*Castanea*). Species in these genera are all woody-stemmed trees and shrubs that produce nuts. They grow in mountainous areas with well-drained, slightly acidic soils (Rutter et al. 1991). The Fagaceae range through the temperate forests of the Northern Hemisphere and usually dominate the forest community (Manos 2001). The basal genus *Fagus* contains 10 species (Manos 1997, 2001), whereas *Quercus* contains the majority of the diversity within the family, with nearly 600 species recognized (The Plant List 2013).

Members of *Castanea* are characterized by their dentate-margined leaves and heavily spined seed coverings, called burs (Sargent 1896). *Castanea* can be superficially split into two groups, chestnuts and chinquapins (or chinkapins). The two groups of *Castanea* species have been classified by the number of nuts per bur, with chestnuts typically having 3 nuts per bur and chinquapins only one. Species within the genus interbreed readily (Rutter et al. 1991).

Contemporary classification of *Castanea* includes nine accepted species (The Plant List 2013), although one is a hybrid (*C.* × *neglecta*). The remaining eight species include four from Asia: Chinese chestnut (*C. mollissima*), Japanese chestnut (*C. crenata*), Chinese chinquapin (*C. henryi*), and dwarf chestnut (*C. sequinii*); one species from Europe: European or sweet chestnut

(*C. sativa*); and three species from North America: American chestnut (*C. dentata*), Allegheny chinquapin (*C. pumila*) and Ozark chinquapin (*C. ozarkensis*).

Based upon the fossil record, *Castanea* is assumed to have diverged from *Quercus* 60 million years before present (mybp). Molecular data suggest the chestnut genus radiated from Southeast Asia to Europe 42.55 mybp and from Europe to North America via the North American Land Bridge 39.14 mybp (Lang et al. 2006). The diversification of species in North America was estimated to have been 24.4 mybp (Lang et al. 2006). Chestnuts did not become plentiful in Northeastern North America until 2500 years ago, based on palynological data (Paillet 2002). That plentiful status remained for centuries, until the arrival of the exotic, invasive disease known as chestnut blight, *Cryphonectria parasitica* (Murr.) Barr. The story of the chestnuts in North America epitomizes how an iconic species that dominates a large part of a continent can be impacted by an invasive species.

***Castanea* Species**

Old World *Castanea*

Two of the Old World *Castanea* species -- Chinese chestnut (*C. mollissima*) and European chestnut (*C. sativa*) -- have received great attention, primarily because of economic value of lumber and nut production.

Chinese Chestnut

The Chinese Chestnut, native to the ancestral home of *Castanea* in eastern Asia, is now grown throughout the United States as an ornamental shade tree due to its high level of blight resistance. Although the Chinese chestnut is smaller in stature than the American chestnut, it is a key species as a parent tree for cross-breeding blight-resistant trees (Clapper 1954a).

European Chestnut

In contrast, the European chestnut (*C. sativa*) showed little or no resistance to the chestnut blight, and so it was greatly affected by the disease. European chestnuts have been known to reach great size. The tree species with the largest girth ever recorded was a European chestnut located in Sicily and named the "Hundred-Horse Chestnut." Measured in 1780, the tree had a circumference of 57.9 m (190 ft) (Guinness World Records 2017). The name refers to a legend: long ago, during a heavy rainstorm, the Queen of Aragon and her one hundred mounted horsemen took refuge under the branches of this tree (Anonymous 1871).

New World *Castanea*

Allegheny Chinquapin

Of the three *Castanea* species in North America, Allegheny chinquapin, *C. pumila* Mill, is the most different in growth habit. Allegheny chinquapin usually grows as multi-stemmed shrubs and is the only member of the genus known to reproduce stoloniferously (Rutter et al. 1991). The multi-stemmed plants can form dense thickets, providing shelter for wildlife (Payne et al. 1994). This species ranges across the eastern United States from Pennsylvania to Florida and west to Arkansas and east Texas (Johnson 1988), though its abundance has been reduced greatly due to its great sensitivity to chestnut blight. Allegheny chinquapin overlaps in overall distribution with the other two *Castanea* species, though *C. pumila* is usually found in less mountainous areas.

American Chestnut

Unlike the shrub-like *C. pumila*, American chestnut trees (*C. dentata*) were some of the largest trees in eastern North America. American chestnut trees comprised 25% of the Eastern Deciduous Forest before 1904, and were referred to as 'the redwoods of the east' (Burnham

1988). It was not uncommon for American chestnut trees to reach a height of 40 m (~131 ft) (Buttrick 1925), with a diameter of 1.5 m (~5 ft) (Burnham 1988), and were believed to live more than 600 years.

American chestnut grew primarily in the Appalachians east of the Mississippi River from Maine to Mississippi (Johnson 1988). They were utilized by the American people for nearly every part of the homestead (Burnham 1988). Shingles, panels, fences, tool handles, telephone poles and railroad ties were all made from rot-resistant chestnut wood. Tannins extracted from the tree were used to process leather. Livestock were turned out into public forests to gorge upon the abundant mast of chestnuts littering the forest floor. The sweet-tasting chestnuts were high in starches and were shipped by train to large cities where they were sold fresh or roasted (Senter et al. 1994).

The story of the American chestnut is one that is tied to the foundations of folklore in the United States. Memories of this once-plentiful tree are seen as names on street signs in nearly every town in the eastern US. Chestnuts are recognized by their prominent place in the Christmas holiday, through the line, “Chestnuts roasting on an open fire.” That line in the holiday tune, "The Christmas Song,” written by Bob Wells and Mel Torme, was made popular through recordings by Nat King Cole.

Ozark Chinquapin

> "The Ozark Chinquapin nuts were delicious and we waited for them to fall like you would wait on a crop of corn to ripen… they were that important. Up on the hilltop the nuts were so plentiful that we scooped them up with flat blade shovels and loaded them into the wagons to be used as livestock feed, to eat for ourselves, and to sell. Deer, bears, turkeys, squirrels, and a variety of other wildlife fattened up on the sweet crop of nuts that fell every year. But, starting in the 1950's and 60's all of the trees started dying off. Now they are all gone and no one has heard of them."
>
> -Harold, 98-year-old Missouri outdoorsman 2004

The Ozark chinquapin, *Castanea ozarkensis*, was first described by W.W. Ashe in 1923 as a tree growing to 12 m with coarsely serrate leaves (Ashe 1923). Ozark chinquapins grow on dry, rocky slopes in deciduous or mixed woodlands of the Ozarks region of southern Missouri, eastern Oklahoma, and mountainous regions of Arkansas (Figure 1) (Johnson 1988). A disjunct population of *C. ozarkensis* was known from north-central Alabama, based on herbarium specimens, but that population is believed to have been extirpated due to the blight (Johnson 1988). Throughout its present range, Ozark Chinquapin is uncommon. Personal observations of the author indicate remaining trees typically grow in clumped stands within mixed hardwood forests.

Tucker (1975) reclassified Ozark chinquapin as a variety of the Allegheny chinquapin, reducing its status to *Castanea pumila* var. *ozarkensis.* Tucker cited field study and herbarium specimens as his basis for the reclassification, but he largely relied on leaf morphologies and the ability of the trees to interbreed readily. He also stated the "*pumila* complex as it exists in most of the eastern United States is an extremely difficult group in much need of modern experimental study," (Tucker 1975). A later revision of the North American chinquapins by Johnson (1988) supported Tucker's classification. However, in a subsequent study, Anagnostakis (2011) opposed Johnson's inclusion of Ozark chinquapin as a variety of Allegheny chinquapin, saying, "They are, however, so different that I find it hard to agree with him."

The studies that Tucker called for now exist, with recent genetic studies of the entire *Castanea* genus, specifically of the North American taxa. Molecular analysis using chloroplast genome sequencing showed the Ozark chinquapin to be the basal member of the North American clade, with the Allegheny chinquapin and American chestnut being sister species (Lang et al.

2006). A second study supported those findings in the North American clade through morphological comparisons, geographic range, and chloroplast sequencing (Shaw et al. 2012).

Before the blight arrived, *C. ozarkensis* was a large tree and dominated the forest canopy (Paillet and Cerny 2012). One of the largest specimens on record was near Fayetteville, Arkansas, and was reported as 52ft (15.85m) tall and 3ft 3in (0.99m) in diameter (Moore 1950). The currently listed World Champion *C. ozarkensis* was located in southern Missouri, with dimensions reported as 11.14 in (28.3 cm) DBH and 62 ft (18.9 m) tall in 2013 (Missouri Department of Conservation 2016). Although Ozark chinquapin trees are slightly more resistant to chestnut blight than the American chestnut, they are still greatly affected (Graves 1950).

