Defining and mitigating the impacts of *Acanthococcus lagerstroemiae* (Hemiptera: Eriococcidae) management on pollinators

by

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Abstract

Crapemyrtle bark scale (CMBS) [*Acanthococcus lagerstroemiae* (Kuwana)] is a recently introduced pest in the United States. CMBS attacks crapemyrtle [*Lagerstroemia* spp. L. (Myrtales: Lythraceae)], a plant ubiquitous in southern landscapes, as the primary host. Neonicotinoid insecticides, including imidacloprid and dinotefuran, are widely used to reduce damage and spread of CMBS. Because bees and beneficial insects commonly visit crapemyrtle flowers, this study evaluated the risk of translocation of imidacloprid and dinotefuran into pollen following a soil application. Soil applications of both insecticides were made using maximum label rate. Three timings: post-bloom/pre-dormancy (autumn), pre-bud break (winter), and post-bud break/pre-bloom (spring) were used. Pollen and leaf samples were collected from trees and analyzed using a modified QuEChERS method of LC/MS. Laboratory assays were also performed with new growth to evaluate treatment efficacy against CMBS crawlers and foliage-feeding crapemyrtle aphids [*Sarucallis kahawaluokalani* (Kirkaldy) (Hemiptera: Aphididae)]. Additionally, abundance and diversity of crapemyrtle's floral visitors were analyzed in highly urban and suburban sites. Pollinators from ten families in two orders (Diptera and Hymenoptera) visited crapemyrtle over two levels of urbanization. Residues in crapemyrtle pollen exceeded the chronic oral lowest observable adverse effect concentration (LOAEC) for adult honey bees, regardless of application timing. Contact exposure to contaminated pollen poses an additional risk to bees visiting systemically-treated crapemyrtles. Application of systemically-applied neonicotinoids to control CMBS in urban landscapes increases exposure risks to pollinators. Pollinator-friendly methods of CMBS control are needed.

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List of Abbreviations

AU	Auburn University
CMBS	crapemyrtle bark scale
DBH	diameter at breast height
QuEChERS	quick, easy, cheap, effective, rugged, and safe
LC/MS	liquid chromatography/mass spectrometry
USDA	United States Department of Agriculture
UPLC	ultra performance liquid chromatography ethylene bridged hybrid
LOAEC	lowest observable adverse effect concentration

Chapter 1: Introduction and Literature Review

1.1 Crapemyrtle Bark Scale

Crapemyrtle bark scale (Fig. 1), *Acanthococcus lagerstroemiae* (Kuwana), is an invasive insect pest recently introduced to the United States (Wang et al. 2016). It feeds in the phloem and xylem of its host plant, reducing flowering, causing branch dieback, and aesthetic damage through colonization of its honeydew by sooty mold fungi (*Capnodium*, *Chaetothyrium*, *Metacapnodium*, and *Scorias* spp.) (Fig. 2) (Wang et al. 2016). Furthermore, heavy infestations of crapemyrtle bark scale (CMBS) may contribute to plant death in smaller containerized crapemyrtles undergoing heat or drought stress (Chen 2018, *personal observation*). CMBS infestations will continue to increase production costs for nurseries (Gu et al. 2014) and may lead to unintended costs for consumers due to increased need for insecticides (Wang et al. 2016).

Among the insect's host range, crapemyrtle, *Lagerstroemia* spp. L. (Myrtales: Lythraceae) is one of several with economic or ecological importance. Pomegranate (*Punica granatum* L.), soybean [*Glycine max* (L.) Merr.], paradise apple (*Malus pumila* Mill.), edible fig (*Ficus carica* L.), brambles (*Rubus* spp.), Japanese persimmon (*Diospyros kaki* Thunb.), and Korean boxwood (*Buxus microphylla* Sieb. et Zucc.) are some of the other important crop and ornamental hosts of CMBS in Asia (Hua 2000 and Park et al. 1999 cited by Wang et al. 2016), and Hungary (Kozár et al. 2013). In the United States, CMBS has only been found on crapemyrtle and native plant beautyberry (*Callicarpa americana* L.) suggesting the insect can feed and develop on plants found outside its native range (EDDMapS 2019). In a no-choice test, Wang et al. (2019a) evaluated the host range and found that *A. lagerstroemiae* can only develop and reproduce on five species other than crapemyrtle: henna (*Lawsonia inermis* L.), sinicuichi (*Heimia salicifolia* Link), pomegranate, winged loosestrife (*Lythrum alatum* Pursh.), and

beautyberry. Crapemyrtle, however, was the highest quality host. Based on the density of gravid females, *Lagerstroemia indica* x *fauriei* (482 ± 92) produced 2.5 times the number of gravid females as *C. americana* (200 ± 70), and over three times more than the other alternative hosts (<150) (Wang et al. 2019a).

Native to Asia, this scale is distributed widely and is present in seven countries including China, Japan, South Korea, Mongolia, England, and the United States (Kozár et al. 2013). First discovered in the United States in 2004 at a commercial landscape in Richardson, TX (Collin County) (Merchant et al. 2014), CMBS is now in 14 states: Arkansas, Alabama, Georgia, Kansas, Louisiana, Mississippi, New Mexico, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, Virginia (EDDMapS 2019), and Washington (Wang et al. 2016) as of October 2019. Within Alabama, the scale has been reported in the northernmost and southernmost parts of the state including Baldwin, Madison (EDDMapS 2019), Limestone, and Lauderdale counties (Held *unpublished data*). According to Wang et al. (2019b), the United States distribution of CMBS is likely only limited by cold weather. Since the predicted northern limit (43° N latitude) overlaps with that of crapemyrtle, it is expected that CMBS will eventually be present in all the same USDA Hardiness zones as crapemyrtle (Wang et al. 2019b).

Acanthococcus lagerstroemiae appear as white, felt-like scales (Fig. 3) on the bark of host plants, and are sessile as adults (Gu et al. 2014, Wang et al. 2016). Adult females are paedomorphic, or resemble the nymphal stage, and covered with a felt-like coating secreted during development. As females lay eggs within the ovisac (Fig. 4), the female's body is replaced with 114 to 320 eggs (Jiang and Hu 1998 as cited by Wang et al. 2016). After the eggs hatch, first instar crawlers disperse across the host and possibly to different plants. CMBS can colonize all above-ground parts of the host including trunk, branches, stems, leaves, and fruits (Wang et al. 2016). The nymphs are mobile and responsible for short-range dispersal. One or two

days following egg hatch, nymphs settle and begin feeding and producing honeydew. Laboratory observations showed that nymphs could relocate to a new feeding site following each molt (Wang et al. 2015, *personal observation*). Following three nymphal instars, the females become sessile adults and the males undergo pre-pupal and pupal stages, then finally emerge as alates. Adult males (Fig. 5) lack mouthparts, have one pair of wings with reduced venation, and have two white caudal filaments (Wang et al. 2016). Reviewing papers from the Asian range of *A. lagerstroemiae*, Wang et al. (2016) concluded that *A. lagerstroemiae* has two to four generations per year in Asia. In the United States, CMBS is predicted to have more than two generations in USDA Hardiness Zone 8 (− 12.12 °C to − 6.7 °C) (Wang et al. 2019a), and up to four generations are expected in Zones 9 and 10 (− 6.7 °C to 4.4 °C) (Gu et al. 2014).

Given its wide and discontinuous distribution across the United States, it is suspected that crawlers utilize several strategies for short- and long-range dispersal; adaptations to the bodies and behaviors of coccoids indicate some of these strategies. Passive dispersal via wind can carry first instars anywhere from a few meters to several kilometers away from the host plant upon which they were born (Greathead 1997, Hanks and Denno 1998). Miller and Denno (1977) hypothesized that the long legs, antennae and lateral setae relative to the small body size of pseudococcids facilitated their aerial dispersal. Wandering behavior has been recorded in a coccid (*Pulvinaria mesembryanthemi*) without access to suitable feeding site (Washburn and Frankie 1985). Washburn and Frankie (1985) and Washburn and Washburn (1984) also note that crawlers from several genera in Coccidae will actively seek wind dispersal, orientating themselves downwind with forelegs and antennae outstretched. Gu et al. (2014) posited that CMBS crawlers might move via birds. To illustrate, hemlock wooly adelgid (*Adelges tsugae* Annand) is capable of transferring to birds that disturb infested branches in an experiment (Russo

et al. 2016). Lastly, human movement of infested plant material is thought to be the primary method of long-distance transport (Wang et al. 2016).

Throughout its global distribution, there are several natural enemies of *A. lagerstroemiae* including parasitoids from two hymenopteran families (Aphelinidae and Encyrtidae) and nine genera, and predators from four families (Anthocoridae, Chrysopidae, Coccinellidae, and Cybocephalidae) and at least seven genera (Wang et al. 2016, Franco et al. 2019). In the United States, five species of lady beetle are associated with CMBS: *Chilocorus cacti* L. lady beetle, *Chilocorus stigma* (Say), *Hyperaspis bigeminata* (Randall), and *Harmonia axyridis* (Pallas), and *Hyperaspis lateralis* Mulsant (Wang et al. 2016). *Chilocorus kuwanae* (Silvestri), a species of twice-stabbed lady beetle which predates on CMBS in Asia, was previously released in the United States to control euonymus scale (*Unaspis euonymi* Comstock) and has since established in USDA Hardiness Zone 7 and colder (Hendrickson et al. 1991). In addition, one species of unidentified hymenopteran parasitoid was reared from CMBS nymphs in Louisiana (Wang et al. 2016). In a laboratory setting, a single fourth instar *Chilocorus cacti* consume up to 400 CMBS eggs in 24 h (Wang et al. 2019). The ladybeetle predators of CMBS in Alabama and ways to recruit them are currently being studied.

Because biological control methods are still being studied, chemical and mechanical methods are the current primary methods of control in the United States (Wang et al. 2016). Crapemyrtle bark scale has proven difficult to control using contact insecticides likely due to its secreted covering and its behavior of settling within bark crevices and pruning scars (Gu et al. 2014). Mechanical removal methods include brushing the bark of an infested tree with a mild dishwashing solution or using high-pressure water, both of which reduce CMBS and sooty mold (Gu et al. 2014). Systemic insecticides like neonicotinoids, particularly those containing imidacloprid, clothianidin, dinotefuran, and thiamethoxam have proven most effective,

especially when applied to the soil (Gu et al. 2014). The insect growth regulators, buprofezin and pyriproxyfen, can reduce crawler density by 70 to 100% for 2 to 4 months after application (Vafaie 2019). Neonicotinoid insecticides provide effective long-term reduction of CMBS crawlers, and bifenthrin bark sprays applied twice over 17 d can also provide a short-term reduction of crawlers (Vafaie and Knight 2017). Merchant et al. (2018) found that imidacloprid (as Mallet®) suppressed *A. lagerstroemiae* throughout the length of their longevity trial, 61 weeks after treatment. The cost of rotating between two neonicotinoid products is about $10 per 3-meter-tall tree (Wang et al. 2016). Unfortunately, a single application likely will not provide complete control, and insecticide applications will need to be reapplied each year (Layton 2019). It is also recommended to treat surrounding crapemyrtles, even if they are not apparently infested (Layton 2019). This can lead to significantly increased costs for landscape managers and homeowners, and greater potential risk to beneficial and non-target insects.

1.2 Crapemyrtle

Crapemyrtles (Fig. 6), are small deciduous summer flowering trees or shrubs used widely in landscapes across the southeastern United States (Chappell et al. 2012). Total sales for crapemyrtle reached over $65.9 million, the highest of any deciduous flowering tree. Over 5.5 million crapemyrtles were sold in nurseries in 2012 (USDA 2014). Crapemyrtle's native range spans from southern China, Korea, and Japan to Oceania (Chappell et al. 2012). After its introduction to the United States in the late 1700's, *Lagerstroemia indica* L. Pers. was initially offered for sale in the Bartram's *1807 Catalogue* (Cothran 2004). Soon thereafter, the crapemyrtle became a staple plant in the southern United States, with its abundance of blooms and great tolerance to heat and drought (Cothran 2004). Since then, *L. indica* has been crossed with *Lagerstroemia fauriei* Koehne, *Lagerstroemia subcostata* Koehne, and *Lagerstroemia limii*

Merr. to produce larger flower panicles with more varied colors, heightened disease tolerance, and desirable bark characteristics (Chappell et al. 2012). Some of the most recent of these hybrids, including 'Natchez', 'Tuscarora', and 'Muskogee' are crosses between *L. indica* and *L. fauriei*. They were produced for their superior resistance to powdery mildew [*Erysiphe australiana* (McAlpine) Braun & Takamatsu (Erysiphales: Erysiphaceae)] and are frequently utilized in the landscape (Chappell et al. 2012). After decades of breeding, crapemyrtle is now available in diverse growth habits, offers year-round landscape interest, has increased disease tolerance, and is adaptable to a variety of soil types (Chappell et al. 2012).

Crapemyrtle is well-adapted to the southeastern United States, and can tolerate temperatures from USDA Hardiness Zones 6 to 10 (− 23.3 °C to − 1.1 °C) (Dirr 1990). Flowering begins in May in Zones 8 and 9 (− 12.12 °C to − 6.7 °C and − 6.7 °C to − 1.1 °C) and continues sporadically in the deep south until first frost (Chappell et al. 2012). According to Dirr (1990), flower panicles develop at the apex of each stem and are about 15.3 cm to 45.8 cm long with hundreds of individual 1.3 cm to 5.1 cm florets. The crepe-like flowers can range in color from shades of pink, red, white and purple (Dirr 1990). Another facet to crapemyrtle's year-round interest is its exfoliating bark, which is exhibited in *L. fauriei* and its hybrid cultivars. The bark exfoliates during summer, revealing an underlying layer that varies in color from cream to cinnamon (Head 2006). Crapemyrtle also offers fall color ranging from yellow, to orange, to red, depending on the cultivar, in USDA Hardiness Zones 8 and colder (Dirr 2002). *Lagerstroemia* spp. grow best in sites with full sun, good air-flow, and well-drained, slightly acidic clay or clay-loam soil. Shady sites lead to weak growth and increased foliar pathogens and wet sites incur increased soil pathogens (Chappell et al. 2012). After it becomes established in a landscape, crapemyrtle requires little to no fertilization or irrigation, and minimal pruning (Chappell et al. 2012).

Crapemyrtle is considered to be low maintenance, however, there are some limiting abiotic factors such as winter damage in colder climates (Chappell et al. 2012) and a low salt tolerance (Francois 1982). Correct placement, choice of cultivar, and yearly maintenance can mitigate these stresses. Appropriate pruning practices performed at the end of winter preserve not only the architecture of the branches (Knox 2003), but also the cold tolerance of crapemyrtle by not encouraging new growth into the colder months (Haynes et al. 1993). In addition, any fertilization or irrigation should be ended by July and August, respectively, to prevent winter damage (Chappell et al. 2012).

