DISSERTATION

INVESTIGATION INTO POTENTIAL NON-TARGET EFFECTS FROM THE USE OF EMAMECTIN BENZOATE TRUNK INJECTIONS FOR THE MANAGEMENT OF EMERALD ASH BORER (COLEOPTERA: BUPRESTIDAE)

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ABSTRACT

INVESTIGATION INTO POTENTIAL NON-TARGET EFFECTS FROM THE USE OF EMAMECTIN BENZOATE TRUNK INJECTIONS FOR THE MANAGEMENT OF EMERALD ASH BORER (COLEOPTERA: BUPRESTIDAE)

A potentially significant source of increased conflict in Colorado involving insecticide use in urban areas and honey bees (*Apis mellifera* L.) recently emerged with the establishment of the invasive species emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). Detected in Colorado in 2013, numerous concerns were quickly raised about potential non-target effects from the enormous increase in insecticide use on urban trees in the state that are used to manage this insect. A series of studies were conducted to address some of these and are presented in three chapters.

Chapter I, focuses on the types of pollen that honey bees collect from urbanized sites in northern Colorado during the onset of flowering during late winter (early March) through mid-spring (early May), giving particular attention to the incidence of pollen collected by honey bees from ash (*Fraxinus*), which flowers at this time. This project found that some ash pollen is collected, averaging 9.9 percent of the total, during the earliest period when pollen sources first become available, in March and when green ash (*Fraxinus pennsylvanica* Marshall) is in flower. Incidence of ash pollen collection then dropped greatly, when a wider range of alternative pollen sources become available in the later blooming species white ash (*Fraxinus americana* L.). Important pollen sources were identified with early blooming *Acer* representing a very high proportion of pollen collected during late winter/early spring. Other notably common sources of pollen in samples included *Prunus*, *Salix*, and Brassicaceae. These first studies can also be used

in plans to improve availability of early season pollen resources for honey bees and to identify plants that bloom coincident with ash and can provide alternative pollen for honey bees during periods of ash bloom.

Chapter II examined whether there would be effects on decomposition of senesced green ash leaves collected during leaf fall from trees previously treated with trunk injections of insecticides (emamectin benzoate, azadirachtin). These involved a series of studies, using litterbags conducted over four years. There was no difference in leaf decomposition in the first two years, when litterbags had a mesh size (5mm) large enough to allow access by earthworms and they were shallowly buried; all leaf matter was completely degraded regardless of insecticide history. Subsequent trials in the following two years used litterbags with a smaller mesh size (2mm) designed to exclude earthworms but allow access by microarthropods involved in decomposition of leaf litter (e.g., Collembola, Oribatida). In addition, differences in litterbag placement (surface, shallow burial) and length of field exposure (90 days, 150 days) were included in the last two study years, which used foliage from two sites. In some, but not all, studies there were significant reductions (compared to the untreated control) in leaf area loss in litterbags of leaves collected from trees with a history of use of emamectin benzoate (TREE-äge) and azadirachtin (Tree-Azin), compared to the untreated control.

Chapter III measured levels of emamectin benzoate residues present in different plant tissues collected from green ash: senescing leaves at leaf fall, flowers, and pollen. All samples of senescent foliage collected in October 2018, approximately four months after emamectin benzoate application, had detectable residues of emamectin benzoate but at levels never exceeding 1.1ppb. Sample collections of the trees made the previous season (October 2017), when a period of 16 months had passed since the insecticide application, detected far lower

levels of residues. Levels of emamectin benzoate appearing in flowers was lower than that appearing in foliage and was detected only in 8 of 22 samples at the levels of quantification allowed in this analysis (0.28ppb). Among the 60 samples of pollen collected from trees that had been treated with emamectin benzoate, emamectin fragment ions were not detected at the Limit of Detection (LOD) attained with this analysis (0.1ppb).

Together, these studies help answer several questions regarding potential non-target effects resulting from certain insecticide uses involved in management of emerald ash borer. These can be used to help in risk assessments and in ways to mitigate potential non-target effects.

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DEDICATION

This thesis is dedicated to my three children, Khalid, Fahad, and Munirah Alogail and my husband Talal Alogail, who have withstood their life circumstances with grace, humor, and compassion. There is not a day that goes by that I have not loved you from the bottom of my heart.