In 1957, when the blight reached the Ozarks, nearly all chinquapin trees were killed above the roots (Paillet 1993). North American *Castanea* species have long been known to reproduce clonally from root buds (Mattoon 1909). Root collar sprouts grow vigorously during a 'release' event after the forest canopy has been opened, growing for 3-5 years until the blight kills them. However, that short duration of growth is seldom long enough for the plant to produce flowers and seeds, thereby preventing sexual reproduction.

Chestnut Blight and *Castanea*

Forests of the eastern United States have changed greatly over the last century. Many dominant species have experienced great loss, each with its own introduced pest or disease. The elms were decimated by Dutch elm disease; hemlocks are under attack by hemlock wooly adelgid; ash are currently being threatened with extinction by emerald ash borer; and the chestnuts are all but gone due to chestnut blight.

Chestnut Blight, *Cryphonectria parasitica* (Murr.) Barr. (Diaporthales: Cryphonectriaceae), is a fungal disease that was first noticed in the United States in 1904 in the

Bronx Zoo, New York, when American chestnut trees began dying (Merkel 1906). The disease was believed to have been imported on Japanese chestnut nursery stock (Murrill 1908). Although the fungus had little impact on *Castanea* in the native range in Asia, North American *Castanea* were greatly affected by the blight because they did not share a long relationship with the fungus, as did the Asian species.

Chestnut blight infects a tree twig, branch, or trunk at a point of injury or where a branch has broken off (Anagnostakis 1982). The fungus then moves into the vascular tissue of the stem and begins to radiate outward. On young trees, a sunken area, known as a canker, will appear around the point of infection (Anagnostakis 1987). As the fungus continues to move outward, it will eventually circle around the branch and girdle it, causing everything above the infection to die (Murrill 1906a). Fungal spores are released from these points and are spread by wind and any other animal that passes over (Anagnostakis and Hillman 1992). Fruiting bodies of the fungus occasionally appear visibly as small, yellow to red colored pustules around the canker (Murrill 1906b).

The fungus does not infect the tree below the surface of the ground. Root systems of infected trees can live for many years sending up new root-collar sprouts, which can continue to grow for 3-4 years until they are overtaken by the blight (Metcalf 1913). Because infected sprouts die within a few years, failing to grow and mature enough to produce seed, North American *Castanea* species are now considered ecologically extinct. Despite the near-absence of chestnut trees as hosts, the blight can persist on various species of oak (Graves 1937).

Plant diseases do not occur in isolation, and infections by multiple causal agents could interact, either enhancing disease progression or inhibiting it. One example of an interaction is hypovirulence, which is when a disease has reduced virulence or when a pathogen has reduced

severity (Grente 1965, translated by Anagnostakis 1987). Hypovirulence in *Cryphonectria parasitica* infecting *Castanea* was first observed in Italy in *C. sativa* orchards, in which it was noted that the blight was no longer spreading (Biraghi 1935). Hypovirulence was thought to be a disease or group of diseases that reduced the virulence of chestnut blight (Anagnostakis 1982). The severity of the strain of hypovirulence in Europe has allowed cultivated *C. sativa* to persist and seeds are still produced at commercial levels.

As soon as hypovirulence was noticed in Europe, researchers wondered if it could be as effective in the United States (Van Alfen et al. 1975). Day et al. (1977) worked to determine the causative agent of the hypovirulence and discovered foreign double-stranded RNA (dsRNA) present in the blight culture. Soon after, Dodds reported that these dsRNAs were virus-like (Dodds 1980). Hypovirulence has been determined to be caused by viruses in the family Hypoviridae, and several strains are believed to exist (Hillman et al. 1995). However, due to vegetative incompatibility, the European strains of the virus were not viable treatments for American chestnuts (Anagnostakis 1982). A North American virus was found attacking chestnut blight in Michigan in 1976 (Anagnostakis 1987). Despite the potential for using hypovirulence to combat chestnut blight, that intensive approach has been used effectively only in commercial orchards (Anagnostakis 2009), but not in remaining *Castanea* trees outside of cultivation.

Insect Pests of *Castanea*

Although a variety of insects can be found on *Castanea* species in North America, only two weevil species (Coleoptera: Curculionidae) are known to be obligate chestnut feeders: the larger chestnut weevil, *Curculio caryatrypes* Boheman, and the lesser chestnut weevil, *Curculio sayi* Gyllenhal. The greater chestnut weevil was reported to be the largest *Curculio* species found in the United States but had not been reported since the mid-1950s (Gibson 1969).

However, Anderson (2017) reported that two specimens had been collected in 1987, having been reared from an American chestnut tree.

The lesser chestnut weevil still occurs, and has been reported to be a major pest of cultivated chestnuts (Keesey and Barrett 2008). Adult females of *C. sayi* possess an extremely long proboscis, which allow them to get past spines on the burs, where they feed, and chew holes in which to oviposit. Female *C. sayi* begin oviposition into the burs when the *Castanea* seeds are nearly mature and the burs begin to split open. Most *C. sayi* complete their life cycle in two years, but a small percentage of weevils require a third season for development (Brooks and Cotton 1929). Nuts infested by weevils usually contain multiple larvae. Adult weevils have two distinct emergence periods: in the spring, during flowering; and in the late summer, when seeds ripen (Keesey and Barrett 2008). In the spring, adult weevils can be found feeding at male catkins. If harassed, the adult weevils will feign death and fall to the ground (pers. obs.).

Two non-native beetles are known to defoliate chestnuts in North America. The Asiatic oak weevil, *Cyrtepistomus castaneus* (Roelofs), was accidently introduced to the northeast U.S. from Japan in 1933 (Evans 1959). Although mainly a defoliator of oaks, these weevils also have been reported to cause up to 35% defoliation of chestnut trees (Johnson 1956). Another non-native defoliator is the Japanese beetle, *Popillia japonica* Newman, which was first noticed in the United States in New Jersey, in 1916 (Fleming 1972). Despite the defoliation or skeletonizing by both these exotic beetles, it is unknown whether their feeding causes harm to chestnut trees.

Biology of *Castanea*

Reproduction

Self-fertilization occurs when a plant is able to produce fertile seeds with its own pollen. The reverse, self-sterility, occurs when a plant fails to produce fertile seeds with its own pollen, or those seeds fail to develop normally. All *Castanea* species are monoecious, with both male and female flowers on the same tree. However, species in the genus *Castanea* are generally considered to be self-sterile (Vilkomerson 1940). Chinese chestnuts have been reported to have a self-fertilization rate of less than three percent (McKay 1942). Morris observed a number of *C. pumila* flowers produced self-fertile seeds, which sprouted but developed abnormally (Morris 1914).

Chestnuts have two types of inflorescences: staminate (male) catkins and androgynous catkins; the latter possess 2-3 pistillate (female) bracts at their base with the remaining portion staminate (Figure 2) (Jaynes 1975). Inflorescences are borne on the distal portion of the twig on the current year's growth. Each bract typically possesses three pistillate flowers (Botta et al. 1995). The bracts develop into burs and each flower will develop into a nut, thus producing 3 nuts per bur. Chinquapins possess more pistillate bracts per catkin than do chestnuts but each bract possesses only one pistillate flower which will then develop into one nut in a bivalvular bur. *Castanea* pollen are oblong with three lobes extending the length of the granule (Chenge-le et al. 2009). Pollen granules of the staminate catkins are miniscule in size at 15-17 µm long and 9-10 µm in diameter (Figure 3) (Johnson 1988).

A study of American chestnut by Vilkomerson (1940) revealed three different types of flowering sequences occurring among separate trees. In the first flowering type, called protandry, two periods of anthesis or pollen release occur up to a week before pistillate flowers

on the same tree are receptive. The second period begins after female flowers are no longer receptive. The second flowering type is protogynous, in which the female flowers bloom first. The third type of flowering is synchronous, in which male and female flowers bloom at the same time. Information about the type of flowering sequence is necessary for ensuring successful breeding techniques as well as for understanding natural pollination (Vilkomerson 1940). The first two flowering sequences are thought to contribute to the self-sterility of chestnuts.