Biotic stresses, although relatively few, include both diseases and insect pests. Most can be managed with cultural control methods (Chappell et al. 2012). Crapemyrtle aphid [*Sarucallis kahawaluokalani* (Kirkaldy) (syn. *Tinocallis kahawaluokalani*) (Hemiptera: Aphididae)], Japanese beetle [*Popillia japonica* (Newman) (Coleoptera: Scarabaeidae)], flea beetles (mainly *Altica* spp.) (Coleoptera: Chrysomelidae), and granulate ambrosia beetle [*Xylosandrus crassiusculus* (Motschulsky) (Coleoptera: Curculionidae)] are the most notable insect pests other than CMBS that infest crapemyrtle (Chappell et al. 2012). Crapemyrtle aphids are sap-sucking insects that are host specific to crapemyrtle (Alverson and Allen 1992). Sooty mold colonization following honeydew deposits can cause yellowing of leaves, premature leaf-drop, and even complete defoliation (Dozier 1926). Mizell and Knox (1993) found that *L. indica* had better resistance to crapemyrtle aphid feeding than *L. fauriei* and its hybrids and that dwarf cultivars had superior resistance out of each growth habit. Mostly, crapemyrtle aphid populations are controlled by natural enemies, but high population infestations may be lowered using a strong stream of water to physically remove them (Blake et al. 2019). Crapemyrtle aphid also can be effectively controlled using horticultural oils as dormant spray after leaf-drop or with insecticidal soaps (Frank et al. 2009). Japanese beetles are generalist herbivores that develop underground

until they emerge as adults to mate and lay eggs June through August. As grubs, they feed on the roots of host plants, and adults chew flowers and foliage, defoliating over 300 species (Held 2004). Crapemyrtle is a preferred host that experiences extensive feeding damage, although there are several cultivars that are resistant or moderately resistant to Japanese beetle damage (Held 2004). Flea beetles also attack their hosts in both their larval and adult stages; both life stages feed on foliage (Phillips and Gillett-Kaufman 2019). Flea beetles rarely become problematic on established landscape plants, however, they can be serious pests for trees in production (Pettis et al. 2004). Granulate ambrosia beetle attacks can cause extreme stress and can even kill its hosts (Chappell et al. 2012). The female, which causes boring damage, selects trees largely based on whether trees are stressed and releasing ethanol, so the first form of control is preventative, in the maintenance of healthy trees. However, abiotic stresses like flood stress, which is the most common, may be unavoidable during wet springs (Frank et al. 2019).

The ecological importance of crapemyrtle, regarding pollinators, has yet to be definitively determined. Since crapemyrtle is an introduced species and does not offer nectar as a floral reward (Kim et al. 1994), it seems of little value to pollinators. Crapemyrtle does offer a reward in the way of pollen; in fact, most crapemyrtles have dimorphic pollen, which includes a feed pollen, specifically for rewarding visiting pollinators (Fig. 7) (Kim et al. 1994). This pollen is exclusively for visiting pollinators and cannot successfully fertilize in other than *in vitro* conditions. It has a ratio of sugars similar to nectar, and its chemical makeup suggests high digestibility for bees (Nepi et al. 2003). Recently, there has been increased interest in experiments evaluating the ecological impacts of crapemyrtle in the United States, including the analysis of bee visitations. Deyrup et al. (2002) was one of the first to report on bees visiting crapemyrtle. They recorded the diversity of pollinators visiting floral hosts, including *L. indica*, at the Archbold Biological Station, a 2,100 hectare area of natural habitat in south central

Florida. Most of the specimens were collected directly from flowers, and some with a malaise trap. In *L. indica* flowers, they collected two species of *Augochloropsis*, one species of *Augochlorella*, two species of *Dialictus* (Halictidae), and one apid (Deyrup et al. 2002). The site used by Deyrup et al. (2002) was not urban, but rather surrounded by Florida scrub and pine flatwoods among other natural habitats. Riddle and Mizell (2016) evaluated bees visiting a block of 224 crapemyrtles representing 14 cultivars at the UF/IFAS North Florida Research and Education Center (Quincy, FL) via transect walks for two summers followed by selective netting to confirm bee identifications. In this block, 10 bee species representing Apidae and Halictidae were found (Riddle and Mizell 2016). It is important to note that Riddle and Mizell (2016) pruned their trees by topping, altering their natural bloom phenology, which may have affected the diversity of bees collected. Bee-preferred cultivars included 'Natchez', 'Byers Wonderful White', 'Miami', 'Osage', 'Apalachee', 'Sioux', and 'Yuma' (Riddle and Mizell 2016). Another study, Braman and Quick (2018), compared bee visitation of crapemyrtle cultivars in Blairsville, GA that varied in plant height and flower color. They created a block of potted trees representing 40 cultivars and sampled blooms over two summers. Visual observations were used mostly, in tandem with capture samples. Three branches, with blooms present, were randomly selected per tree and observed for 2 min per observation. Since visual observations did not allow for precise identification, all bees other than honey bees [*Apis mellifera* L. (Hymenoptera: Apidae)], bumble bees [*Bombus* spp. Latreille (Hymenoptera: Apidae)], and carpenter bees [*Xylocopa* spp. Latreille (Hymenoptera: Apidae)] were grouped into a category called "small bees." Twelve species within Apidae, Halictidae, and Megachilidae were recorded at that location in GA (Braman and Quick 2018). Regarding flower color, dark purple, dark pink, and white flowers were the most visited of the nine categories (Braman and Quick 2018). Interestingly, bees were most often observed on the smaller growth forms (shrubs) with fewest observations on the small

tree growth forms (Braman and Quick 2018). While none of the papers sampled trees in an urban landscape, data suggest that the context of the crapemyrtle trees may be important contributors to pollinator assemblages.

The literature has one published paper that sampled floral visitors to crapemyrtle trees established in urban landscapes. Mach and Potter (2018) rated 72 species of flowering woody plants in urban landscapes in Kentucky and Ohio using snapshot counts and selective netting from the 45 plants with the highest bee attractiveness. Their goal was to determine relative attractiveness of flowering woody plants, and not specifically focused on crapemyrtle. Of the 45 plants from which bees were collected, crapemyrtle was rated in the lowest quartile relative to other woody plants in their study. In their pollinator sampling, mostly Halictidae, followed by Apidae, and a few Colletidae were found on crapemyrtle flowers (Mach and Potter 2018).

Time of sampling also likely affects pollinator diversity found in each experiment. Most of the aforementioned studies (Deyrup et al. 2002, Riddle and Mizell 2016, Braman and Quick 2018) did not report the time of day when they sampled. Deyrup et al. (2002) depended on their expertise and knowledge of pollinators to know when they had collected a sufficient representation of diversity for each plant. Mach and Potter (2018) surveyed in an 8 h period between 10:00 to 18:00, which likely affected the abundance and diversity of bees they recorded. It is possible that these papers are not likely representative of the pollinator diversity for established urban crapemyrtles sampled at peak foraging time.

1.3 Bees in Urban Landscapes

Urban landscapes present unique challenges to pollinators. Reductions in bee health, or species richness and abundance, have been widely reported (Williams et al. 2010). Changes in land-use and increased urbanization are potentially contributing factors. As a result of

urbanization and agricultural intensification, pollinators are facing habitat loss, and consequently, a patchy availability of foraging and nesting sites, as well as pests, transmission of diseases, and pesticides (Goulson et al. 2015). An urbanized area is characterized by a high percentage of impervious surfaces, like buildings, roads and hardscapes (Held 2019a). Eighty-two percent of North America's population lived in urban areas in 2018 (UN 2018). As urban landscapes are developed, they become isolated fragments of their original habitat, similar to the principles of island biogeography (Fattorini et al. 2018). Czech et al. (2000) found that when the category of habitat loss is subcategorized, the three leading causes of endangerment to species in the United States are as follows: interactions with non-native species, urbanization, and agriculture. Obviously, these causes are not independent; urbanization ranked highest in total number of associations with other causes of endangerment. Additionally, urbanization and agriculture were also the two causes most frequently associated with one another, likely due to the "drastic modification of habitat at the urban-rural interface" (Czech et al. 2000). Urbanization is a source of global biotic homogenization, as cities are exclusively built to serve the purposes of humans. Consequently, urban expansion often promotes the substitution of native plant species with non-native ones, resulting in reduction of native plant species richness (McKinney 2006, Held 2019a). However, the effects of non-native species on native congeners are not always negative, and are certainly not straightforward (Stout and Morales 2009, Clem and Held 2018). As an example, it was previously thought that competition for pollination services between non-native plants and their native counterparts would negatively affect the pollination success of native plants (Morales and Traveset 2009). In their meta-analysis, Harrison and Winfree (2015) suggest studies were persistently flawed due to inclusion of non-native ornamental plants bred for novel or showy flowers that can be expected to elicit greater effects. Later studies comparing pollinator visitation across multiple species of native and non-native

plants found that non-native plants were not preferred over native plants (Harrison and Winfree 2015, Mach and Potter 2018). Furthermore, suburban bees interact with non-native plants more often than forest-dwelling bees, but at a rate proportional to the higher abundance of non-native plants (Williams et al. 2011). Chrobock et al. (2013) analyzed pollinator visitation across urbanized and semi-natural landscapes with native and non-native plants in the same family or genus. While average visitation was higher in the semi-rural sites, they found overall, that pollinator visitation was higher for native plants (Chrobock et al. 2013). A similar result was shown by Garbuzov and Ratnieks (2014) who found no consistent difference between horticulturally-modified cultivars and their wild-type counterparts. Mach and Potter (2018) also found no differences in amount of bee visitation or diversity in their quantification of bee assemblages on woody landscape plants based on provenance. They did, however, find less visitation on heavily modified cultivars.

Urban drivers like warming and environmental contaminants, in addition to habitat fragmentation and the introduction of non-native species, also make urban environments invaluable as study systems for more global change drivers affecting plant-pollinator interactions (Williams et al. 2011). One study, in Raleigh, NC, United States, showed that urban warming was a good predictor of bee abundance and community composition. For each degree of warming, bee abundance declined 43%, regardless of floral density (Hamblin et al. 2018). The same study also noted the local percentage of impervious surface (within 200 m surrounding each site) and floral density were also predictors of bee abundance. Species richness increased with floral density, however, floral density was only shown to benefit large bees (Apidae), and sites with higher temperatures and percentages of impervious surfaces still present limitations to their pollinator communities (Hamblin et al. 2018).

Diverse populations of bees live and can be supported in urban landscapes (Hall et al. 2017) and oftentimes, the diversity and abundance of pollinators can vary within the different habitats of the same city. Matteson et al. (2013) visited both greenspaces and highly developed residential areas in New York, based on number of buildings, herbaceous cover, and canopy cover in a 30 m radius among other measures. They evaluated the effects of vegetation, development intensity, and median income on floral resources and insects. Matteson et al. (2013) found that there was a reduction of both floral resources and their visitors in highly-developed areas with less canopy and herbaceous cover, but that some low-density residential areas supported high levels of both floral resources and visiting insects. Their results led them to assert that urban landscapes are heterogeneous and therefore, generalizations about them are inappropriate without acknowledging the differences between land use classifications (Matteson et al. 2013). Hall et al. (2017) cited several instances in which cities from across the world displayed higher diversity and abundance of pollinators than their rural counterparts (Cane et al. 2006, Matteson et al. 2008, Osborne et al. 2008, Frankie et al. 2009, Verboven et al. 2014, Baldock et al. 2015, Sirohi et al. 2015). Larson et al. (2014) used percentage area of impervious surface to characterize their sites comparing pollinator assemblages on weeds in turfgrass and found similar diversity across their urban, suburban, and periurban-rural sites. Not only diversity and abundance, but colony health can also be differentially affected in landscapes of varying land-use intensification (Goulson et al. 2002).

A consequence of urbanization and agricultural intensification is the increased use of pesticides (Held 2019a). The class of insecticides most implicated with the declining health of bees, neonicotinoids, are the most widely used in the world (Jeschke et al. 2011, Casida and Durkin 2013). Neonicotinoids are water-soluble, systemic pesticides, which protect plants against herbivorous feeding (Wood and Goulson 2017). Neonicotinoids target the insect central

nervous system. More specifically, they act as acetylcholine mimics and bind to the post-synaptic nicotinic acetylcholine receptors, causing continuous action potential, which results in paralysis and eventual death (IRAC 2019). Once they are applied via either soil application, bark application, trunk injections, or foliar spray, the active ingredient and its biologically active metabolites are translocated throughout the plant's tissues including pollen and nectar. Effects of neonicotinoids on non-target insects, especially bees, have been widely recorded (Girolami et al. 2009, Blacquière et al. 2012, Godfray et al. 2014, Goulson 2014, Lundin et al. 2015, Morrissey et al. 2015, Pisa et al. 2015, Goulson et al. 2015, Rundlöf et al. 2015, Wood and Goulson 2017, Basley 2019). Neonicotinoids are acutely toxic to honey bees (U.S. EPA 2014). Imidacloprid, for example, has an acute oral LD_{50} of 0.0039 μg a.i./bee for adult honey bees and acute contact LD_{50} of 0.043 μg a.i./bee (Housenger et al. 2016). The acute contact exposure levels for honey bees would be less since bumble bees, are about 7 times more sensitive to contact exposures than honey bees (Sanchez-Bayo and Goka 2014).

There are many exposure routes for pollinators including direct contact, ingestion of contaminated pollen and nectar, and ingestion of guttation fluids, contaminated ground water (Housenger et al. 2016), and even honeydew from sap-sucking insects on treated plants (Calvo-Agudo et al. 2019). Sublethal effects have also been noted for bees chronically exposed to neonicotinoids including effects on foraging (Henry et al. 2012), reproduction and behavior (Blacquière et al. 2012). Only a few studies have considered exposure routes to pollinators in urban landscapes. Urban bee communities are often mainly composed of polyectic species which feed on nectar and pollen from a variety of plants (Mach et al. 2018) including ornamental trees like crapemyrtle. Neonicotinoid residues in urban landscapes can come from treated ornamental plants when purchased from garden centers (Lentola et al. 2017), flowering weeds in treated

turfgrass (Gels et al. 2002, Larson et al. 2015), established plants in the landscape (Mach et al. 2018), dew (McCurdy et al. 2017) and guttation fluid from treated turfgrass (Larson et al. 2015).