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SUMMARY

A study was conducted was conducted during 2014 and 2015, in coordination with backyard beekeepers, to identify the types of pollen collected by honey bees (*Apis mellifera* L.) in urbanized areas of northern Colorado. The focus of the study was on pollen collected during the earliest period of pollen availability (March-April), and there was particular attention given to incidence of pollen collected from ash (*Fraxinus*) as this is the host of emerald ash borer, *Agrilus planipennis* Fairmaire, an invasive species recently established in the study area that now regularly receives applications of systemic insecticides for its management. During the study period pollen from a wide range of plants were found to be collected used including 28 plant families and at least 38 genera. During the very earliest period of collections (March 2-22), pollen from trees that are wind pollinated made up over 72 percent of the collected pollen including Sapindaceae (*Acer*), Oleaceae (*Fraxinus*, *Forsythia*), and Betulaceae (*Alnus*). Of these pollen from early blooming *Acer* was predominantly collected, comprising 61.6 percent of the total pollen brought into hives during this period. As spring bloom progressed a much more diverse range of pollen was collected with *Prunus*, *Salix*, and Brassicaceae being taxa that were most common in samples. No pollen from *Fraxinus* was observed in 2014 samples. In 2015, most (85%) collections of pollen from *Fraxinus* occurred during the first four weeks (8 March-4 April) when only the early flowering species *F. pennsylvanica* (green ash) was in flower and averaged 9.9 percent of total pollen collected during this time. *Fraxinus* pollen collections then

dropped greatly, when later flowering *F. americana* (white ash) was in bloom and an abundance of alternative pollen sources became available. The identification of pollen sources used by honey bees in these sites can be used to help conserve/promote important early season sources of pollen used by honey bees, assess potential exposure of honey bees to pesticides associated with *Fraxinus*, and to develop strategies to reduce *Fraxinu*s pollen collection by increased use of alternative pollen sources coincident with *Fraxinus* bloom.

INTRODUCTION

Plant pollen is critical food resource required by all of the 16,000-plus species of bees that occur worldwide (Iwanami et al. 1988, Michener 2007), not including the oil collecting bees (Buchman 1987), and for all of the 946 species present in Colorado (Scott et al. 2011). Aside from sources of carbohydrates, usually provided by floral nectar and, less commonly, honeydew, pollen is the source of essentially all other nutrients bees require.

The importance of pollen in the nutrition and health of bees has been best studied with the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), and is reviewed by Brodschneider and Crailsheim (2010). The honey bee is the world's most important managed pollinator and in the United States it is critical to the production of many important fruit, nut and fruiting vegetables (Morse and Calderone 2000), particularly those that originated from areas where the honey bee (Africa, Europe, Middle East) or other *Apis* species (Asia) are native (Han et al. 2012). Morse and Calderone (2000) provided an estimation of the added value honey bee pollination provided to agricultural crops in the United States of $14.6 billion/year, a figure that would be much higher today (Calderone 2012). In Colorado, the most valuable crops that are highly dependent on honey bee pollination are pome fruits (apple pear) and stone fruits (peach, nectarine, cherries).

Pollen is the only natural source of protein and lipids used by honey bees, and also provides many water soluble vitamins and minerals. The availability of pollen to honey bees, and the quality of the pollen, can have a wide range of effects acting on larvae, adults, or the overall functioning of the colony (Brodschneider and Crailsheim 2010).

The greatest proportion of pollen is used in the brood rearing (Herbert et al. 1970). Most all of the pollen is processed within the colony before it is fed to larvae. The

majority is ingested by nurse bees that tend and feed the larvae, converting it to a highly nutritious jelly produced in their hypopharyngeal glands and then provided as larval food (Moritz and Crailsheim 1987). Other pollen is provided to the developing larvae in the form of "bee bread" a mixture of pollen, regurgitated nectar, and glandular secretions of adult bees. This is further colonized by microorganisms that improve the nutritional value, compared to pollen (Gilliam 2007, Dharampal et al. 2019). Babendrier et al. (2004) estimated that less than 5% of the protein provided to honey bee larvae was directly provided in the form of pollen.

Relatively little pollen is stored in a honey bee colony, as compared to honey, and it can be rapidly depleted when new sources are not available (Schmickl and Crailsheim 2002). Variations in the availability of pollen resources, both quantity and quality, can have myriad effects that may vary through the season (DiPasquale et al. 2016). Peak brood rearing, and related pollen consumption, typically occurs in March and April (Anonymous 2019), stimulated by the first appearance of floral resources. At this time pollen stores collected the previous season may become critically depleted, particularly if poor weather delays the onset of spring pollen sources or freezing events kill spring flowers. Supplemental feeding of pollen or pollen substitutes is a practice that can provide for protein needs at this time (Seshadri et al. 2019). In Michigan, it is recommended that pollen be fed around mid-February to early March and Huang (2018) further comments that "bees will be desperately seeking protein sources in April and May when it is warm enough, but few flowers are open yet".