Flowering in American chestnuts begins in mid-June, and lasts about a month. Timing varies due to location, elevation, and other environmental factors. The reproductive season is the time from first flowering to seed drop (Clapper 1954a). Pistillate flowers become receptive when the styles have turned to a straw-yellow color and have bent over at a right angle (Clapper 1954a). Staminate inflorescences are aborted and drop from the tree when anthesis is complete.

Oaks and most other hardwoods bloom in early spring, before leaves are present or while leaves are still small. In contrast, chestnuts blossom when leaves are fully developed. One advantage to late-season flowering is that the likelihood that flowers will be destroyed by a late, harsh frost, is greatly reduced. Thus, the seed mast of chestnuts is consistent and large every year, unlike oaks, whose seed production varies greatly from year to year (Rutter et al. 1991). However, late-season flowering also shortens the reproductive season for chestnuts, compared to oaks and hickories; therefore, chestnuts have less time for seeds to mature (Graves 1941).

Pollination

Studies of pollination strategies of chestnut – accompanied by speculation – have persisted for longer than a century. The desire to understand pollination strategies ignited when imperilment of the chestnuts was initiated by arrival of the blight. Each species differs in some way, and each species seems to have its own story. Despite the similarity of methods employed

by each researcher, with each encountering similar problems, the questions remain: "How are these species pollinated? By wind? By insects? Both?" Each study has produced results that tend to support one of the two pollination strategies: wind (anemophily) or insects (entomophily).

Wind pollination characteristics

Crane (1937) stated unequivocally, “Insects have nothing to do with pollination of the chestnut,” thus directing subsequent studies toward anemophily. A literature review by Clapper (1954a) summarized the list of floral characteristics of *Castanea* that are typically associated with wind pollination: male and female flowers are borne separately, they are freely exposed to air currents, stamens produce large quantities of pollen, pollen is small and lightweight, female flowers are inconspicuous and possess no odor or nectar, styles are sticky and well adapted to collect windborne pollen. A pollination study conducted by Clapper (1954a), enclosed Chinese chestnut, *C. mollissima*, flowers in cheesecloth and mosquito netting to evaluate wind pollination and left a third group of flowers open to allow both wind and insects access to flowers. From his study, he concluded that chestnuts were mainly wind pollinated and that wind pollination was more effective than insect pollination. Johnson conducted a similar study and reached the same conclusion: North American chestnuts and chinquapins are primarily wind pollinated and any contribution to pollination by insects would be indirect (Johnson 1988).

Insect pollination characteristics

In the same 1954 study, Clapper also listed possible insect pollination characteristics of chestnuts: male catkins are highly odorous, possess nectar, are colorful, and erect, pollen is sticky, male catkins are in immediate proximity to female flowers that possess stiff styles, and bees and flies frequently visit these flowers (Clapper 1954a). DeOliviera et al. (2000) evaluated

the importance of insect pollinators for seed production in the European chestnut, *C. sativa*. Their study used three treatments: 1) one that allowed insects and wind; 2) another with large-mesh muslin bags that allowed 80% of wind to pass through, while preventing access by most insects; and 3) small-mesh muslin bags that allowed only 20% of wind currents and excluded insects. Their study showed that, in the presence of insects, seed set increased by 16-73%. The increase in seed production led them to propose that European chestnut is entomophilous and insects provide the majority of pollination. Another study (Giovanetti and Aronne 2011) showed that honey bees collected pollen and nectar from *C. sativa* flowers. They suggested insects pollinate *C. sativa* when abiotic conditions are unfavorable to wind pollination, supporting the entomophilous conclusion, stating "Pollination of chestnut by insects is clearly established, although wind pollination may occur under certain conditions" -- exactly the opposite of the conclusion reached by Johnson (1988).

Objectives of the Study

Because of the threats from chestnut blight to Ozark chinquapin, and the lack of knowledge of the mode of pollination of this species, this study was conducted to assess the importance of insects as pollinators of *C. ozarkensis*. Such knowledge would be essential to contribute to conservation of this range-limited species, and would aid in efforts to breed blight-resistant *Castanea* species.

The main objectives of this study were:

1) to discover the role and importance of insects in pollination of Ozark chinquapin; and

2) to describe the insect community found on the flowers of Ozark chinquapin.

The results of the study are presented, offering some insight into the importance of insects for pollination, as well as a discussion of the findings and their implications for conservation of the Ozark chinquapin.

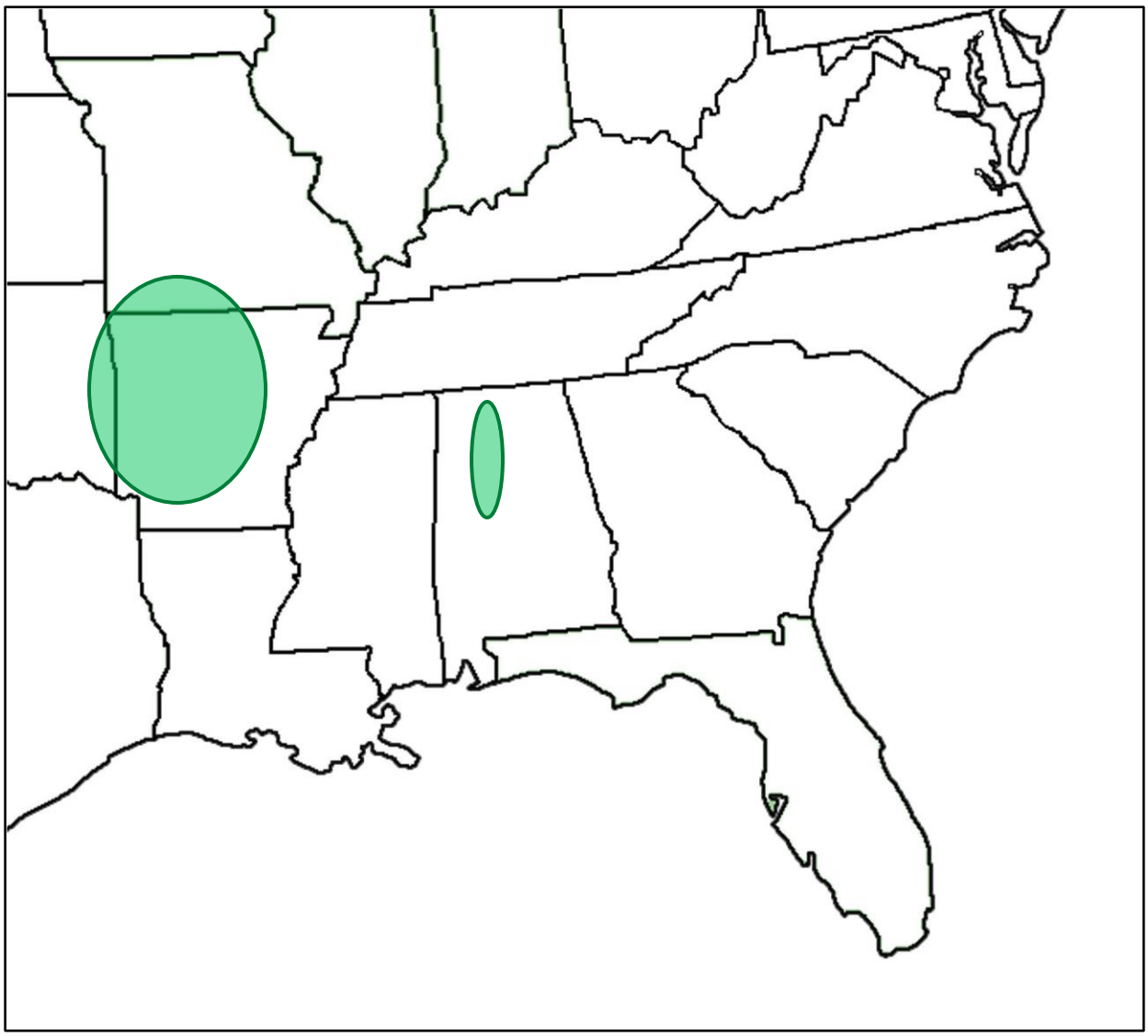

Figure 1. Range map of the Ozark chinquapin, *Castanea ozarkensis*, in the southeastern United States, based on Johnson's (1988) report of herbarium specimens. The disjunct population in Alabama is thought to be extirpated.

Figure 2. Inflorescences of the Ozark chinquapin, *Castanea ozarkensis*, showing catkins typical of the genus. The brightly-colored catkins at the base of the twig are staminate, with pollen produced at the tips of the stamen. Androgynous catkins are present at the apex of the branch, with pistillate bracts at the base and staminate flowers at the tip. Photo by the author.