Pesticide application rates used for ornamental plants are much higher than for field crops (Krischik et al. 2015, Mach et al. 2018). Cowles and Eitzer (2017) asserted that the high rates allowed for ornamental nursery crops translocate into pollen and nectar and often create hazardous conditions for pollinators. They tested three neonicotinoids applied at various times before bloom on two model plants, both highly bee-attractive, and found that soil applications applied at high rates caused the most hazardous residues. Cowles and Eizter (2017) predict that as plant size increases, like with trees, the likelihood of dangerous residues in pollen and nectar decreases due to large biomass. Regardless, many insecticide labels restrict their application during bloom or when pollinators are present as fresh residues on blooms are highly likely to expose bees (Larson et al. 2014). Otherwise, as with neonicotinoids, if a plant is not in bloom, products may be applied at any time of year. Mach et al. (2018) attempted to decrease the risk of hazardous residues in nectar of ornamental plants treated with two soil-applied neonicotinoids by manipulating timing of application. They used spring, summer, and autumn timings and found that only the summer application was able to mitigate imidacloprid, but not dinotefuran residues in nectar. Therefore, even when used according to the label, systemically-applied neonicotinoids can translocate into pollen and nectar at levels harmful to bees, and other non-target beneficial insects (Gels et al. 2002, Larson et al. 2014, Mach et al. 2018). Additionally, there may be added risk to bees in urban landscapes as they tend to revisit the same patches of floral resources (Osborne and Williams 2001). If those resources are contaminated at dangerous levels, floral visitors may reach dietary LD_{50} for neonicotinoids over time, otherwise known as the T50 (Sanchez-Bayo and Goka 2014). Therefore, it is important to attempt to lessen the risk to bees and other non-target insects visiting urban ornamental landscape plants.

Chapter 2: Hazards to pollinators from use of neonicotinoids on crapemyrtle

2.1 Introduction

Crapemyrtle bark scale (CMBS) (*Acanthococcus lagerstroemiae*), an invasive felt scale, feeds on crapemyrtle (*Lagerstroemia* spp.) and several other economically important ornamental and crop hosts throughout its global range (Wang et al. 2016). In the United States, the geographic range is mostly in the southern states, but is predicted to overlap with the range of the primary host crapemyrtle. Despite the common name, *A. lagerstroemiae* can complete development on at least five host plants in two families, but has the best fitness on crapemyrtle (Wang et al. 2016). Crapemyrtle is a ubiquitous component, and often a majority tree species, in southern urban landscapes (Stewart et al. 2002, Martin et al. 2011). This non-native plant is adaptable to various soil types, can tolerate the heat and drought associated with high levels of urbanization, and has relatively few pests (Chappell et al. 2012). *Acanthococcus lagerstroemiae* can greatly affect the aesthetic value of crapemyrtle, decreasing its bloom size and vigor, causing branch dieback, and decreasing efficiency of photosynthesis via sooty mold colonization (Wang et al. 2016). Crapemyrtle bark scale likely has more than two generations per year in USDA Hardiness zone 8 (− 12.12 °C to − 6.7 °C) (Wang et al. 2019), and females are highly fecund, each laying up to 320 eggs (Wang et al. 2016). First instar crawlers are responsible for distribution and are likely the life stage most vulnerable to insecticides (Wang et al. 2016). In China, in USDA zone 8, nymphs emerge in late May and then again in early August (Gu et al. 2014). In Arkansas, crawlers have been observed emerging in mid-May to June (Wang et al. 2015), suggested their life cycle may be similar to CMBS from China.

Crapemyrtle has few other severe pests, but one of the most notable, crapemyrtle aphid, is host specific to crapemyrtle and can similarly produce honeydew, which often leads to sooty

mold. Soil-applied or trunk injected neonicotinoid insecticides are commonly used for control of crapemyrtle bark scale and crapemyrtle aphids (Gu et al. 2014). A yearly reapplication is often required to control or prevent crapemyrtle bark scales, increasing costs for what was previously a low-input landscape tree (Layton et al. 2019). Neonicotinoids move via phloem and xylem fluid acropetally from the application site and provide control where contact insecticides fail to reach (Bonmatin et al. 2015). Systemic application is advantageous, as well, because it decreases pesticide exposure to humans and non-target insects (Mach et al. 2018). Dinotefuran and imidacloprid are two widely used neonicotinoids that differ in their water solubility and sorption to soil particles, therefore their uptake and dissipation in the plant differ. Dinotefuran (39.8 g/L) has a higher water solubility than imidacloprid (0.61 g/L) (Bonmatin et al. 2015) and also has a lower sorption rate to soil organic matter than imidacloprid (Kurwadkar et al. 2013). Dinotefuran, therefore, provides faster control, but is less stable, as *in planta* residues peak and decline faster than imidacloprid (Mach et al. 2018).

Unfortunately, when systemically applied, neonicotinoids are also translocated into the nectar and pollen of ornamental plants (Krischik et al. 2015, Cowles and Eitzer 2017, Mach et al. 2018). Soil-applied imidacloprid and dinotefuran at labeled rates produces nectar containing levels of residues harmful to bees (Mach et al. 2018). Imidacloprid and dinotefuran have acute oral LD_{50}'s of 0.0039 µg a.i. (Housenger et al. 2016) and 0.023 µg a.i. (U.S. EPA 2017) per bee respectively, for adult honey bees. When imidacloprid is metabolized in plants, two metabolites, olefin and 5-hydroxy imidacloprid, can be as toxic to honey bees as their parent compound (Suchail et al. 2001). Because urban landscapes contain a wide diversity of woody plants, few studies have analyzed insecticide residues in pollen and nectar in plants treated under field conditions (Lundin et al. 2015, Mach et al. 2018). Because the neonicotinoid insecticides vary in within plant uptake and movement, manipulating application timing may mitigate effects of

residues on pollinators while still maintaining effective control against sap feeding pests (Mach et al. 2018)

Several stressors to insect pollinators, like habitat loss and fragmentation, pesticide exposure, and nutritional stress are intensified within urban bee populations (Goulson et al. 2015). Pollinators provide important ecological services in urban areas like pollination of ornamental, garden crops, and wild plants, so it is imperative those floral resources do not expose them to dangerous levels of pesticides (Mach et al. 2018). Crapemyrtle flowers are nectarless, but produce a large amount of reward or feeder pollen. This pollen is exclusively for visiting pollinators and cannot successfully fertilize except in *in vitro* conditions. This pollen has a ratio of sugars similar to nectar, and its chemical makeup suggests high digestibility for bees (Nepi et al. 2003). Bees (Apidae and Halictidae) are the common pollinators foraging in crapemyrtle flowers (Riddle and Mizell 2016, Braman and Quick 2018), with nectar feeding pollinators (Lepidoptera) absent in flowers. For these reasons, crapemyrtle is considered to have a low ecological value among woody landscape plants for pollinators (Mach and Potter 2018). However, only one study (Mach and Potter 2018) evaluated tree-pollinator assemblages in an urban landscape context. The long bloom season and abundant flowers on crapemyrtle may provide pollinators protein and energy during the summer dearth especially in highly urbanized sites when there are few other options (Riddle and Mizell 2016). Within the same city, bee diversity can vary greatly between habitats due to availability of floral resources (Matteson et al. 2013).

Increasing occurrences of crapemyrtle bark scale escalate the need for neonicotinoid applications to manage or prevent infestations (Layton 2019). Applications at label rates even to soil may expose visiting pollinators to increased levels of contaminated pollen. This study was conducted to understand the potential risks to pollinators associated with application of systemic

insecticides for management of crapemyrtle bark scale. Using trees from varying levels of urbanization, we quantified pollinator assemblages on crapemyrtle on suburban and urban sites. Trees in these locations were also treated with soil-applied neonicotinoids at three timings to determine the efficacy against two common pests of crapemyrtle and the levels of residues in new leaves and pollen of crapemyrtles in urban landscapes.

2.2 Materials and Methods

2.2.1 Sites and trees used in the study

Tests were conducted at five sites on or near the Auburn University campus. all within approximately 3.5 km of each other (Table 1). The sites included crapemyrtles located in campus landscapes, along a street, in a parking lot, and at the Turfgrass Research Unit. All sites were classified as either urban or suburban based on the percentage of impervious surfaces within a 200 meter radius (Larson et al. 2014, Held 2019a), which ranged from 41 to 82%. To characterize the surrounding landscape, images (Google Maps 2019) were taken of each site and overlaid with circles with 200 m radii. Then the area with impervious surfaces, like buildings and roads, were measured and recorded following Larson et al. (2014) using Image J software (Rasband 2019). Soil samples from each site were analyzed by the AU Soil Testing Laboratory to determine soil characteristics. The study trees were *Lagerstroemia* × 'Natchez' as confirmed by an expert, Dr. Allen Owings (LSU AgCenter) ranging from about 3 to 7.6 m in height. The trees were well established in their site and have been planted for at least five years, most for decades. For each tree, cumulative diameter at breast height (DBH) was also taken by adding the diameter at 1.37 m for each bole in accordance with the instructions for multi-bole trees on the insecticide labels. The trees ranged from 25 to 107 cm diameter breast height (Fig. 8).

Table 1. Five research sites on or near the Auburn University campus with established crapemyrtle trees.

Site	Turfgrass unit	CASIC	Funchess/Rouse	Magnolia	Athletics
GPS Coordinates	32°34'41.6"N 85°29'57.4"W	32°35'10.1"N 85°29'35.8"W	32°35'57.8"N 85°28'57.1"W	32°36'23.4"N 85°29'01.8"W	32°35'51.4"N 85°29'29.0"W
Site Description	landscape planting	landscape planting	landscape planting	street trees	parking lot planting
PIS[y]	40.94	42.70	64.36	81.77	41.08
Avg. Tree Height (m)	5.89	5.69	5.63	6.62	4.24
Avg. DBH[z] (cm)	77.98	50.80	58.67	67.82	41.66
Soil Texture	sandy loam/sandy clay loam	sandy loam/sandy clay loam	sandy clay loam/sandy loam	sandy clay loam/sandy loam	sandy clay loam/sandy loam
Soil pH	5.82	6.42	6.66	6.31	6.42
% Organic Matter	2.29	2.11	4.17	6.98	4.00

[y]Percent Impervious Surface
[z]Average diameter at breast height

2.2.2 Floral visitors to crapemyrtle

The diversity and abundance of crapemyrtle floral visitors were quantified with two, 30-second "snapshot" counts per tree from our untreated trees (Garbuzov and Ratnieks 2014), followed by selective netting to confirm the identification of each insect collected (Mach and Potter 2018). Each tree was an individual sample; 10 trees were sampled from four of our sites. Sampling occurred throughout the first bloom flush of crapemyrtle, beginning in late May, through June, July, and early August. Bloom phenology was variable across sites, so bloom intensity was visually estimated for each tree. It was then rated on a scale of 0 (absence of bloom), 1 (<⅓ of maximum), 2 (⅓–⅔ of maximum), or 3 (full bloom, >⅔ of maximum) (Anderson and Hubricht 1940, Garbuzov and Ratnieks 2014). Counts and sampling were only performed on days with ideal weather for pollinators, sunny or sparsely cloudy with winds less than 15 kph (Larson et al. 2014, Mach and Potter 2018). Temperature data for each count were retrieved from an AU Mesonet weather station on campus which had temperature recordings at

every 30 min (Auburn University Mesonet 2019). Temperatures ranged from 23.3 to 29.7°C at the times of each count.

Separate counts were performed on the same three trees at two of the sites at 07:30, 08:30, 09:30, 10:30, and 11:30 to determine the optimal time to perform pollinator counts. Previous literature has given varying times for the anthesis of *Lagerstroemia* flowers, with one report citing 08:30 (Harris 1914), and another at 10:00 to 11:00 (Nepi et al. 2003). Our own observations showed that on clear and sunny days, pollinators began foraging on crapemyrtle as soon as daylight reaches the flowers. Dr. Yan Chen (LSU Agcenter, Hammond LA) had prior experience collecting pollen from crapemyrtles which showed that all the pollen was collected by bees by 13:00 (Y. Chen, *personal observation*). Bees stop visiting floral resources once they are depleted of rewards (Rivera et al. 2015). Counts were therefore performed between 7:30 and 10:30 CST. Two people, each on opposite sides of the crapemyrtle, counted insects actively foraging on flowers within sight during one 30-second period, scanning from one side to the other to avoid counting the same insect twice. The numbers from each side were then totaled, giving the count of visiting pollinators for that tree. Immediately following each count, insects actively foraging on flowers were selectively netted (Fig. 9) with aerial nets and transferred to plastic bags to be transported to the laboratory. An aerial net with a 4.5 m PVC handle was used to reach pollinators on the highest blooms of sample trees. Counting and netting took approximately two to three hours per tree. Initially, 50 insects were collected per tree, but by the second sampling date, the time required to collect that amount was prohibitive, and therefore was reduced to 25 per tree. In the laboratory, insects were frozen, then pinned, labeled, and organized loosely into groups of morphospecies. Identification to genus was confirmed and vouchers were submitted to the Auburn University Biodiversity Learning Center.

2.2.3 Insecticide applications

Study trees were selected on all five sites to be treated with one or two different neonicotinoid insecticides applied at three timings representing different phenological stages of crapemyrtle. Imidacloprid (Mallet® 2F T&O Insecticide, Nufarm, Alsip, IL) and dinotefuran (Zylam™ Liquid Insecticide PBI Gordon, Shawnee, KS) were applied at the maximum label rate to different trees at post-bloom/pre-dormancy (31 Oct, 2 and 4 Nov, 2018), pre-bud break (24 to 25 Feb and 1 Mar), and post-bud break/pre-bloom (26 and 30 Apr, and 1 May) timings. These insecticides are the two most commonly recommended systemic insecticides used for management of crapemyrtle bark scale (Layton 2019). The insecticide rates were calculated according to their label based on cumulative inches of DBH as recommended for multi-bole trees. Trees were blocked by size (DBH), and then randomized into treatment replicates with trees within a replicate having similar trunk sizes. The assumption was that DBH, or tree size, would be the best indicator of bloom phenology, additionally that size would dictate the maximum amount of blooms per tree, likely influencing pollinator visitation. Seven biological replicates (trees) per treatment were treated at 11.83 mL per 2.5 cm DBH for imidacloprid, and 23.66 mL per 2.5 cm DBH for dinotefuran using soil injection (Figs. 10 and 11). Soil injection deposits insecticide solution in water below the surface and into the rootzone for plant uptake. Injection holes were spaced evenly around the base of the trunk, no more than 30.5 cm (12 inch) away from the tree base. A CO_2 pressurized soil injector (R&D Sprayers, Opelousas, LA) equipped with a metal probe was inserted to a depth of 46 to 64 cm (18 to 24 in) and one quart of dilute solutions per 2.5 cm (1 in) of DBH was applied as recommended by the product label. Average soil moisture for experimental sites ranged from 9.39 to 22.63%.