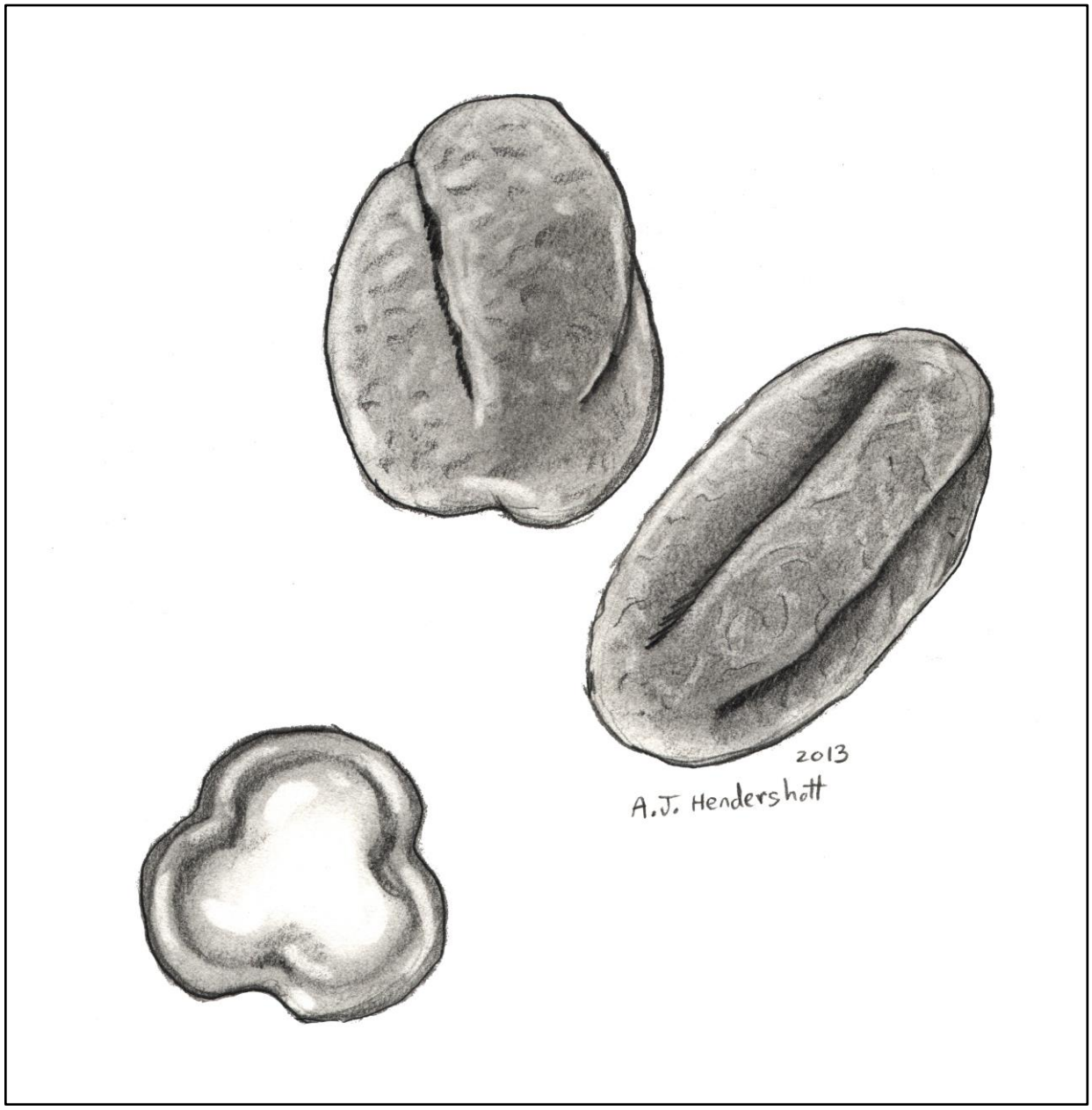

Figure 3. Illustration of *Castanea* pollen, showing polar and lateral views. Drawing provided courtesy of A. J. Hendershott.

II. Materials and Methods

Study Site

The selected site was located in Barry County, Missouri, USA, 6.75km southeast of Cassville, and is approximately 0.75 km^2. Contacts for potential study sites were provided by the Ozark Chinquapin Foundation. The research plot exists on the Springfield Plateau near the border of the Salem Plateau. The ridgetops have been cleared of most trees to allow cattle grazing but a few oaks, walnuts, and chinquapin remain (Figure 4). The plot is bisected and bordered by forested valleys. The surrounding land is similar with hay fields and national forest.

There were 40 known *Castanea ozarkensis* trees at the study site in 2015-2016. Plants near the trees flowering at the same time as chinquapins include: raspberries, blackberries, dewberries, multiflora rose, poison hemlock, clovers, asters, sensitive plant, and thistle. All but one of the *C. ozarkensis* trees at the site had visible indications of the blight disease, appearing as large, swollen cankers usually indicative of a type of hypovirulence. Trees varied in growth form from small, stump-sprout shrubs to 15m tall trees with 25cm diameter at breast height. Canopy diameters ranged from 1.8-5.5 m. The trees occurred individually or in clusters of 4-5 trees, with distances between clusters ranging 130-400 m. The ages of the trees were unknown. All appear to have recruited naturally and not been planted or cultivated. The site was scouted to find specific chinquapin trees meeting these criteria: be easily accessible, possess both male and female catkins, have enough branches with female catkins to place treatments, and recently produced seeds (indicated by presence of burs from the previous season on the ground beneath the trees). The study was conducted from May to September in both 2015 and 2016.

2015 Methods

For the 2015 field season, three treatments were placed: insect exclusion, insect and wind exclusion, and a control (no exclusion). Insect exclusion was accomplished through the use of plastic mesh bags, with 1mm openings (Pentair Aquatic Ecosystems, Apopka, Florida, USA). The mesh was folded to form a bag, and the edges were folded over and stapled to prevent the mesh from unraveling. The finished size of the mesh bag was 13x20x22cm. White paper bags, measuring 9x13x23cm (Sam's Club, Bentonville, Arkansas, USA), were used to exclude both insects and wind.

Five trees were selected in 2015, and 10 replications of each of the three treatments were placed on each tree. A random-number generator was used to decide the order of placement for each treatment. The two exclusion treatments consisted of enclosing the terminal end of a twig in the respective bag type, secured by a zip tie. Colored zip ties were used to denote treatment type. Before bags were placed over the end of the twig, all leaves were removed except for the apical leaves. Leaf removal was conducted to allow bag placement and to allow air circulation around the catkins. No reproductive structures were removed from the twigs. Bags were placed on trees prior to flowering to prevent uncontrolled pollination. Twigs used for the control were treated identically, as to removal of leaves and addition of a colored zip tie to mark the twig; however, the no-exclusion control had no bag placed over the twig. In 2015, treatments were applied to trees on May 22, 25, and 26 depending on maturity of catkins on each tree. For each individual tree, all treatments were applied the same day.

In the two exclusion treatments, all bags were removed approximately one month after placement (June 21), after flowering was completed, to allow burs to develop naturally. Color-coded zip ties were replaced loosely during bur development to mark twigs used for treatments.

Water-resistant, paper pollination bags, measuring 12x17x34cm (#404, Lawson Pollination Bags, Northfield, Illinois, USA) were applied to each treatment twig that had burs present on September 7. These bags were placed to prevent nut removal by squirrels, and to minimize damage from chestnut weevils and lepidopteran caterpillars. Burs were collected September 17 at the time of bur split, by breaking the cluster off the twig, placed in bags, and returned to the laboratory for examination to determine seed set, indicative of successful pollination.

2016 Methods

Methods were adjusted in 2016. The number of replicates per tree was reduced from 10 to 3, meaning each tree had 9 bags (3 of each of the 3 treatments), which allowed increasing the number of trees used from 5 to 12. The slightly larger and water-resistant pollination bags (Lawson #404) replaced the paper bags used in the previous season. Slightly larger mesh bags were used (14x28x28cm). Treatments were applied on May 19, 23-25, 27, and 31 in 2016 and removed after the conclusion of flowering on June 18, 24, 29, and July 7. Pollination bags were reapplied prior to bur split on September 7 and 15 and remained on the trees 5-10 days, until the majority of burs split open.