2.2.4 Sampling of leaves and pollen for insecticide residue analysis

Trees were sampled for first flush leaves and pollen. In other studies, concentrations of neonicotinoid insecticides in the first flush leaves of woody plants correlated with levels in pollen or nectar (Mach et al. 2018). Leaf samples were taken between 21 Mar and 6 Apr from control trees and those treated with either insecticide, but only for trees that have been treated (post-bloom/pre-dormancy and pre-bud break application timings). The post-bud break and pre-bloom application had not been made by this time. Five grams of new growth were collected from five trees for each treatment using gloved hands. Leaves were collected at the "mouse-ear stage," when the leaf just begins to unfold (Fig. 12) (Meier 2003). Leaves were then placed into a labeled paper bag and into a cooler with dry ice to flash freeze them until they were transferred to a -20° C freezer to await shipment for analysis. Flowers were collected from approximately 06:00 to 09:30 from control and treated trees. Flowers were harvested using hand shears then placed into containers lined with aluminum foil (Fig. 13). Samples for residue analysis were collected with extreme care to avoid contamination. Nitrile gloves were always worn when handling leaf and pollen samples, and tools that were not disposable were cleaned with acetone between samples. The flowers were taken to the laboratory and allowed to sit in their ventilated, but covered, containers for 4 to 24 h while the anthers released their pollen. Crapemyrtle produces relatively small amounts of pollen; approximately 300 to 600 individual flowers were required to obtain the 120 mg pollen needed for each sample. Individual flowers were removed from the panicle and petals were removed before flowers were repeatedly tapped onto an aluminum-foil-covered workspace to dislodge the pollen from the anthers. Pollen was then collected using a sterile 1000 µL filtered pipette tip connected at the end by tubing to a vacuum pump (Fig. 14) (Gould 2018). The collected pollen for each sample was stored in sterile 50 mL centrifuge tubes in a -20° C freezer. The amount of pollen released from the flower samples was extremely variable. Sometimes, the flowers collected from a tree did not yield enough pollen for

a complete sample in a single day, and had to be revisited again within the following week to harvest more flowers. If a tree did not yield enough pollen for a complete sample, pollen from multiple trees in the same treatment were pooled to make a complete sample. Three samples per treatment were collected. Approximately 2 g of additional pollen from flowers from untreated control trees was also collected to be used for spike and retrieval analyses.

2.2.5 Efficacy of treatments against two common pests

Colony of A. lagerstroemiae *and* S. kahawaluokalani

Cuttings from crapemyrtles infested with *A. lagerstroemiae* were collected in Foley, AL (30°20'24.3"N 87°40'51.4"W), in December 2017 and transported in floral water tubes in a sealed cooler to the laboratory in Auburn, AL. Upon arrival, two to three 15.24 cm (6 inch) infested cuttings were then tethered to each of six, 25-gallon containerized *Lagerstroemia* × 'Tuscarora' trees in a research greenhouse. Each tree was maintained in the greenhouse, receiving a yearly application of granular, slow release fertilizer (Scotts Osmocote 15:9:12, Scotts Co, Marysville, OH) and placed on micro-irrigation. Once the initial trees were well infested, eight additional 15-gallon containerized *L.* × 'Tuscarora' trees were infested in the same manner using cuttings from our initial colony and kept in the same greenhouse. These new trees were similarly fertilized annually with slow release granular fertilizer (Osmocote 15:9:12) and watered using micro-irrigation. Due to space constraints, the initial colony was moved into an enclosed outdoor shade house on the Auburn University Campus and watered daily during the summer and as needed during cooler parts of the year. A pre-existing population of *S. kahawaluokalani* were maintained on colony trees along with *A. lagerstroemiae.*

Mortality Tests

The efficacy of each product (imidacloprid and dinotefuran) applied at each timing was evaluated by challenging cuttings from trees with either crapemyrtle aphids or crapemyrtle bark scale crawlers. Four terminal cuttings of approximately 15.24 cm long were taken from each of the five trees in each treatment. Cuttings were placed in a cooler on ice, then immediately stuck in water agar (5.5 g/L) in 30 mL disposable plastic containers with lids to maintain their turgidity upon arrival at the laboratory (Fig. 15). Cuttings were infested with ten immature crapemyrtle aphids taken from our colony trees by gently moving them to the leaves with a fine-bristled paintbrush. Infested cuttings were placed into a translucent, 18 oz (532.32 mL) plastic drink cup and a second cup with a vented and screened hole was placed on top. The cups were then sealed together with Parafilm® and placed in a growth chamber under a 14:10 (L:D) photoperiod at 22°C/20° C day/night temperature cycles based on bionomics of crapemyrtle aphid (Alverson and Allen 1992) (Fig. 16). Mortality of aphids was calculated 24 and 48 h after infestation during the final week of May.

This experimental setup was repeated on 10 to 21 Jun except cuttings were challenged with crapemyrtle bark scale nymphs. As before, four cuttings were taken from five trees per treatment, and kept on ice until they were stuck in containers of water agar in the laboratory. For infestation of cuttings with crapemyrtle bark scale, one two-inch length of infested stem and a single 3.2 cm^2 piece of infested leaf with crawlers present were taken from our colony trees and lashed with Parafilm® to 15.24 cm cuttings (Figs. 17 and 18). The crawlers were allowed to settle and feed for one week in the same sealed and ventilated containers described earlier. They were placed in a growth chamber under the sample temperature and photoperiod as before. Mortality was determined based on appearance of individual scale insects. Dead insects appeared dried, shrunken, and darkened in color. Mortality was then calculated for crawlers after one week of feeding.

The growth of scales used for the CMBS mortality test was measured to verify that they were feeding and growing during the experiment. Three trees were selected as replicates from each treatment. Each of these trees had four subsamples, or cuttings, as described earlier. Once the cuttings were inoculated with scale insects, 72 h were allowed for scale dispersal and settling to occur before three scale insects per cutting were selected as representatives for growth. Each scale insect selected was denoted with a small mark directly adjacent to it with a blue fine-point permanent marker (Sharpie, Atlanta, GA) (Fig. 19). Images were then recorded of each scale from directly above with a camera (Teledyne Lumenera Infinity 2-1C, Ottawa, Ontario, Canada) mounted to a dissecting microscope. Images were taken using image capture software (Lumenera Infinity Capture software v. 6.3.1, *Infinity Capture* 2019). The scale insects were kept in a growth chamber under the same conditions as crapemyrtle aphids, with a 14:10 (L:D) photoperiod and a 22° C/20° C day/night temperature cycles for 2 wk. Then, a second image was taken of the same scale from the same angle and same magnification. The before and after images of each scale were then uploaded to Image J (Rasband 2019) and their length and width were measured in millimeters following calibration with an image of a ruler taken at the same magnification. The area for each photographed nymph was calculated using the formula for the area of an ellipse.

2.2.6 Analytical methods for residue analysis

Leaf and pollen samples were shipped overnight on dry ice in a temperature-controlled package to Dr. Chris Ranger (USDA, Wooster, Ohio) for analysis where they were immediately transferred to a -80° C freezer until analysis. Extraction protocols were modified from David et al. (2015).

Leaf and Pollen Extraction Protocol

Leaf samples were removed from storage at -80° C and 2 g of tissue per sample was weighed in a 30 mL polypropylene screw cap tube pre-filled with 1.4 mm ceramic beads (Omni International, Kennesaw, GA). Five mL of chilled acetonitrile was added to each tube and the tissue was then macerated using a bead mill homogenizer (Omni Bead Ruptor Elite) at 4 m/s for two 30-sec cycles. The following QuEChERS extraction salts were then added to each tube corresponding to the use of 2 g of tissue: 1.2 g $MgSO_4$ and 0.3 g NaCl. The tubes were immediately shaken by placing horizontally on a BenchMixer XLQ QuEChERS shaker for 10 min at 2,250 rpm (Benchmark Scientific, Edison, NJ). The tubes were then centrifuged for 5 min at 4,000 rpm using a Restek Q-Sep 3000 (Restek Corp., Bellefonte, PA). A 1 mL aliquot of the acetonitrile supernatant was then transferred into a dispersive solid phase extraction tube containing 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18-EC, and 7.5 mg GCB (Restek Corp.). The dSPE tubes were immediately shaken using a BenchMixer XLQ for 2 min at 2,250 rpm, and then centrifuged at 3,000 for 5 min. A 0.1 mL aliquot of the acetonitrile supernatant was then transferred to a Thomson SINGLE StEP outer shell vial (Restek Corp.) and mixed (1:1) with 0.1 mL of ultra-pure water containing 100 ng/mL of deuterated internal standard (dinotefuran-d3 or imidacloprid-d4). A 0.2 μm polyvinyldifluoride Thomson filter with a pre-slit polytetrafluoroethylene/silicone cap was then used to remove particulates. Extracts were immediately analyzed by UPLC-MS after filtering.

Pollen samples were removed from storage at -80° C and 0.1 g of tissue per sample was weighed in a 2 mL polypropylene screw cap tube pre-filled with 1.4 mm ceramic beads (Omni International) (Fig. 20). An aliquot of 0.4 mL of ultra-pure water was added to each tube, and then mixed rapidly at 4 m/s sec for two 30-second cycles (Omni Bead Ruptor Elite) to hydrate the pollen (Figs. 21 and 22). An aliquot of 0.5 mL chilled acetonitrile was then added to each tube followed by rapid mixing at 4 m/s sec for two 30-sec cycles (Omni Bead Ruptor Elite) (Fig.

23). QuEChERS extraction salts were then added to each tube (0.1 g of tissue: 0.2 g $MgSO_4$ and 0.05 g NaCl) followed by immediate hand shaking to prevent clumping of the $MgSO_4$ (Fig. 24). The tubes were subjected to additional shaking by placing on a BenchMixer XLQ QuEChERS shaker for 10 min at 2,250 rpm (Benchmark Scientific, Edison, NJ) (Fig. 25). The tubes were then centrifuged for 5 min at 13,000 rpm. A 0.5 mL aliquot of the acetonitrile supernatant was then transferred into a dispersive solid phase extraction tube containing 0.03 g $MgSO_4$, 0.01 g Supelclean PSA, 0.01 g DSC_{18} (Sigma-Aldrich, City, State) (Figs. 26 to 28). The dSPE tubes were placed on a BenchMixer XLQ for 2 min at 2,250 rpm, and then centrifuged at 13,000 for 10 min. A 0.1 mL aliquot of the acetonitrile supernatant was then transferred to a Thomson SINGLE StEP outer shell vial (Restek Corp.) and mixed (1:1) with 0.1 mL of ultra-pure water containing 100 ng/mL of deuterated internal standard (dinotefuran-d3 or imidacloprid-d4) (Fig. 29 to 31). A 0.2 μm polyvinyldifluoride Thomson filter with a pre-slit polytetrafluoroethylene/silicone cap was then used to remove particulates. Extracts were immediately analyzed by UPLC-MS after filtering.

LC-MS Conditions

Filtered extracts were analyzed using a Waters Acquity UPLC H-class system coupled with a Xevo tandem quadrupole mass spectrometer operating in positive electrospray ionization mode. Samples were separated using an Acquity UPLC BEH C_{18} (2.1 mm i.d. x 50 mm) reverse-phase column maintained at 30° C. Mobile phase solvents were (A) water with 0.1% formic acid and 5 mM ammonium formate and (B) methanol with 0.1% formic acid with 5 mM ammonium formate. The following conditions were used at 0.3 mL/min: 0−2.00 min from 90% to 10% A, 2.00−4.00 min at 10% A, 4.00−4.30 min from 10% A to 90% A, and a conditioning period from 4.30−7.00 min at 90% A. The mass spectrometer used a capillary voltage of 3.50, desolvation temperature of 350° C, and desolvation gas at 650 L/h. High purity argon was used as the

collision gas and nitrogen was used for atmospheric pressure ionization gas. Multiple reaction monitoring was used to detect native and deuterated imidacloprid and dinotefuran in the extracts (Table 2).

Recovery Assessment

The extraction efficiency associated with the QuEChERS technique was assessed by determining pesticide recovery (%) from spiked leaf tissue and pollen (Table 3). Extraction efficiency of imidacloprid and dinotefuran from leaves was assessed by spiking 2 g of leaf tissue from non-treated trees with 0.1 mL of 100 ng/mL (i.e. equivalent to 5 ng/g); 1,000 ng/mL (50 ng/g); 5,000 ng/mL (250 ng/g); 10,000 ng/mL (500 ng/g); and 20,000 ng/mL (1,000 ng/g) of native analyte. Tubes were allowed to sit for 1 h and then extracted as described above with the addition of 5 mL of acetonitrile. A calibration curve was prepared using matrix matched standards, and recovery (%) was determined by comparing expected and detected concentrations of analyte.

Extraction efficiency of imidacloprid from pollen was assessed by spiking 0.1 g of pesticide-free pollen with 0.4 mL of 10 ng/mL and 100 ng/mL native imidacloprid in water to achieve 40 ng/g and 400 ng/g, respectively. After spiking, the 2 mL tubes were rapidly mixed at 4 m/s for two, 30-sec cycles (Omni Bead Ruptor Elite) to hydrate the pollen, and then allowed to sit for 10 min. The pollen was then extracted as described above starting with the addition of acetonitrile. A calibration curve was prepared using matrix matched standards. A calibration curve was prepared using matrix matched standards, and recovery (%) was determined by comparing expected and detected concentrations of analyte.

Table 2. Multiple reaction monitoring parameters used for analyte detection.

Analyte	Parent (m/z)	Product (m/z)	Cone (V)	Collision (V)	Type
Imidacloprid	256.06	209.10	18	14	Quantitation
	256.06	175.10	18	14	Confirmation

Imidacloprid-d4	260.00	213.10	18	18	Quantitation
Dinotefuran	203.00	129.10	20	20	Quantitation
	203.00	157.00	20	20	Confirmation
	256.06	175.10	18	14	Confirmation
Dinotefuran-d3	206.00	132.00	20	20	Quantitation

m/z=mass/charge number of ions; V=volt.

Table 3. Average recoveries (and standard deviations) of analytes in nectar and leaf tissues.

	Leaf Tissues					Pollen	
	5 ng/g	50 ng/g	250 ng/g	500 ng/g	1,000 ng/g	40 ng/g	400 ng/g
Analyte	(n=6)	(n=6)	(n=6)	(n=6)	(n=6)	(n=4)	(n=3)
Imidacloprid[a]	97.77 (2.53)	91.5 (1.63)	94.32 (2.56)	89.62 (1.69)	87.32 (1.22)	90.75 (4.61)	93.7 (0.51)
Dinotefuran[b]	86.75 (2.79)	88.78 (2.54)	95.77 (1.89)	97.1 (0.83)	96.75 (1.22)	-	-

[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient [AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.
[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.
Recoveries for dinotefuran were 10x higher than expected, and were removed from recovery results.