In addition to the three treatments, an additional trial was set up to assess insect pollination while excluding wind. Four trees were selected to receive three water-resistant pollination bags (as above). Honey bees, *Apis mellifera*, were collected from *C. ozarkensis* catkins at the site during peak flowering on June 7. The honey bees were collected from trees on the opposite side of the study site, to allow the greatest chance of viable pollination. These honey bees were observed prior to collecting to confirm pollen presence in their corbiculae. One honey bee was enclosed in each bag and allowed to move freely inside the bag. The enclosed

honey bees were observed to ensure they were actively visiting the enclosed flowers. The bees stayed in the bags until they expired. Those bags were removed from the trees the same day the other treatment bags were removed. As with the other treatments, the pollination bags were reapplied prior to bur split on September 7 and 15 and remained on the trees 5-10 days, until the majority of burs split open.

A survey was conducted during the flowering period of *C. ozarkensis* to determine the presence of insect species that could potentially be pollinators of the trees. Insects were collected by hand or by sweep net directly off chinquapin flowers. Sampling occurred for 2-3 hours in each of two daylight periods (June 2, and 7, 2016) and one period after dark (June 8-9, 2016), to collect both diurnally and nocturnally active insects. The collected insects were pinned and identified to the lowest possible taxonomic level (species, genus, family). All insect specimens were deposited into the University of Arkansas Arthropod Museum, Fayetteville, AR, USA.

Ozark chinquapin twig and leaf samples were also collected for vouchers. They were collected with blooming catkins in the spring and with mature burs in the fall. These pressed specimens were deposited in University of Arkansas Herbarium, Missouri State University Herbarium, and Ozark Chinquapin Nature Center, Roaring River State Park, Cassville, MO, USA.

Data Analyses

After the flowering period, pistillate flowers have three possible outcomes:

1. Abortion or death where the catkin falls off the tree and produces no burs or seeds,
2. Develop into empty burs, or
3. Develop into burs with seeds.

Two trees from 2016 were excluded from all analyses due to no production of burs or seeds within or outside of experimental treatments. The fourth treatment, to exclude wind but allow one honey bee, was excluded from Analyses 1 and 2.

Analysis 1

Evaluation of the outcome of flowering was based on catkin abortion (outcome 1), empty burs (outcome 2), and burs with filled seeds (outcome 3). The number of occurrences of each outcome was calculated for each treatment across tree and year. For analysis, data for outcomes 2 and 3 were combined to categorize the data into two outcomes: bur absence or presence. Using an assumption of equal likelihood of bur absence or presence, the data for each treatment were analyzed by chi-square, using Microsoft Excel 2016.

Analysis 2

Bur presence was assumed to indicate pollination. The number of burs in each treatment for 2016 was compared and evaluated as a proxy for pollination to include data from the honey bee only treatment. Numbers of burs per unit were assumed to be approximately normally distributed and subjected to analysis of variance. Differences among the means were determined using least significant difference (LSD) procedure at $\alpha=0.05$.

Analysis 3

The difference between years of seed production data was analyzed and found to not be significantly different ($P=0.722$). For that reason, data from 2015 and 2016 were grouped together for analysis. The proportions of burs with seeds (outcome 3) were analyzed using a generalized linear mixed model with a binomial distribution and a logit link function. Treatments and years were considered fixed effects and trees as random effects.

Seed set analysis, or the proportion of seeds per burs, was conducted using SAS (version 9.4, analytics 13.2) and PROC GLIMMIX. Significance was evaluated at $\alpha=0.05$.

Figure 4. Aerial view of the study site in Barry County, Missouri, showing the cleared ridgetops with scattered *Castanea ozarkensis* trees. Image from Google Earth, taken May 8, 2012.

Figure 5. A screen bag placed on the apex of a twig of Ozark chinquapin, *Castanea ozarkensis*, as a treatment to exclude insects but allow wind access to evaluate pollination. Photo by the author.

III. Results

Flower Outcome

Flower outcome varied as a response to treatment (Table 1). When both insects and wind were excluded, 71.6% of bags produced no burs or seeds. When only insects were excluded, 64.9% of bags produced neither burs nor seeds. However, when flowers had access to both wind and insects, only 24.4% failed to produce burs or seeds.

Bur Production 2016

The numbers of burs produced in each treatment differed significantly ($F=21.15$; $df=3,97$; $P< 0.0001$; Table 2). The mean number of burs per unit for the treatment excluding both wind and insects was 1.53, versus 2.66 for treatment that excluded only insects. In contrast, the treatment that included an individual honey bee produced 5.92 burs per unit and the control treatment with access to wind and insects averaged 18.0 burs per unit. In addition, the percent of treatment units that produced burs aligned with the numbers of burs: 13.1% of the bags excluding insects and wind produced burs, and 34.5% of bags excluding only insects produced burs. In contrast, 58.3% of bags containing bees produced burs and 83.3% of the units in the open control produced burs. Mean separation tests using LSD showed the control treatment to be different from the exclusion treatments but failed to find a difference between the exclusion treatments.

Seed Production

Seed production for the two combined years differed significantly among treatments ($F=39.11$; $df=2,13$; $P<0.0001$; Table 3). Seed production in the control group, which allowed access by both insects and wind, averaged 33.2%, which was significantly greater than the other two treatments ($P= <0.0001$). Seeds were produced in 7.6% of flowers in which insects were

excluded, versus 5.7% of flowers for which both insects and wind were excluded; those two percentages did not differ.

The fourth treatment type in 2016, wind exclusion allowing one honey bee per bag, produced 22.7% seed set.

Insects Collected

Insects found on flowers represented 122 species or morphospecies classified in six orders (Table 4). The most speciose orders were Coleoptera with 39 species across 16 families and Lepidoptera with 30 species in 9 families. The remaining insects collected were: Thysanoptera, 1 species in 1 family, Hemiptera, 15 species in 7 families; Hymenoptera, 18 species in 8 families; and Diptera, 19 species in 6 families.

Insect larvae were also collected from Ozark chinquapin seeds. Lesser chestnut weevil, *Curculio sayi* (identified by Robert Anderson, Canadian Museum of Nature), were collected while they were drilling into seeds. Coleopteran larvae were also collected from seeds but not reared to adulthood. Lepidopteran larvae were also found inside Ozark chinquapin seeds with frass chambers extending into the bur. Infestation occurred at only one lepidopteran larva per seed and larvae were only present in some of the seeds from the tree producing the largest seeds at the study site. One adult moth was successfully reared from the seeds but has not yet been identified.

Table 1. a) Outcome of flowering, as indicated by the mean proportions of treatment units containing *Castanea ozarkensis* flowers that produced seeds, produced burs but no seeds, or produced neither (empty). Treatment units contained flowers that were either bagged to exclude both insects and wind, bagged to exclude insects only, or remained without bags as a control, allowing access by both insects and wind. b) Outcome of flowering, as indicated by the mean proportions of treatment units containing *Castanea ozarkensis* flowers that were not pollinated (as evidenced by absence of burs) or were pollinated (as indicated by presence of burs or burs with seeds). Treatment units were the same as above.

a)

		Outcome (proportions)		
Treatment	n	empty	burs only	burs with seeds
exclude insects and wind	74	0.716	0.162	0.122
exclude insects only	77	0.649	0.169	0.182
open, no exclusion	78	0.244	0.038	0.718

b)

	Outcome (proportions; mean ±SE)	
Treatment	no pollination (absence of burs)	pollination (burs or burs with seeds)
exclude insects and wind	0.716 (0.024)	0.284 (0.024)
exclude insects only	0.649 (0.026)	0.351 (0.026)
open, no exclusion	0.244 (0.021)	0.756 (0.021)

Table 2. Mean (±SE) numbers of burs produced by *Castanea ozarkensis* flowers in treatment units, and the proportion of treatment units that produced any burs, for 2016 only. Treatment units contained flowers that were either bagged to exclude both insects and wind, bagged to exclude insects only, bagged to include one *Apis mellifera* adult, or remained without bags as a control, allowing access by both insects and wind.

Treatment	Number of burs	Proportion of units with burs
exclude insects and wind	1.533a (0.783)	0.133
exclude insects only	2.655a (0.784)	0.345
include honey bee	5.917a (1.649)	0.583
open, no exclusion	18.00b (2.708)	0.833

Table 3. Mean (±SE) proportions of treatment units containing *Castanea ozarkensis* burs that produced seeds. Means followed by different letters are significantly different at P=<0.0001. Data from both 2015 and 2016 were combined.