2.2.7 Statistical analyses

All tests were designed as an augmented factorial design (Piepho et al. 2006), with three timings and two products plus the control treatment. All datasets were analyzed using linear or generalized linear models using the GLIMMIX procedure of SAS (v. 9.4; SAS Institute Incorporated, Cary, NC). Aphid mortality data were analyzed using a generalized linear model with a Poisson distribution and log link function. The 24- and 48-h datasets were analyzed separately. Crapemyrtle bark scale mortality data were analyzed using a generalized linear model, with a Negative Binomial distribution and log link function. CMBS growth data were analyzed using a mixed linear model, with a Gaussian distribution. All pollinator count data were analyzed using a generalized linear model with a log link function. The analysis to establish peak time for pollinator sampling was done with a Poisson distribution. Pollinator counts based on level of urbanization were analyzed with a Negative Binomial distribution. Least squares means

were analyzed using bloom intensity as a covariate. When there was a significant interaction between product and timing, simple effects were run. All tests were analyzed with a significance level of <0.05.

2.3 Results

2.3.1 Floral visitors to crapemyrtle

Timepoints that yielded average counts >5 (range 4 to 22) per 30-sec count were 07:30 and 08:30 (Fig. 34). Counts between 9:30 and 11:30 average less than 5 per count, with counts of 1 to 3 per 30 sec count by 11:30. Bloom intensity (F=26.4, df 1. 7, P=0.0013) and urbanization level (F= 7.88, df = 1. 7, P=0.026) were significant. When controlled for bloom intensity, crapemyrtles at highly urbanized sites had 27.3 ± 8.0 floral visitors per tree compared to 6.9 ± 1.85 per tree on suburban sites. Level of urbanization was significant. Suburban and highly urban trees had genus diversity scores of 0.87 and 0.95, respectively (Simpson's Diversity Index, 1-D). Genus richness was proportionally equal for highly urban (11 genera) and suburban (18 genera) pooled samples. Additionally, the underlying species abundance distribution was similar for both suburban and highly urban sites (Fig. 35). Apids were most prevalent (≈60%) across both levels of urbanization, including mostly *Apis mellifera* L., then *Bombus* spp., *Xylocopa micans* Lepeletier and *virginica* L. and *Melissodes* sp. (Table 4). *Lasioglossum* spp. (Halictidae) were also prevalent (app. 30%) in highly urban and suburban samples. The remaining 10% is comprised of other halictids (*Halictus ligatus* Say, *Dieunomia nevadensis* Cresson), syrphids or hoverflies (*Pelecinobaccha costata* Say, *Palpada* sp., *Toxomerus* sp., *Meromacrus* sp., *Allograpta obliqua* Say, and *Eristalis* sp.), a few wasps (*Polistes Carolina* L., *Scolia nobilitata* Fabricius, *Sceliphron caementarium* Drury), other flies (*Condylostylus* sp., *Physocephala* sp., *Lucilia cuprina* Wiedemann), and three species of megachilids (*Megachile mendica* Cresson,

Megachile sculpturalis Smith and *Megachile rotundata* Fabricius) were the least abundant. *Eristalis sp.*, *L. cuprina*, *M. mendica* and *S. caementarium* were only found in highly urban samples. *Condylostylus* sp., *D. nevadensis*, *H. ligatus*, *M. sculpturalis*, *Melissodes* sp., *Meromacrus* sp., *Physocephala* sp., and *Polistes carolina, Scolia nobilitata*, *Toxomerus* sp., and *Xylocopa* spp. were only found in suburban samples.

Table 4. Pollinator taxa distribution and diversity.

	Urbanization Level	Highly Urban	Suburban
	Trees (n)	4	6
	Pollinators (n)	122	199
	Simpson's Diversity (1-D)	0.95	0.87
Bees	*A. mellifera*	49.18	51.25
	Bombus	13.93	10.05
	Total Apidae	63.11	64.32
	Halictidae	28.69	27.64
	Megachilidae	1.64	1
Flies	Calliphoridae	0.82	0
	Conopidae	0	0.5
	Dolichopodidae	0	1
	Syrphidae	3.27	4

Wasps	Scoliidae	0	1
	Sphecidae	2.45	0
	Vespidae	0	0.5

Pollinator taxa distribution is presented as percentage of total bees

Diversity is calculated as the 1 - Simpson's D, which generates a number between 0 and 1 with higher values indicating more genus–rich and even samples

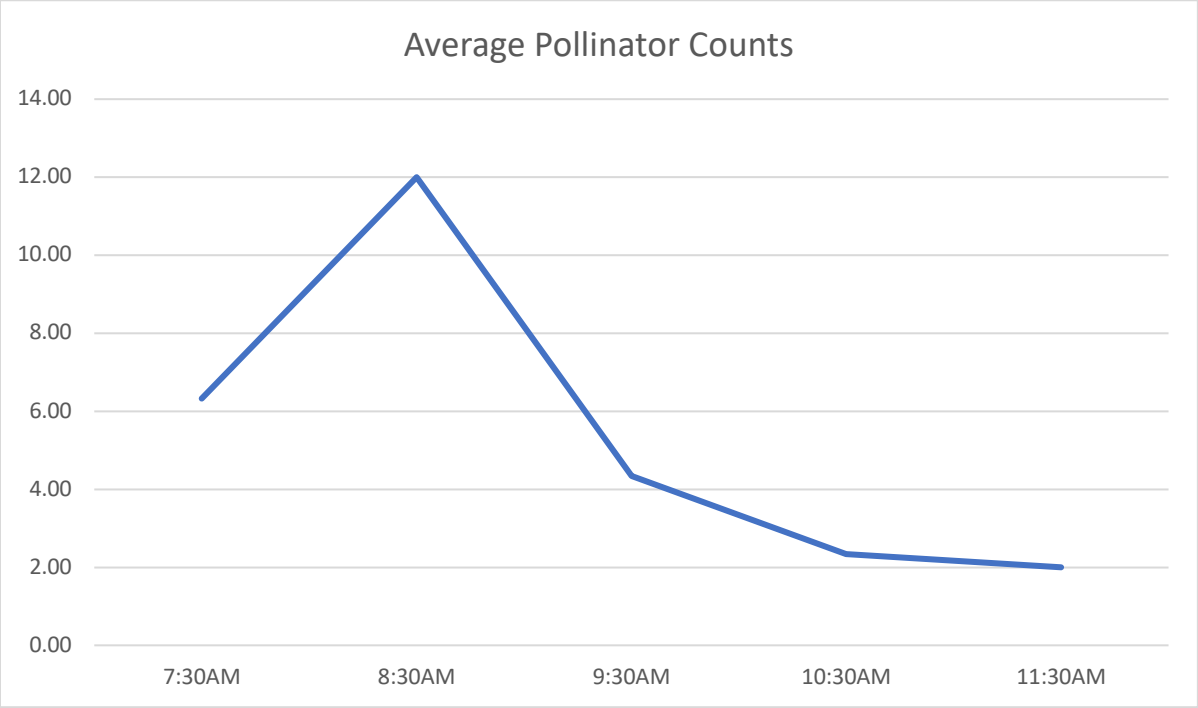

Fig. 32. Pollinator counts of pollinators in flowers of three Natchez crapemyrtle trees from three trees over five timepoints, in 2019 (Auburn, AL).

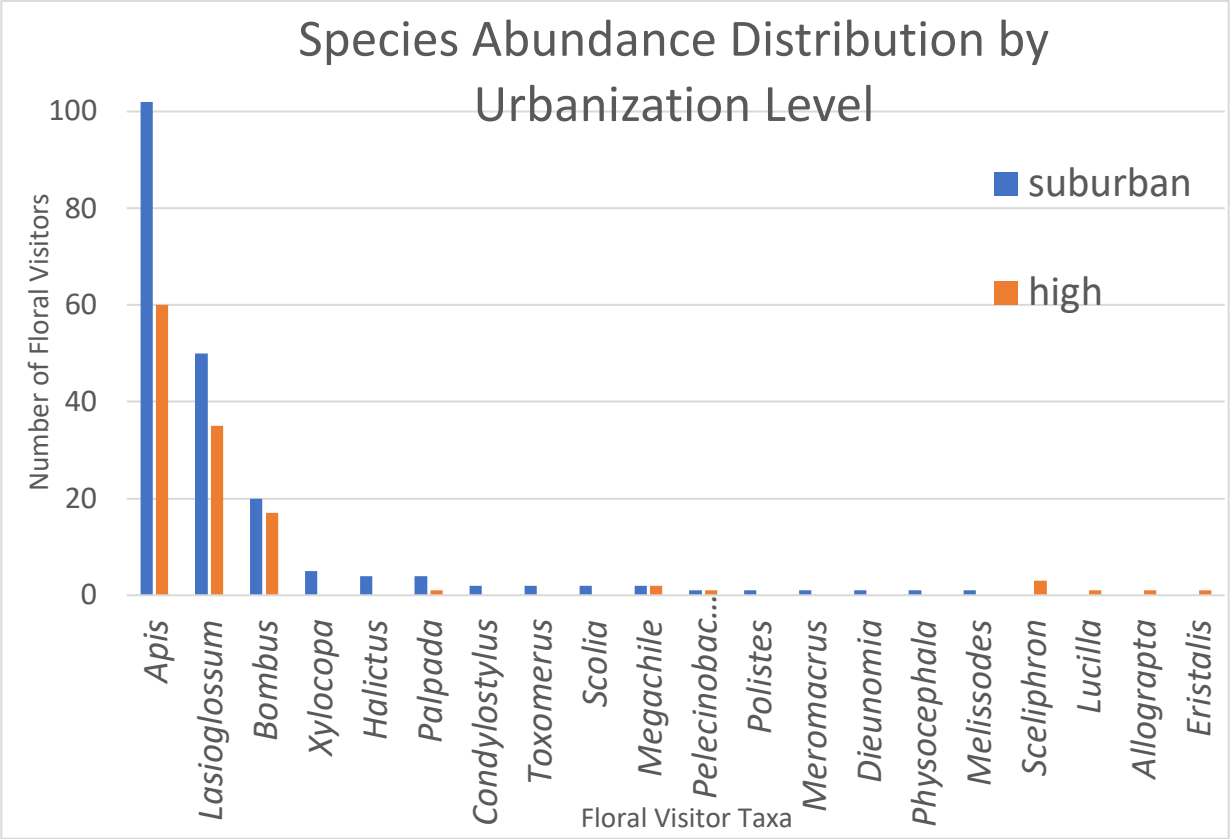

Fig. 33. Species abundance distribution for suburban and highly urban pollinators.

2.3.2 Efficacy of treatments against two common pests of crapemyrtle

Aphid Mortality

After 24 h, crapemyrtle aphids on cuttings from trees treated with either imidacloprid or dinotefuran at post-bud break/pre-bloom had significantly higher mortality than the control group ($P<0.05$) (Table 5). Aphids mortality ranged from 64 to 71% across all application timings for imidacloprid and were not significantly differ from one another. The application timings for dinotefuran, however, were all significantly different from one another. Residues of dinotefuran applied after bud break were more effective against aphids at 24 h (Table 5). After 48 h, mortality of aphids on cuttings from plants treated with dinotefuran and imidacloprid incurred significantly higher mortality than control ($P<0.05$) (Table 6). Irrespective of timing,

imidacloprid provided better 75.4 ± 5.7% mortality of crapemyrtle aphids compared to 51.1 ± 4.2 % mortality from dinotefuran. Additionally, all three timings for each product provided significantly greater mortality than the control. Irrespective of product, the post-bud break/pre-bloom (spring) applications resulted in the greatest mortality (72.5 ± 6.7%), while the other two timings resulted in 56-58% mortality. None of the three timings was significantly different from each other.

Table 5. Mortality of crapemyrtle aphids, *Sarucallis kahawaluokalani* (Kirkaldy) after exposure to foliage from trees treated with neonicotinoid insecticides.

	Mean (±SEM) mortality of aphids (%)		
Timing	Active ingredients	24 h exposure	
Post-boom drop/pre-dormancy (autumn)	Imidacloprid[a]	64.31 ± 8.10	A*
Pre-bud break/ pre-bloom (late winter)		65.77 ± 8.95	A*
Post-bud break/ pre-bloom (spring)		71.11 ± 9.11	A*
Post-boom drop/pre-dormancy (autumn)	Dinotefuran[b]	31.45 ± 4.95	a
Pre-bud break/ pre-bloom (late winter)		44.72 ± 6.36	b
Post-bud break/ pre-bloom (spring)		62.07 ± 8.05	c*
	Control (non-treated trees)	33.87 ± 4.09	

An asterisk denotes $P < 0.05$, or significance from control. When the interaction term in the model is not significant ($P > 0.05$), the main effects for levels within each treatment factor followed by the same lower-case or upper-case letter are not significantly different. When the interaction term in the model is significant ($P < 0.05$), the simple effects means (treatment means for application timing grouped within product) followed by the same lower-case or upper-case letter are not significantly different.

[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient [AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.

[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.

Table 6. Mortality of crapemyrtle aphids, *Sarucallis kahawaluokalani* (Kirkaldy) after 48 h exposure to foliage from trees treated with neonicotinoid insecticides.

		Least squares means for main effects	
Timing	Product	Mean (±SEM) dead aphids (%)	
Post-boom drop/pre-dormancy (autumn)		58.01 ± 5.24	A*
Pre-bud break/ pre-bloom (late winter)		56.82 ± 6.13	A*
Post-bud break/ pre-bloom (spring)		72.53 ± 6.69	A*

	Imidacloprid[a]	75.42 ± 5.75	a*
	Dinotefuran[b]	51.07 ± 4.18	b*
	Control (non-treated trees)	26.62 ± 3.18	

An asterisk denotes P < 0.05, or significance from control. When the interaction term in the model is not significant (P > 0.05), the main effects for levels within each treatment factor followed by the same lower-case or upper-case letter are not significantly different. When the interaction term in the model is significant (P < 0.05), the simple effects means (treatment means for application timing grouped within product) followed by the same lower-case or upper-case letter are not significantly different.

[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient [AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.

[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.

CMBS Mortality

The main effects of products (P=0.26) or application timings (P=0.30) were not significant for mortality of CMBS crawlers on cutting from those trees. Average mortality in the control group was 48.13 ± 4.52%; imidacloprid and dinotefuran treatments resulted in 52.34 ± 4.11% and 46.48 ± 3.85% mortality, respectively. Post-bloom drop/pre-dormancy and pre-bud break/pre-bloom treatments incurred 46.10 ± 4.43% and 47.36 ± 4.59%, and the post-bud break/pre-bloom treatment 55.15 ± 5.15% mortality.

CMBS growth test

The increase or decrease in size of CMBS nymphs was measured to determine if sublethal effects of exposures to aged residues in treated trees were occurring. The main effect of application timing was significant at P<0.1 (F= 2.56, DF= 2 , P=0.081), but product was not significant (F= 1.02, DF=1, P=0.31). Only the post-bud break/pre-bloom (spring) group had significantly less growth (P=0.01) as compared to the CMBS nymphs on cuttings from control trees (Table 7). While nymphs on cuttings did not readily die, they were the only group of CMBS scales to lose size during the 2 wk experiment. Those nymphs confined on either trees

treated at post-bloom/pre-dormancy (autumn) or pre-bud break (late winter) had 7.43% and 32% smaller final areas, respectively, than the control after 14 d.

Table 7. Effect of application time and insecticide on growth of crapemyrtle bark scale over 2 wk.

		Least squares means for main effects	
Timing	Product	Mean (±SEM) Change in Area (%)	
Post-boom drop/pre-dormancy (autumn)		33.81±15.44	A
Pre-bud break/ pre-bloom (late winter)		9.26±18.04	A
Post-bud break/ pre-bloom (spring)		-2.4±16.46	A*
	Imidacloprid[a]	7.6±13.48	a
	Dinotefuran[b]	19.52±16.38	a
	Control (non-treated trees)	41.24±17.67	

An asterisk denotes $P < 0.05$, or significance from control.
When the interaction term in the model is not significant ($P > 0.05$), the main effects for levels within each treatment factor followed by the same lower-case or upper-case letter are not significantly different.
[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient[AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.
[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.

2.3.3 Residues in leaves and pollen

Imidacloprid and dinotefuran were detected in early season foliage regardless of application timing (Table 8). Four of the 19 non-treated trees had detectable levels of insecticides in leaf tissue. Two control trees had levels of imidacloprid at 12.15 to 28.4 ng per g leaf tissue in foliage. Similarly, two control trees had detectable levels of dinotefuran at 12.4 to 14.35 ng per g leaf tissue. Those four trees were excluded from the data set.

Table 8. Neonicotinoid residues in newly expanded leaves of crapemyrtle trees treated with neonicotinoid insecticides.

Treatment and timing of application	**Mean (range) Concentrations in leaf tissue (ng/g or ppb)**	n
Imidacloprid[a], post-bloom/pre-dormancy (autumn)	3.64 (0-11.50)	9
Imidacloprid, pre-bud break/ pre-bloom	12.25 (0-84.70)	9

(late winter)		
Dinotefuran[b], post-bloom/pre-dormancy (autumn)	291.18 (0-1530.80)	7
Dinotefuran[b], pre-bud break/ pre-bloom (late winter)	29.63 (0-150.85)	7
Control (non-treated trees)	0	15

[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient[AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.
[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.
Limit of Quantitation (LOQ) = 5 ng/g based on matrix-matched standard.

Regardless of application timing, imidacloprid and dinotefuran were detected in the pollen of crapemyrtle (Table 9). The levels of each active ingredient in pollen were lower on average the closer the application date was to bloom. For dinotefuran, detectable residues ranged on average from 1,019 to 1,634 ng per g of pollen. For dinotefuran, all three replicated samples had detectable levels for trees treated post-bud break. Two samples had detectable levels for trees treated pre-bud break and only one sample had detectable levels when treatments were applied the previous fall (pre-dormancy). For imidacloprid, all three samples at each application timing had detectable residues with averages ranging from 437 to 1,786 ng per g of pollen. Based on these residue data, it would be helpful to know the realistic concentrations per European honey bee (*A. mellifera*). To estimate these doses, an average pollen consumption rate of 4 mg pollen per day was determined for honey bee workers (Crailsheim et al. 1992). For imidacloprid, a western honey bee would have oral doses between 1.75–7.14 ng of imidacloprid per worker per day (Table 10). All of these concentrations are near or exceed the currently reported chronic NOAEC/LOAEC LD_{50} values for imidacloprid and western honey bee adults and larvae, but would not exceed the acute oral or contact LD_{50} values (Table 11). For dinotefuran, the per worker daily dose would range from 4 to 6.5 ng. All concentrations of dinotefuran in pollen are near the lower limit of the acute oral LD_{50} values but none exceeds either of the published acute oral or contact LD_{50} values (Table 11).

Table 9. Neonicotinoid residues in crapemyrtle pollen.

Treatment and timing of application	Mean (range) concentrations (ng/g or ppb) of insecticide in pollen	n
Imidacloprid[a]: post-bloom/pre-dormancy (autumn)	1,786.28 (1,762.30-1,810.25)	2
Imidacloprid[a]: pre-bud break/ pre-bloom (late winter)	437.15 (198.05-664.50)	3
Imidacloprid[a]: post-bud break/pre-bloom (spring)	485.68 (337.4-609.15)	3
Dinotefuran[b]: post-bloom/pre-dormancy (autumn)	1615.62 (0-4846.85)	3
Dinotefuran[b]: pre-bud break/ pre-bloom (late winter)	1634.7 (0-3866)	3
Dinotefuran[b]: post-bud break/pre-bloom (spring)	1,019.7 (672.13-1,714.05)	3
Control (non-treated trees)	0	3

[a]Applied as the liquid formulation of Mallet® (21.4% Active ingredient[AI], Nufarm, Alsip, IL) at 239.653g AI per liter water.
[b]Applied as the liquid formulation of Zylam™ (10 % Dinotefuran [AI], PBI Gordon, Shawnee, KS) at 105.44g AI per liter water.
Limit of Quantitation (LOQ) = 5 ng/g based on matrix-matched standard.

Table 10. Realistic concentrations per bee based on residues in crapemyrtle pollen**.**

Treatment	Estimated mean oral amount of A.I. from crapemyrtle pollen per honey bee worker per day
Imidacloprid: post-bud break/pre-bloom	1.94 ng
Imidacloprid: pre-bud break/pre-bloom	1.75 ng
Imidacloprid: post-bloom/pre-dormancy	7.14 ng
Dinotefuran: post-bud break/pre-bloom	4.08 ng
Dinotefuran: pre-bud break/ pre-bloom	6.54 ng
Dinotefuran: post-bloom/pre-dormancy	6.46 ng

Concentrations from average crapemyrtle pollen residue levels by treatment converted to per bee concentrations. Pollen consumption rate based on 4mg/day rate of honey bee workers from "Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): Dependence on individual age and function" (Crailsheim et al. 1992).

Table 11. Published[a] LD_{50} values for honey bees for imidacloprid and dinotefuran.

	LD_{50} Values
Imidacloprid chronic oral 10-day NOAEC adult honey bee	0.16ng/bee
Imidacloprid chronic oral 10-day LOAEC adult honey bee	0.24ng/bee
Imidacloprid larval chronic 21-day NOAEC/LOAEC	1.8ng/bee/>1.8ng/bee
Imidacloprid acute oral	39 ng/bee
Dinotefuran acute oral	7.6-32ng/bee
Imidacloprid acute contact	43ng/bee

Dinotefuran acute contact	24-47ng/bee

[a]LD_{50} Values for dinotefuran and imidacloprid from the Environmental Protection Agency.

2.4 Discussion

Crapemyrtle trees in urban landscapes are visited by pollinators, and those pollinators can be at risk when insecticides are applied for management of crapemyrtle bark scale. As noted in previous studies (Deyrup et al. 2002, Riddle and Mizell 2016, Braman and Quick 2018, Mach and Potter 2018), the primary pollinators visiting flowers of crapemyrtles are honey bees and sweat bees (Halictidae). Pollinator assemblage data presented here as well as Mach and Potter (2018) are the only studies to report floral visitors in context of an urban landscape. Level of urbanization significantly influenced abundance, but not pollinator diversity (Table 4). Trees within highly urban sites had more pollinators than suburban sites, but genus richness was equivalent between levels of urbanization. In this study, we collected six genera of hover flies (Diptera: Syrphidae) as floral visitors in crapemyrtle. Previous pollinator surveys focused on bees and this is the first report of hover flies associated with crapemyrtle. Since all of these pollinators consume pollen, all would potentially be at risk when trees are treated with neonicotinoid insecticides to control sap-sucking insects.

Systemic insecticides like neonicotinoids are used to manage crapemyrtle bark scales and aphids in urban landscapes. When treated with either imidacloprid or dinotefuran, residues from the most recently applied treatment reduced crapemyrtle bark scale (Table 7). Crapemyrtle aphid was controlled most effectively with imidacloprid (Table 5 and 6). At 48-h post-infestation, imidacloprid residues from every timing had higher mortality than the control group (Table 6). Twenty-four hours post-infestation, dinotefuran-treated residues were only significantly effective against aphids for the most recently applied treatment. The crapemyrtle bark scale growth test gives the first indication as to the growth rate of CMBS. Scales in the control group increased

40% in size after 2 wk of feeding. Additionally, we noticed the number of scale insects in the control group was much lower than in the other treatments. Based on the observations of Wang et al. (2015), nymphs are able to move to new feeding locations after each molt. The growth of scales in the treated groups was reduced by sublethal exposures to imidacloprid and dinotefuran. Imidacloprid treated cuttings had the most scales remaining in their original feeding positions, although imidacloprid was not a significant treatment effect in scale growth. This may be due to the added potency of imidacloprid's metabolites, some of which maintain their insecticidal properties, like the parent compound (Nauen et al. 1998).

Time of application can affect the amount of translocation into leaves and pollen. Applications of the same two active ingredients to evergreen hollies (*Ilex*) or deciduous sweetshrub (*Clethra*) at three similar timings also affects residues in leaves and nectar of those two woody shrubs (Mach et al. 2018). Applications of imidacloprid made post-bloom drop/pre-dormancy (autumn 2018) resulted in relatively low residues (3.64 ng/g) in leaf tissues immediately following bud-break (Table 8). Applications of dinotefuran at the same timing resulted in high residues (291.18 ng/g) in leaf tissues. The oldest residues in pollen from the post-bloom/pre-dormancy treatment were similar in concentration for imidacloprid (1,786.28 ng/g) and dinotefuran (1615.62 ng/g), however, there was a large variation between samples in dinotefuran (Table 9). Leaf samples from the pre-bud break/ pre-bloom (late winter 2019) treated trees show low residue concentrations for imidacloprid (12.25 ng/g) and somewhat higher concentrations for dinotefuran (29.63 ng/g). Residue concentrations for pre-bud break/pre-bloom pollen show lower relative concentrations for imidacloprid (437.15 ng/g) than dinotefuran (1634.7 ng/g). The pollen residue concentrations for the post-bud break/pre-leaf flush (spring 2019) were over 2x greater in dinotefuran (485.68 ng/g) treated samples compared to imidacloprid (1,019.7 ng/g) treated samples. The differences in residue concentrations could be

due to differences in solubility and speed of translocation, but also in application rate. Imidacloprid was applied at a rate of 239.65 g AI per liter of water, and dinotefuran was applied at 105.44 g AI per liter of water. Mach et al. (2018) found a correlation between neonicotinoid residues in leaf tissues and nectar in two woody landscape plants. We were unable to perform a correlation between residue concentrations in our leaf tissue and pollen samples. Pollen samples (n=3) were under-replicated relative to leaf samples (n=7). Additionally, there was high variability in residue concentrations in both leaf and pollen samples.

Labeled insecticide application rates for ornamental plants are much higher per plant than crop systems (Krischik et al. 2015, Cowles and Eitzer 2017). However, multi-stemmed plants like crapemyrtle may further accentuate high application rates. For a multi-stemmed plant, the labels recommend adding the DBH of each trunk or stem (Fig. 8). It is as if each trunk is aligned side by side and measured across, rather than gathered together, as with a handful of pencils, then measured, which would give a more accurate analysis of the tree's size. Therefore, the label-recommended protocol for calculating insecticide rates for multi-bole trees likely overestimates their size. However, as it is illegal to disregard current label instructions, the label or use rates on multi-stemmed trees like crapemyrtle may require further revision to reflect a more accurate measurement dose.

All residue concentrations in pollen were high enough to harm visiting pollinators, regardless of timing or product. Cowles and Eitzer (2017) use a 100 ppb threshold for pollen concerning dietary toxicity of neonicotinoid residues in honey bees inferred from the 25 ppb NOEL (no observable effect level) for colony-level effects from chronic exposure to nectar containing imidacloprid. This threshold takes into consideration that more nectar is used by honey bees than pollen. This 100 ppb (ng/g) threshold was vastly surpassed in every treatment. Additionally, when placed into context of realistic daily pollen consumption for honey bees of

different ages: on average, workers- 4 mg, young (4 to 9 d) nurse bees- 12 mg, and larvae- 1 to 6 mg (Crailsheim et al. 1992), all of our treatments exceed the chronic NOAEC and LOAEC for imidacloprid (Table 10 and 11). The average residues for dinotefuran at post-bloom/pre-dormancy (autumn) and pre-bud break/pre-bloom (late winter) timings were nearing the low end of the acute oral LD_{50} values, although concentrations were variable for dinotefuran (Tables 10 and 11). As Mach et al. (2018) indicated, dinotefuran may be more persistent in plant tissues than generally thought.

Bumble bees and solitary bees, present in crapemyrtle blooms, may be exposed to higher risk, as they are more sensitive to neonicotinoids than honey bees (Sanchez-Bayo and Goka 2014). Even more concerning is that time-cumulative effects of neonicotinoids cause shorter times than expected for bees to reach T50 (time in which LD_{50} values are met) (Sanchez-Bayo and Goka 2014). Time-cumulative effects of neonicotinoids are due to irreversible binding of the toxicant to post-synaptic nicotinic acetylcholine receptors (Tennekes and Sánchez-Bayo 2013). Imidacloprid and dinotefuran, for example, have high risk for exposure to honey bees based on contact with 1 g of pollen contaminated at average levels found in bee-collected pollen from several surveys. Average T50's were 3 d for imidacloprid (1 d for bumble bees) and 1 d for dinotefuran (Sanchez-Bayo and Goka 2014). Worse, is that bumble bees in fragmented habitats tend to revisit the same patches of flowers day after day (Osborne and Williams 2001). In addition to honey bees, impact on other floral visitors may produce a resurgence effect for the scale insects. Hover flies and *Orius* spp. (Hemiptera: Anthocoridae) are common in flowers of crapemyrtle are also relative to biological control. Minute pirate bugs are important predators (Franco et al. 2019) and we have observed larvae of hover flies feeding on crapemyrtle bark scale. If these two groups of biological control organisms are exposed to lethal or sublethal doses of neonicotinoids in the flowers, it may result in fewer larvae (flies) or predation events on

treated trees. Since levels in the pollen are relatively high in the following spring, there may be multiple year effects on beneficials and pollinators from a single, legally applied insecticide treatment.