Treatment	Proportion of units
exclude insects and wind	0.057a (0.021)
exclude insects only	0.076a (0.021)
open, no exclusion	0.332b (0.041)

Table 4. Insects collected, by hand, from flowers of *Castanea ozarkensis* in 2016. Insects were identified to lowest taxonomic unit possible and are listed by order, family, and species. Taxa identified as morphospecies are listed by family or genus and numbered as distinct morphotypes. *Collected in 2014 using the same methods as 2016. **Photographed but not collected. ***Specimens recognized as distinct morphospecies but not identified to family.

Order	**Family**	**Species**
Thysanoptera	Thripidae	*Frankliniella** **
Hemiptera	Cicadellidae	1 unidentified species
	Coreidae	*Acanthocephala terminalis*
	Lygaeidae	*Lygaeus kalmii**
		Lygaeus turcicus
	Miridae	2 unidentified species*
		Hyaliodes harti
	Pentatomidae	*Chinavia hilaris*
		Proxys punctulatus
		Stiretrus anchorago
		1 unidentified species*
	Reduviidae	*Phymata americana*
		Phymata hilaris
		Sinea spinipes
	Thyreocoridae	*Corimelaena lateralis*
Coleoptera	Anobiidae	1 unidentified species
	Buprestidae	1 unidentified species
	Cantharidae	*Chauliognatus marginatus*
		1 unidentified species
	Cerambycidae	*Euderces picipes*
		Obrium maculatum
		1 unidentified species*
		2 unidentified species
	Chrysomelidae	*Amblycerus*
		Diabrotica undecimpunctata
		Diachus chlorizans
		Sennius abbreviatus
	Cleridae	*Enoclerus ichneumoneus*
	Coccindellidae	*Harmonia axyridis*
	Curculionidae	*Curculio sayi*
		1 unidentified species
	Elateridae	3 unidentified species
	Lampyridae	1 unidentified species*
	Lycidae	*Calochromus perfacetus**

Table 4. (cont.)

Order	Family	Species
Coleoptera	Melyridae	*Collops quadrimaculatus**
(cont.)	Mordellidae	1 unidentified species
		1 unidentified species*
	Nitidulidae	*Epuraea**
	Scarabaeidae	*Callistethus marginatus*
		Euphoria sepulcralis
		Macrodactylus subspinosus
		Popillia japonica
		Trichiotinus lunulatus
		*Trichiotinus piger**
		Valgus canaliculatus
	Tenebrionidae	*Isomira sericea*
		Lagriini (1 unidentified species)
Hymenoptera	Andrenidae	1 unidentified species*
	Apidae	*Apis mellifera*
		Bombus bimaculatus
		Bombus impatiens
		*Xylocopa virginianica**
	Colletidae	*Hylaeus* 2 species
	Formicidae	*Camponotus*
		Formica
	Gasteruptiidae	*Gasteruption floridanum*
	Halictidae	2 unidentified species
	Ichneumonidae	1 unidentified species
	Vespidae	*Monobia quadridens*
Lepidoptera	Crambidae	*Blepharomastix ranalis*
		Desmia
		Herpetogramma
		Palpita
	Erebidae	*Caenurgina crassiuscula*
		Euclidia cuspidea
		Halysidota tessellaris
		Haploa reversa
		Renia flavipunctalis
		1 unidentified species
	Geometridae	*Eusarca confusaria*
		Prochoerodes lineola

Table 4. (cont.)

Order	Family	Species
Lepidoptera	Geometridae	*Scopula*
(cont.)	Hesperiidae	*Epargyreus clarus*
		1 unidentified species*
		1 unidentified species
	Noctuidae	*Elaphria grata*
		Spragueia leo
	Nymphalidae	*Asterocampa celtis**
		*Charidryas nyceteis**
		Speyeria cybele
	Pterophoridae	4 unidentified species
	Pyralidae	*Condylolomia participalis*
		Hypsopygia
	Zygaenidae	*Acoloithus falsarius*
Diptera	Anisopodidae	*Sylvicola*
	Calliphoridae	1 unidentified species
	Limoniidae	1 unidentified species
	Tabanidae	*Goniops chrysocoma*
	Tachinidae	*Archytas**
		*Trichopoda pennipes**
	Syrphidae	*Eristalis flavipes*
		Eristalis stipator
		Eristalis transversa
		*Mallota postica**
		Toxomerus geminatus
		Sphaerophoria
		1 unidentified species

***Morphospecies with unidentified families: Coleoptera (4), Hymenoptera (4), Lepidoptera (2), and Diptera (6).

IV. Discussion

Each of the comparisons in the present study showed that insects play an important role in pollination of Ozark chinquapin. Comparing the outcome of flowering (Table 1b), the difference between successful (burs or burs with seeds) and unsuccessful pollination (no burs or seeds) was 2-3 fold: more treatment units had successful pollination when wind and insects were not excluded from flowers (open control), and less successful pollination in the treatment units in which either insects or wind and insects were excluded. Moreover, successful pollination itself varied among the treatments: the percentages of treatment units that that produced burs and seeds versus those that only produced burs (Table 1b). Nearly 95% of the treatment units that produced burs in the open control also produced seeds, whereas the treatments that excluded insects only and those that excluded both insects and wind produced burs in only 51.9% and 43.0% units, respectively.

The evaluation of bur numbers in 2016 used burs as a proxy for pollination: any bur production was considered successful pollination. As with the flower outcome results, the total number of burs (18.0) produced per unit in 2016 was greatest in the open control, which was nearly 10-fold greater than either exclusion treatment (Table 2). The treatment that included an individual honey bee in each bag produced an intermediate number of burs per bag (5.82) and 58.3% of the bags had burs, showing greater pollination than the wind-only (insect exclusion) treatment, which produced 2.65 burs per bag. Also, the bee enclosure treatment included only one bee, which was unable to sustain itself on other resources or acquire new pollen. Even with limited opportunity for the bee, the number of burs was double that of the wind-only treatment.

Seed set, measured as the percent of individual burs that produced seeds, was significantly greater when insects and wind were allowed access (Table 3). Nearly one-third of

the burs in the open control produced seeds. Although some seeds were produced in the two treatments that excluded insects, those percentages (7.6% and 5.7%) of burs producing seeds were significantly less than the open control.

A similar study examined pollination of the Chinese chestnut, *C. mollissima*, in Maryland, USA (Clapper 1954a). Clapper used mesh bags (mosquito netting) enclosing emasculated flowers to exclude insects but allow wind, and found bur production in 43% of flowers in which insects were excluded. The value from Clapper's (1954a) study was comparable to the 35% bur production in the analogous insect-exclusion treatment in the present study. Clapper's study did not report values for bur production in open pollination, although did compare seed set in the presence and absence of insects. He reported 90% seed set for Chinese chestnut in the presence of insects, versus 33% seed set when insects were excluded.

Clapper's (1954a) four conclusions on pollination of chestnut are as follows: "[1] chestnuts are largely wind pollinated; [2] wind pollination is natural and normal; [3] wind pollination is more efficient than insect pollination in species of *Castanea* that are separated by sex; [4] insects are not essential for pollination of chestnut." The results found in the present study dispute Clapper's conclusions 1-3 on every count, while the present study's results support conclusion 4. The results from the present study showed that Ozark chinquapin is largely insect pollinated, but insects were clearly not required for pollination, as seen by the number of burs produced (Tables 1 and 2) and seed set (Table 3) when insects were excluded.

Differences in conclusions about the importance of insects to pollination may be attributable to several factors. For one, Clapper (1954a) studied Chinese chestnut (*C. mollissima*), whereas the present study examined pollination in Ozark chinquapin (*C. ozarkensis*). The results in the present study were more similar to results from a study on the

European chestnut, *C. sativa* (De Oliveira et al. 2001) than to Clapper's study or reports from American chestnut, *C. dentata*. Perhaps the similarity in results could be tied to the phylogenetic relationships among species. Johnson (1988) suggested entomophilous characters in *Castanea* species were remnants of past entomophily. It could be that *C. ozarkensis*, the basal member of the North American clade – and closest relative to *C. sativa* – shares entomophilous characters with *C. sativa*, whereas the more derived *C. dentata* has lost those characters.