Hover flies and bees which can consume honeydew from scale insects may also be exposed to insecticides that pass through sap sucking insects feeding on the trees (Calvo-Agudo et al. 2019). This is possible considering our laboratory data. Some application timings of each neonicotinoid insecticide in this study reduced the growth of CMBS but were not lethal. The CMBS were growing at a slower rate, but were still feeding and capable of passing ingested residues onto leaves as honeydew where pollinators or natural enemies may be exposed. These two scenarios could result in a resurgence of CMBS on treated trees because of a negative impact on beneficials.

The spread and ensuing control of crapemyrtle bark scale using systemically-applied neonicotinoids like imidacloprid and dinotefuran can cause harm to the visiting pollinators of crapemyrtle. Effective control methods for crapemyrtle bark scale that reduce effects no non-target insects like pollinators are needed to preserve the economic and ecological value of crapemyrtle and pollinator health. To mitigate risk to visiting pollinators, utilization of systemic pesticides with relatively low toxicity to bees, the lowest effective application rates, and foliar spray application as opposed to systemic application may be used to reduce residues in nectar and pollen (Cowles and Eitzer 2017). Alternate methods of crapemyrtle bark scale control, including recruitment of natural enemies, is currently being examined and may be an efficient and bee-friendly means of CMBS control in the future.

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Appendix 1: Images

Figure 1. Colony tree heavily infested with crapemyrtle bark scale.

Figure 2. Crapemyrtle infested with crapemyrtle bark scale, heavily covered with sooty mold.

Figure 3. Crapemyrtle bark scale on infested branch in the lab.

Figure 4. Female crapemyrtle bark scale and eggs under scale covering.

Figure 5. Male crapemyrtle bark scale under pupal covering.

Figure 6. Experimental *Lagerstroemia* x 'Natchez' crapemyrtles at the Hubbard Center for Advanced Science, Innovation and Commerce.

Figure 7. Honey bee visiting dimorphic anthers of crapemyrtle flower.

Figure 8. Example of "breast height" or 1.37 m on one of our test trees.

Figure 9. Collecting pollinator samples on Auburn University's campus.

Figure 10. Soil injection using CO_2 sprayer.

Figure 11. CO_2 tank and soil injector rod.

Figure 12. Crapemyrtle leaves at mouse-ear stage.

Figure 13. Collecting flowers for pollen samples at AU Turf Research Unit.

Figure 14. Pollen aspiration process.

Figure 15. Mortality test setup.

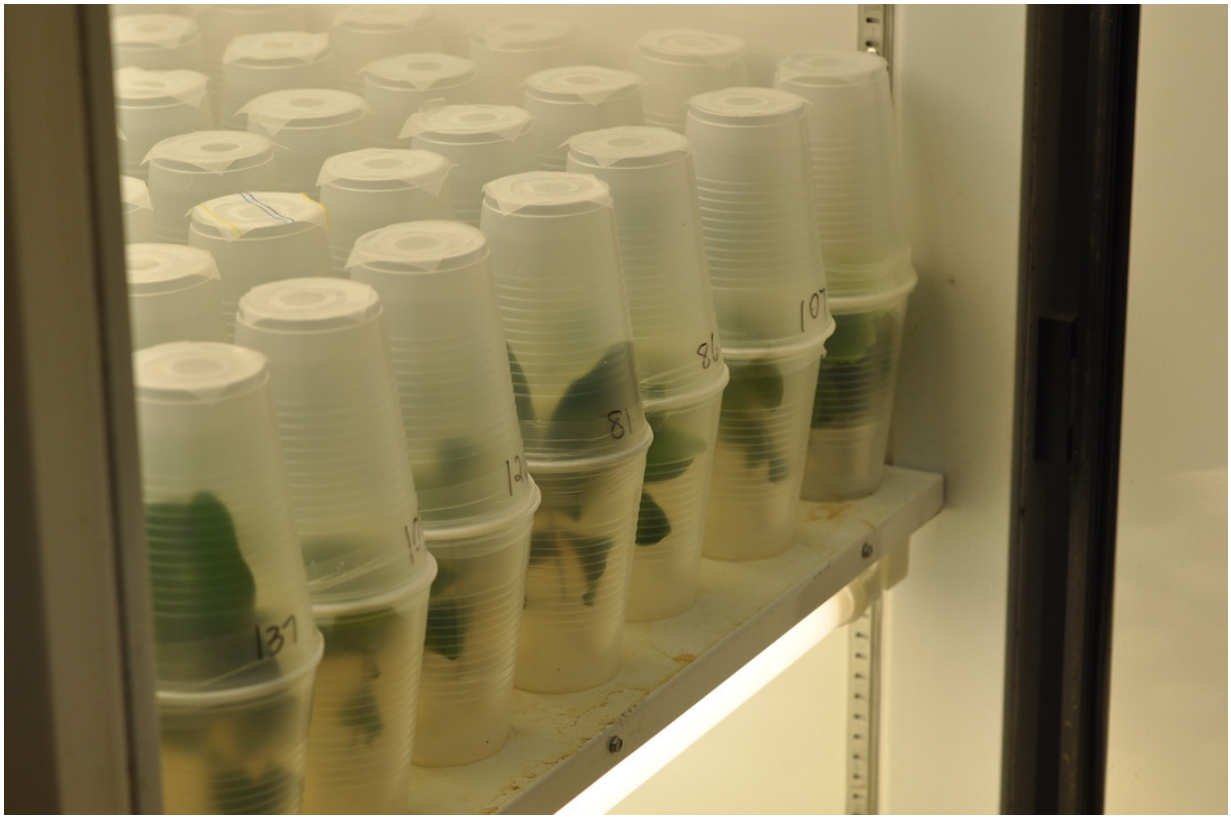
Figure 16. Mortality test containers in growth chamber.

Figure 17. Infested leaf midveins for crapemyrtle bark scale mortality tests.

Figure 18. Crapemyrtle bark scale infestation of crapemyrtle cutting.

Figure 19. Marking a scale for crapemyrtle bark scale growth analysis.

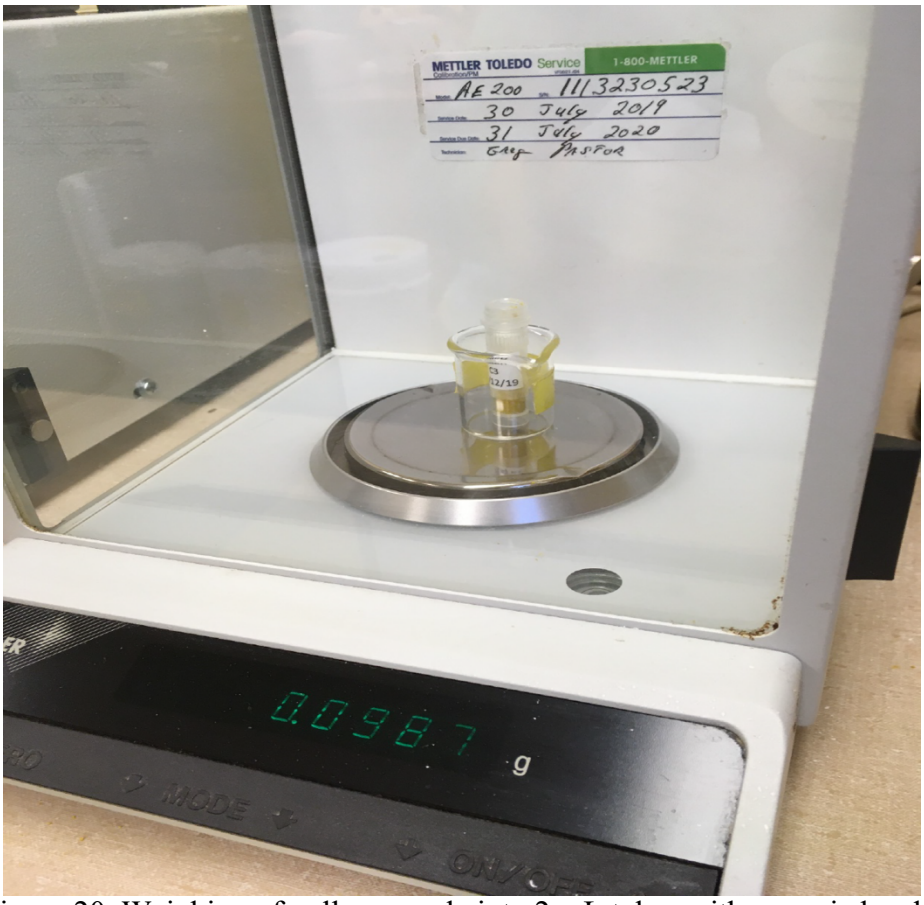

Figure 20. Weighing of pollen sample into 2 mL tubes with ceramic beads.

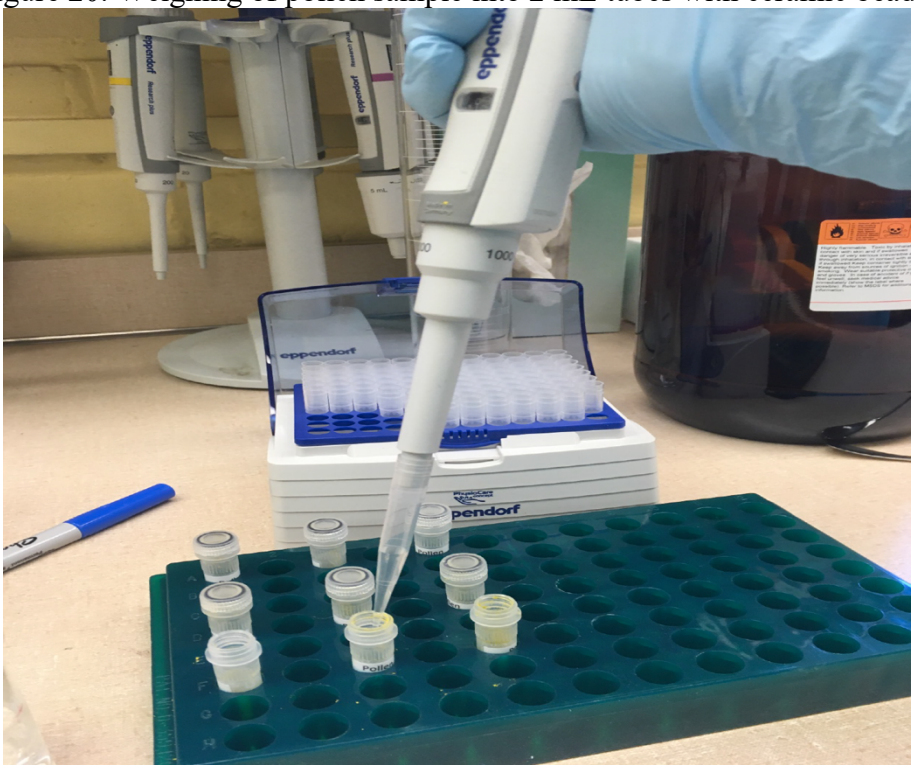

Figure 21. Addition of water to hydrate pollen prior to extraction.

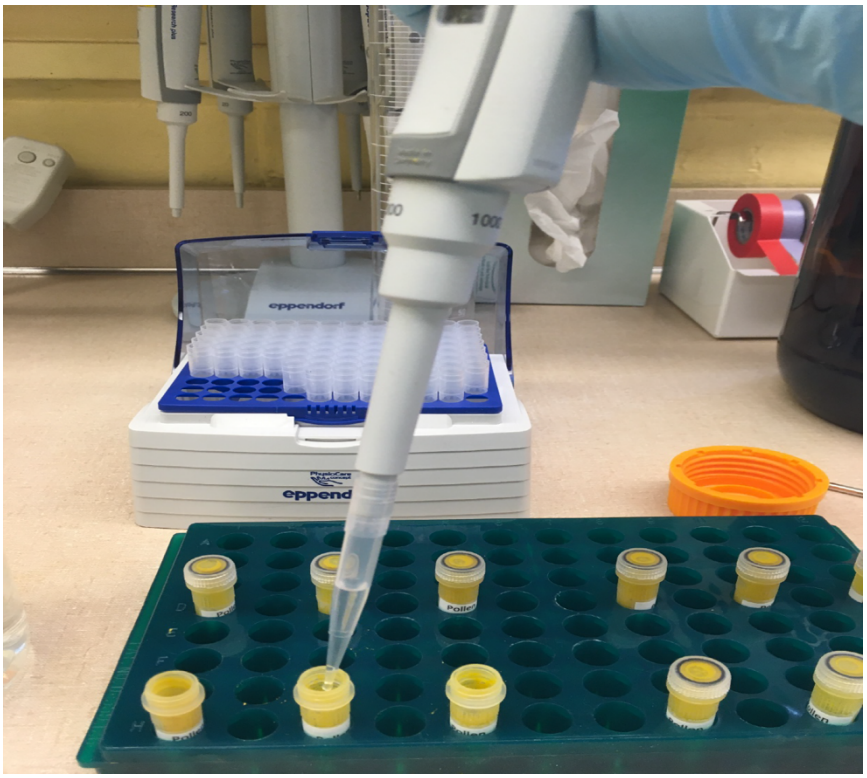

Figure 22. Addition of acetonitrile to mixture.

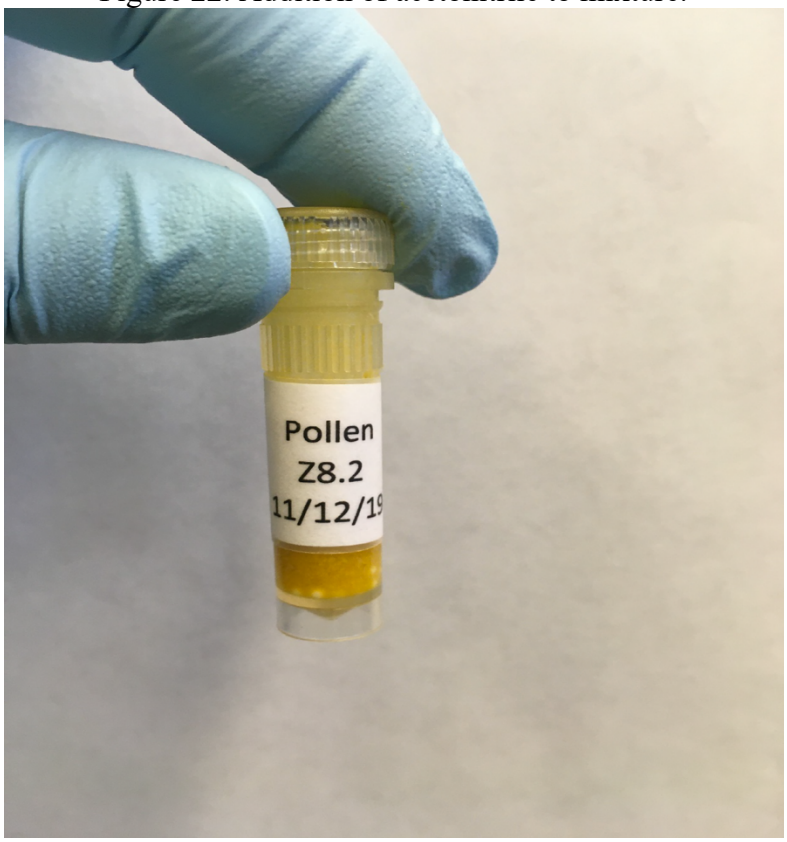

Figure 23. Water, acetonitrile, and pollen emulsification after mixing.

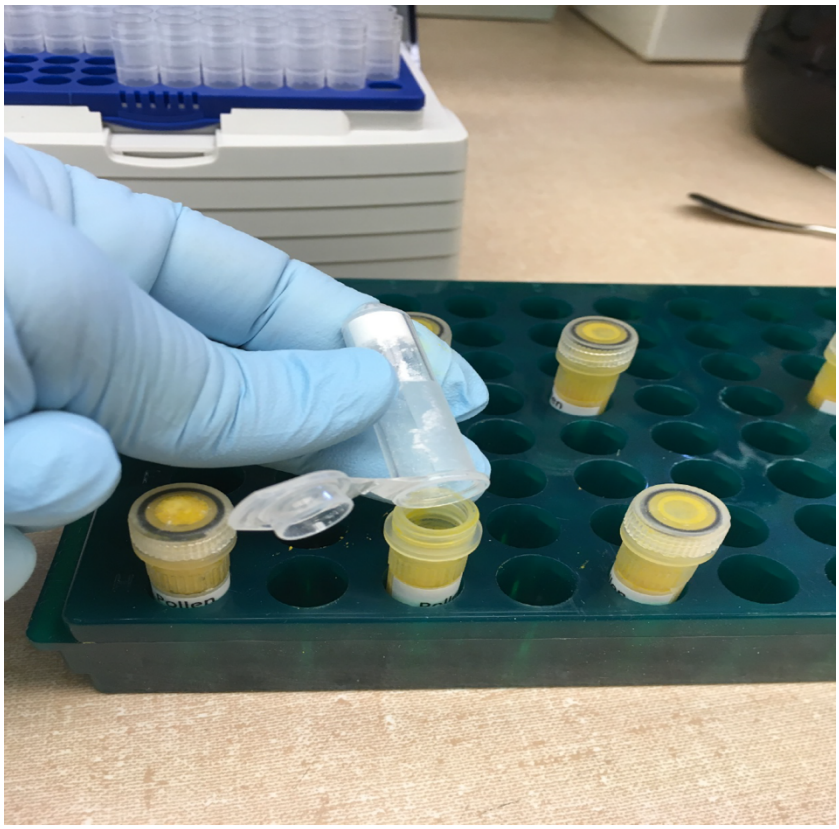

Figure 24. Addition of QuEChERS extraction salts.

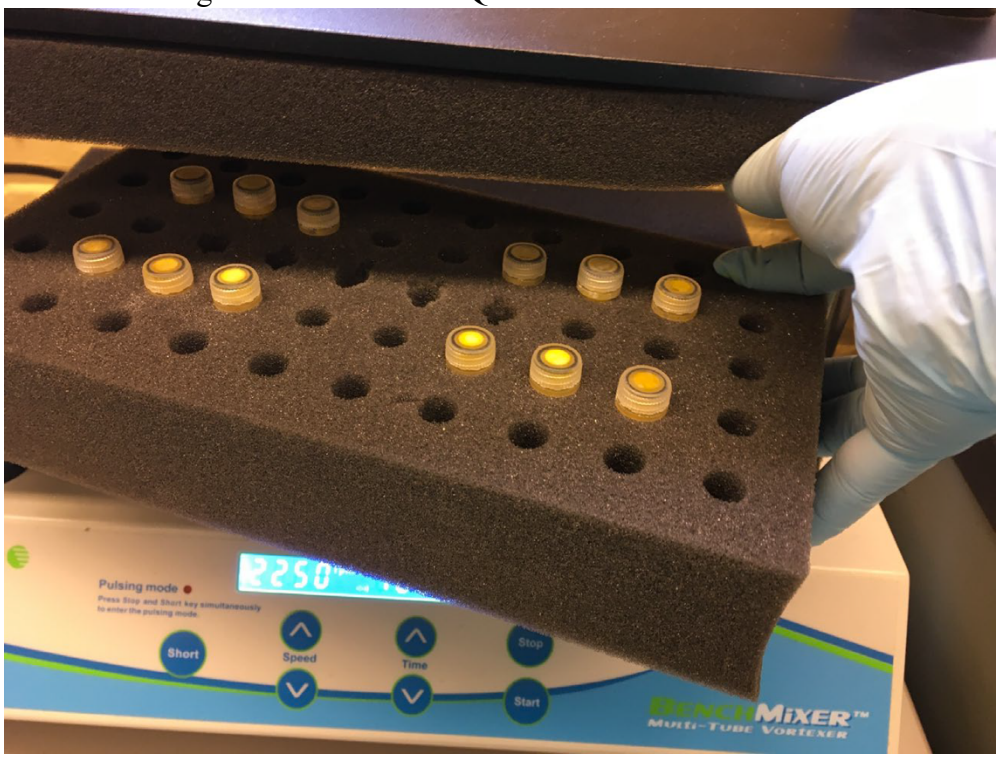

Figure 25. Additional shaking of tubes after addition of QuEChERS extraction salts.

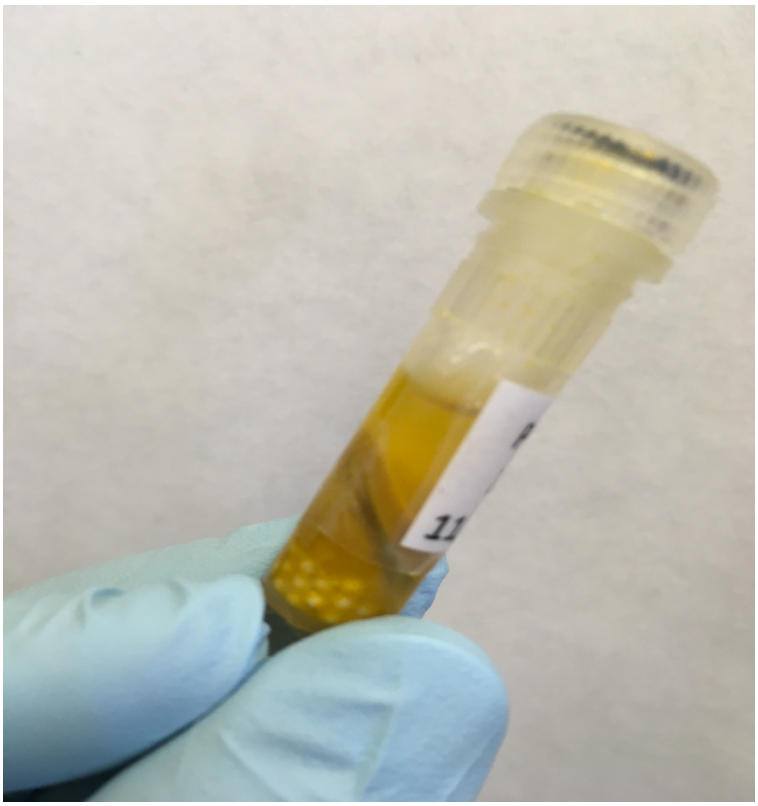

Figure 26. Note the presence of ceramic beads and the upper layer of acetonitrile containing extracted analytes after mixing.

Figure 27. Removal of acetonitrile supernatant.

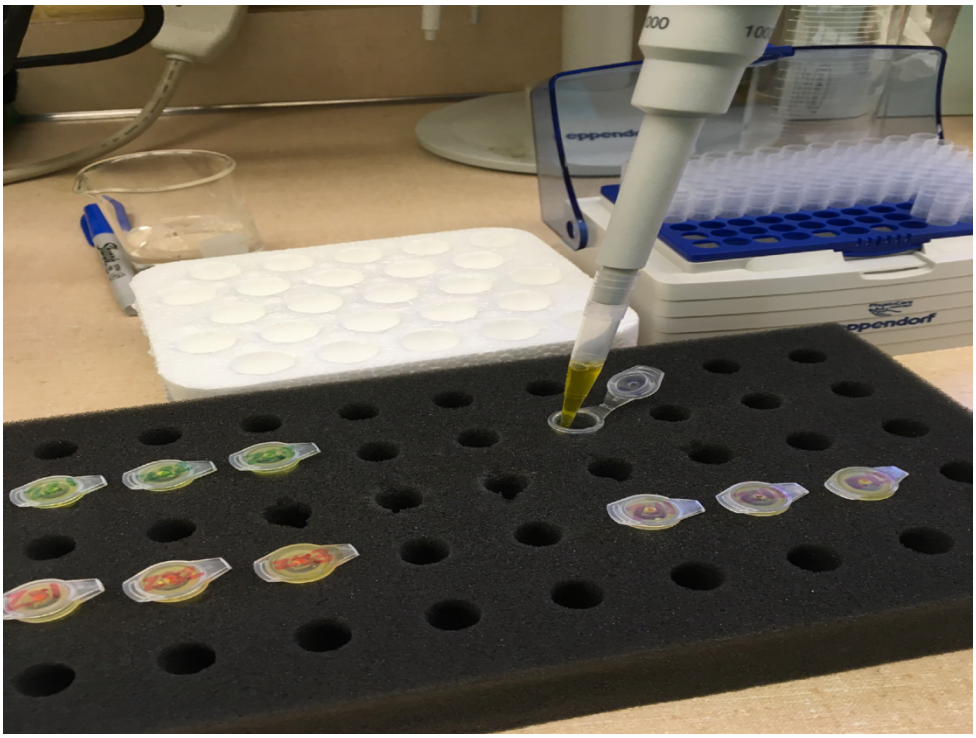

Figure 28. Transfer of supernatant to dSPE tubes for pigment cleanup.

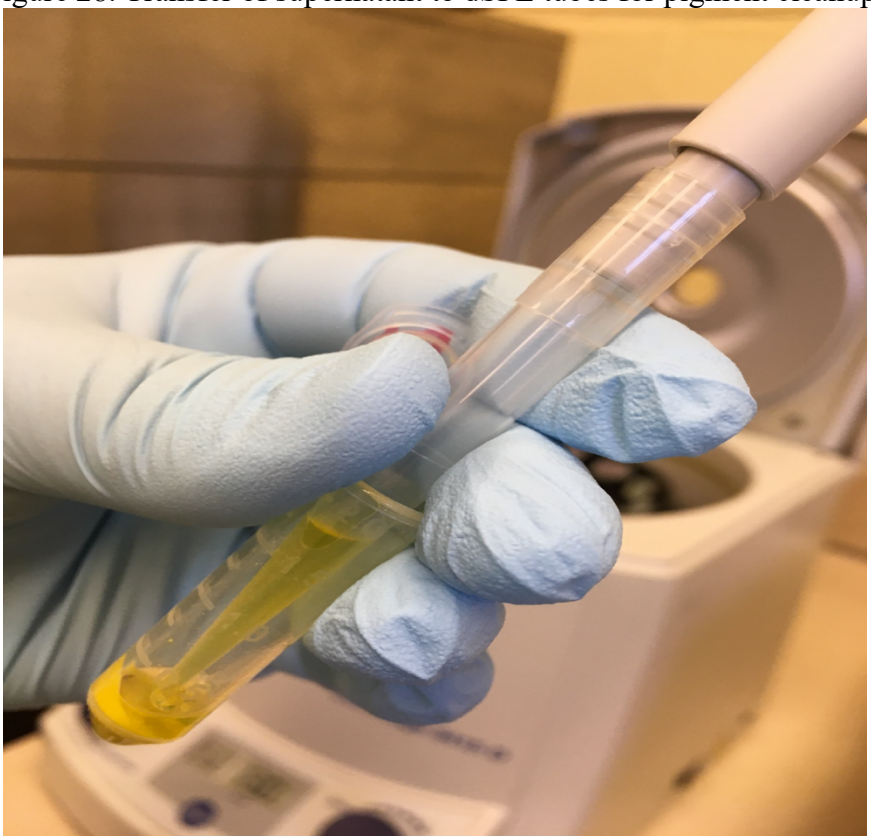
Figure 29. Removal of acetonitrile supernatant.

Figure 30. Transfer of supernatant to glass LC-MS vials for storage prior to injecting.

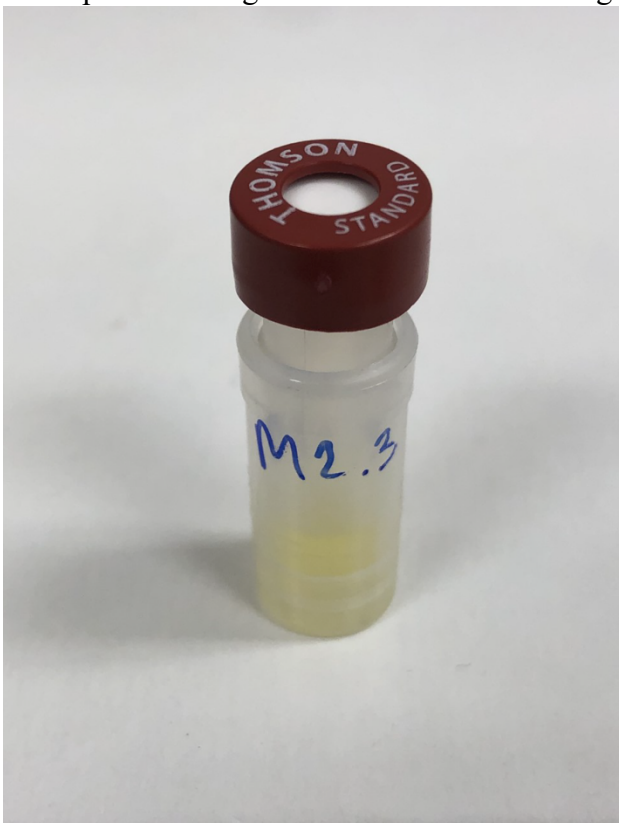

Figure 31. Filtered pollen extracts ready for analysis by LC-MS.