Difference between the present study and past studies in the magnitude of the role of insects may also be due to focus and perspective of the studies. Most studies of chestnut species and their pollination strategies have focused on plant characteristics and propagation in the face of chestnut blight. As such, those studies (e.g., Clapper 1954a, Johnson 1988) had primarily a botanical focus, rather than entomological. The difference in focus of the studies is evident in the great difference in the reports of the numbers of insects collected on flowers. Clapper (1954a) mentioned only three species that occurred in notable numbers on *C. mollissima* in Maryland, USA, citing them as pollen feeders having no incentive to move between trees because of the large quantities of pollen present. Two of those species (*Chauliognatus marginatus* (Coleoptera: Cantharidae) and *Macrodactylus subspinosus* (Coleoptera: Scarabaeidae)) were also observed in the present study. He also noted bees and flies feeding on nectar but cited those as "comparatively rare." A more recent study by Johnson (1985) surveyed diurnal and nocturnal insects present on flowers of Allegheny chinquapin, *C. pumila* in North Carolina, USA. Johnson observed many more insects (56 species) than did Clapper, but fewer than half as many species as were collected in the present study (122 species, Table 4). This could be due to regional differences in insect biodiversity among the studies. The same six insect orders were observed in both the present study and Johnson's, but only six species were

the same in both studies. The most numerous insect species found in Johnson's study, *Anaspis rufa* (Coleoptera: Scraptiidae), was not observed on *C. ozarkensis* in the present study. Differences in insect species reported in the studies could be a result of range.

Observing behavior of insects on *Castanea* flowers is critical to the conclusions derived. Most of the species observed on *C. ozarkensis* flowers in the present study (Appendix) were not known to be pollinators, but were pollen feeders, nectar feeders, predators or incidental visitors. However, several species, most notably *Apis mellifera, Bombus bimaculatus,* and *B. impatiens* (Hymenoptera: Apidae) were observed with pollen present on their hind tibiae and collecting pollen as they moved among flowers on different twigs. Johnson's study (1988) reported not witnessing insects present on pistillate flowers, whereas the present study included an observation of *A. mellifera* moving from staminate flowers on one twig and landing on the pistillate flowers of another twig, brushing across the female flowers as the bee moved toward staminate flowers (Appendix).

In the case of *C. ozarkensis*, pollination by insects requires visiting both staminate flowers to collect pollen and pistillate flowers to deposit pollen. Pollen was detected on all insects that were collected in this study that were viewed under a dissecting microscope. The structure and characteristics of flowers may give clues to the importance of insect pollination. Clapper (1954a) stated, "insects would not be attracted to the female flowers." That conclusion was likely influenced by his finding that seed set from twigs that were emasculated, removing male flowers, dropped from 90% to 56%. Based on observations in the present study and others, female flowers possess no bright colors, odor, nectar, nor pollen, suggesting that pistillate catkins do not draw the attention of insects, however, wholly pistillate catkins rarely exist. The majority of the length of an androgynous catkin of Ozark chinquapin is pistillate. Nevertheless, the

staminate portion of the catkin may have enough resources to attract potential insect pollinators and give them cause to drag their pollen-coated bodies over the exposed and upright stylets of pistillate flowers.

The structure of flowers on *C. ozarkensis* may engage insect visitors, as well. Pistillate flowers are borne upright on the distal portion of twigs while male flowers are more proximal. As a result, pistillate catkins present an ideal take-off and landing platform for flying insects, particularly insects that have moved from the proximal staminate, pollen-containing flowers. Clapper (1954a) postulated that insects play a secondary role in pollination by dislodging pollen from staminate flowers to be carried away by the wind. Amphiphily -- wind and insects playing dual, important roles in pollination -- is suggested for some trees (Wallander 2008). However, the contributions of insects and wind will differ among species, even closely related ones as shown in a study on cultivars of *C. sativa* (De Oliveira et al. 2001). Even the characters of *Castanea* pollen suggest the duality. Giovanetti and Aronne (2011) observed *A. mellifera* collecting and transporting pollen from *C. sativa*, but maintained that the pollen had anemophilic characteristics. Pollen can be characterized as anemophilic or entomophilic but the dichotomy, like the importance of wind and insects, is not likely quite as strict.

A major factor influencing the roles of insects and wind in pollination is the experimental setting. All of the previous studies cited in the present study occurred in orchards and plots of cultivated *Castanea* trees. An orchard presents better opportunities for both insects and wind to pollinate. Trees in orchards are planted closer together than in a natural situation and less-related trees are planted in adjacent rows, to enhance cross-pollination. The present study was made on trees in naturally-sprouted remnant patches, rather than in an orchard. In non-cultivated settings, such as forest or the remnant patches in the present study, the *C. ozarkensis* trees are not always

in close proximity to other conspecifics and presumably the relatedness among trees decreases with increasing distance from one another. Also, natural growth habits of chinquapin changed after arrival of chestnut blight. Before the blight, chinquapins grew as a canopy-dominant tree (Paillet and Cerny 2012), but today they grow mostly as shrubby root collar sprouts. In a historical setting, insects likely played a great role in pollination, especially in forest understory conditions where air flow would have been restricted. Regardless, the site of the present study also gives a clue as to the importance of insects. The site of the present study would not be described as "natural." Forest on the ridgetops was bulldozed and the majority of trees and shrubs were removed, leaving sparse chinquapin clusters and individual trees. Wind would have had less restriction on the exposed ridgetop than in the typically dense forest of the surrounding habitats. If wind was the primary factor in pollination of *C. ozarkensis*, the site of the present study would have been the most ideal site for wind pollination to occur. The results showed that, even when flowers were accessible by wind, pollination success was far less than when insects could access the flowers. In both a forest with naturally dispersed trees and limited wind, and in exposed settings with dispersed trees, insects would likely increase cross pollination by carrying pollen from sources further away than could wind.

Finally, the results of any studies on *Castanea* trees outside of Asia need to be considered in light of chestnut blight. Dispersion and isolation of trees, changes in forest composition and health of remnant trees all likely influence the results. Chestnut blight infections can cause any part of the tree above ground to wither and die at any part of the season. Not only can infection compromise tree health and defenses, it can also decrease productivity. The relatively lower values for seed set and bur production in the present study compared to other studies certainly could have been influenced by tree health. It is not known what role tree health and defenses

play in abortion of seeds at any stage (e.g., Woodroof and Woodroof 1928). Also, remnant patches of *C. ozarkensis* may be affected more by other meteorological factors, in addition to wind. The occurrence of heavy rains during flowering induces "atmosphere washing" (Sabugosa-Madeira et al. 2007), which leads to decreased dispersal of pollen by wind. The loss of treatment bags in 2015 due to heavy rain is an indication of the role that rain can play in success or failure of pollination.

Future studies

Numerous questions remain, and future studies that address the role of insect pollination can contribute to conservation of the Ozark chinquapin. An intensive and quantitative study of pollinator behavior would greatly increase our understanding of insect pollination of Ozark chinquapin. Observations of pollinators in the present study were very limited. Methods similar to those of Giovanetti and Aronne (2011) concerning honey bee activity on *C. sativa* flowers could be used to determine the importance of honey bees as well as other insect species for pollination of *C. ozarkensis.* A more-extensive survey, collection, and focused observation of insects on flowers of Ozark chinquapin across its range, throughout the flowering period, and with greater emphasis on possible nocturnal pollinators, could yield additional findings that would aid in conservation of this species. The physiology underlying seed and bur production, in the presence and absence of pollinators may be worthwhile, especially for trees compromised by blight.

Other potential studies include alterations to methods. Although the present study included treatments excluding insects and both wind and insects, and included one treatment with inclusion of honey bee, the study did not have a true treatment that included insects, while excluding wind. Whether an experimental setup could be devised that allowed insects to freely

access flowering trees but exclude the effects of wind is not known. If honey bees are used as the pollinator of interest, allowing them to move more freely would be important. The present study constrained individual bees and did not allow them to move freely or access other resources needed for survival. It is also possible that the presence of bags alone affected seed production. An open pollination treatment that had a constriction present where a bag would normally be tied, but remained open to wind and insects, would help determine the influence of the bag. Additional treatments of artificial pollination inside of paper and screen bags could be added to evaluate the influence of bags on seed production. To do so, pollen should be gathered from trees least related to the study trees and applied during peak receptivity of the pistillate flowers. That study could be expanded to assess the importance of cross-pollination, by artificially pollinating flowers with pollen from adjacent twigs or sprouts versus pollen collected from flowers from distant trees. A weather station present at the study site would be helpful to understand how wind speed and direction, rainfall, temperature, and humidity affect pollination.

In 2015, one tree nearly died from chestnut blight causing a loss of nearly 20% of the data for that year. The number of trees in the study was increased in 2016 to reduce the probability of losing an entire tree's data due to the blight. In order to increase the number of trees in the study, the number of bags had to be decreased because many of the trees added in 2016 did not produce enough flowers to host sufficient numbers of treatment units. Finding sites with sufficient healthy trees to allow large numbers of replications would be difficult, but may be necessary.

One other aspect worthy of a follow-up study is seed size. Seed size and weight were recorded in 2016, but not analyzed. Size was not analyzed due to xenia which occurs when the source of the pollen determines the size of the nuts (McKay and Crane 1939). Seed weight could

not be analyzed due to rapid drying and infestation of insect larvae. Seed size and weight can also be influenced by precipitation prior to bur split (Clapper 1954b). Finding an appropriate method to assess size and weight of seeds would add to explanations of the roles of insects in pollination.

Potential insect pests of *C. ozarkensis* seeds will need to be evaluated to increase viable chinquapin seed production as part of any conservation efforts. The lepidopteran and coleopteran larvae that were found in this study could pose threats to seed production. It can be inferred from the drilling behavior of adult lesser chestnut weevils observed in this study, coupled with reports in the literature, that the coleopteran larvae collected from *C. ozarkensis* seeds were *Curculio sayi*. Although unlikely, detailed studies of the seed-feeding insects might reveal the presence of the greater chestnut weevil (*C. caryatrypes*).

V. Conclusion

Conservation of threatened or endangered species will require successful reproduction to retain or supplement existing populations. Ozark chinquapin (*Castanea ozarkensis*), like the other North American species in the genus, was decimated during the past century after accidental introduction of chestnut blight, *Cryphonectria parasitica*. Restoration and conservation efforts are underway, but pollination strategies of *C. ozarkensis* are not well understood. Studies on the importance of wind or insect pollination with other species in the genus have yielded results showing both strategies. Chinese chestnut, *C. mollissima*, is believed to be an anemophilic species, whereas the European chestnut, *C. sativa*, is proposed to be an entomophilic species. Both are Old World species, but *C. mollissima* has been cultivated and planted throughout the United States, and the closest relative of *C. sativa* is *C. ozarkensis*.

This study found that exposure to both wind and insects enhanced pollination, as shown by a greater proportion of successfully pollinated flowers, greater numbers of burs, and a significantly greater proportion of burs that produced seeds. When flowers were not exposed to insects or to both insects and wind, pollination occurred at significantly lower levels. Pollination still occurred when insects were absent, albeit at much lower levels, but the lack of difference between the treatments that excluded wind or both wind and insects showed the importance of insects to pollination of *C. ozarkensis*. In the presence of both insects and wind, fewer pistillate (female) flowers failed to develop, a greater number of burs were produced, and a greater proportion of burs produced seeds.

This study demonstrated that *Castanea ozarkensis* is an amphiphilic species. Not only do insects play a role in pollination, they are the dominant contributors, whether measured as bur production or seed set, which is the production of seeds in burs. Wind pollination can be less

reliable under some abiotic conditions, such as during rain, high humidity, or lack of wind. However, wind pollination can add to successful seed production, especially in weather conditions that are less favorable to insects. Insect pollinators, such as honey bee (*Apis mellifera*), are likely influenced by different climatic phenomena, such as temperature extremes.

The previous studies on pollination of *Castanea* species all occurred after chestnut blight had reached Europe and North America, thus study sites and specimens were limited. The healthiest and most accessible trees for study were typically in cultivated stands used to breed for blight resistance. The results of those studies were most likely influenced by the study sites and conditions. Cultivated stands of *Castanea* are usually planted with genetically diverse trees in close proximity to one another, to enhance cross-pollination. The proximity of genetically diverse individuals may have enhanced wind pollination, but that type of genetic diversity is antithetical of habits of *Castanea* likely to occur in nature. Remnant stands of *C. ozarkensis* are typically clustered either in a forest or in open settings, presumably with the closest trees being more closely related than to trees in distant clusters. The relative roles of insect and wind pollination are almost certainly different for remnant stands than for trees in cultivation.

The data presented in this study show that insect presence increases seed set in Ozark chinquapin, thus efforts to conserve *C. ozarkensis* need to include tactics to enhance insects. Honey bee hive boxes could be placed near the trees during flowering from mid-May to mid-June, to increase pollination success. In addition, increasing or enhancing habitats around natural or cultivated stands of chinquapins, especially habitats that are conducive for pollinators, would aid in the increase of successful pollination. Planting herbaceous species that flower at times before or after flowering by *C. ozarkensis* can aid in establishment and retention of pollinators.

The present study illustrates the importance of insects and their role in pollination. Many more insects were found on Ozark chinquapin than were previously reported on other *Castanea* species; that difference may not be solely due to associations with *C. ozarkensis*. Previous studies, in addition to being conducted in cultivated stands, were focused on plant characteristics and insects were incidental to those studies or largely overlooked. Studies similar to the present one could also be conducted for those remnant stands of American chestnut, *C. dentata*. The status of pollination strategies of American chestnut is equally poorly represented in current literature as *C. ozarkensis*. However, American chestnut may be affected differently than the smaller-stature Ozark chinquapin, thus the need for explicit study and not simply transfer of findings from congeneric species. Any further attempts to conserve and restore the genus *Castanea* in North America need to proceed with better consideration and understanding of insects and their roles in the reproductive biology of the trees. Likewise, translation of the methods and perspective from this study could be of value as the health of other species in the family Fagaceae may become threatened.

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VII. Appendix

Insects are known to be important in pollination of chestnut in Europe (De Oliveira et al. 2000). Clapper (1954) discussed many entomophilous characteristics of *Castanea* flowers: bright coloration, odorous, nectarous, pollen is sticky, male and female catkins are borne in immediate proximity to one another. However, a study that focused on *C. pumila* failed to observe any insects on pistillate flowers (Johnson 1988).

The present study observed insect behavior on Ozark chinquapin, *Castanea ozarkensis*. Observations took place on June 2 and 7, 2016, for two hours, late morning. Both mornings were cool, around 20°C, and overcast. Female flowers were highly receptive with stigmas bending near right angles from the base of the flower and were yellow in color. Wholly staminate catkins were beginning to brown at the bases but male portions of androgynous catkins had not yet begun to bloom. Male flowers were highly fragrant. Insects observed on flowers were categorized into five non-exclusive groups: potential pollinators, non-pollinators, nectar feeders, pollen feeders, and predators. Categorization was based on insect behavior and species feeding ecology. As examples: ants would not be effective pollinators as they have no cause to travel between trees; beetles that bypass anthers on male catkins to seek pollen at the base of the flowers were categorized as nectar feeders.

The most numerous insects observed were various species of flies. Hymenopterans were also plentiful. Many honey bees, *Apis mellifera*, were observed moving frenetically between staminate catkins on separate twigs. They were not observed to pause and take nectar, but moved quickly across the length of the catkin, raking pollen. The bees then hovered near the flowers, packed the pollen into their corbiculae, and resumed moving quickly across another male catkin. Bumble bees were also observed with pollen collected on their hind tibiae (Figure

A1). They showed similar behavior to the honey bees but spent less time at each catkin and then moved more quickly to catkins on another part of the tree. A small flower scarab, *Valgus canaliculatus*, was observed on staminate catkins at the tips of the anthers, feeding on pollen. Tumbling flower beetles (Mordellidae), flies, and butterflies and skippers all ignored pollen and probed at the base of male flowers, apparently feeding on nectar. A cerambycid beetle, *Euderces picipes*, was observed landing on an androgynous catkin and then moving to a staminate catkin to feed (Figure A2). A honey bee was observed similarly landing on a pistillate catkin and moving quickly to male flowers to collect pollen. Predaceous insects, such as ladybeetles and assassin bugs, were also found on flowers.

Insects were found mostly on staminate inflorescences feeding on pollen or nectar, and were observed infrequently on pistillate catkins. A more intensive study, similar to Giovanetti and Aronne's (2011) study on European chestnut, *C. sativa*, would be valuable to enhance our knowledge of the roles insects play in pollination of Ozark chinquapin.

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Figure A1. A bumble bee, *Bombus sp.*, approaches a staminate catkin of Ozark chinquapin, *Castanea ozarkensis*, with corbiculae (pollen baskets) on hind tibiae full of pollen. Photo by the author.

Figure A2. *Euderces picipes* (Coleoptera: Cerambycidae) landed on the apex of an androgynous catkin and walked down the spike over pistillate flowers to feed on staminate catkins. Photos by the author.