For decades there have been numerous studies that have identified plants that are of value to honey bees in different regions of the world. These have generated various lists of plants and among those found in North America these lists may cover the continent (Anonymous 2020a), countries (Oertel 1980, Lindtner 2014, Anonymous 2017), or regions within a country (Tew

1998, Sanford 2003, Anonymous 2020b). In addition, in recent years there have been countless publications produced about ways to generally improve habitat for pollinators that are not specific to the honey bee. Examples of ones adapted to be Colorado include some produced by Colorado State University Extension (Arathi 2018) and the Xerces Society (Anonymous 2020c).

A limitation of these types of lists is that they generally do not distinguish between whether the plant use by the pollinator is as a source for nectar, pollen, or both. Most lists provided for honey bees focus on nectar plants and there have been far fewer that have looked at sources of pollen. The latter requires quantitative identification of pollen, a tedious process but is an essential method to understand in studies of pollination biology (Kearns and Inouye 1993; Wilson et al. 2010; Forcone et al. 2011; Girard et al. 2012; Cusser and Goodell 2013) as well as for use in identifying high value plants for apicultural production (Dimou and Thrasyvoulou, 2007). Notable examples of where this has been done in the United States include (Severson and Parry 1981) in Wisconsin and (Richardson et al. 2015a) in Ohio.

There have been two studies in Colorado that have attempted to identify plants that are used as pollen sources. The largest was within the thesis study of Wilson (1957), later developed into a Colorado Agricultural Experiment Stations Bulletin (Wilson et al. 1958), which provided a broad list of plants used by honey bees throughout the state, particularly as nectar sources. Subsequently there was a study by McElwey (2004) of pollen brought into hives during midsummer in northeastern Colorado.

Both of these studies, as well as those from Wisconsin and Ohio were conducted in agricultural areas. Only within the last few years has there been attention to plants that are utilized by pollinating insects in urbanized areas. Urbanized sites, while greatly disturbed by

human activity, may be designed in ways that improve compatibility with some species, including certain bees (Rosenzweig 2003; Winfree et al. 2009).

Studies are often based on field observations of pollinator visitation to plants, of which the study by Mach and Potter (2018) is particularly notable. Most studies collect data on all bees, usually including honey bee along with several genera of common native bees. Some studies are further restricted by limiting study to pollinators visiting native plants (Mason 2018). Recently, Lau et al. (2019) produced the first study that quantified pollen use by honey bees in urban and suburban areas, with measures provided from four states (California, Florida, Michigan, Texas).

Not only can identification of plants that have high-value as pollen sources for honey bees be used to improve honey bee habitat, but it can also be used to identify plants where pesticide use on the plant would result in potential hazard to honey bees. Conflicts between insecticide use and accidental poisoning of honey bees has a very long history, with the subject first reviewed by (Shaw 1941). Included as a first report was an 1881 use of the insecticide Paris green (copper acetoarsenite) applied to pear trees in bloom. In agricultural crops studies on potential conflicts of pesticide use and pollinators, particularly the honey bee, have most often involved fruit crops where honey bee pollination is required and key insect pests are present that often require insecticide use to manage. A summary of information widely used to guide U.S. producers on the subject for over two decades is the Pacific Northwest Extension publication *How to Reduce Bee Poisoning from Pesticides*, most recently revised by Hooven et al. (2016). Until recently similarly complete information has been lacking regarding hazards of specific insecticides adapted for use in urbanized areas where pesticides are used most frequently on shade trees, turfgrass and gardens. However, interest in this subject has greatly increased in

recent years and such publications have been produced by some states and regions (Andrews and Rose, 2018; Smitely et al. 2019).

A potentially significant source of increased conflict involving insecticide use in urban areas and honey bees recently emerged in North America with the establishment of the invasive species the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). First detected in Michigan in 2002 (Herms and McCullough 2014) this insect has since spread so that it is presently known to be present in 35 states and five Canadian provinces (Emerald Ash Borer Network, 2020). First detection of the insect in Colorado was confirmed in September 2013 in Boulder. Since that detection Colorado has remained the only western state with confirmed infestations of emerald ash borer, with the closest infestations currently present in extreme eastern Nebraska and Kansas. Within Colorado, the known distribution of emerald ash borer has since spread about 40.2 kilometers so that it presently known from six Boulder County communities and, in 2019, was found outside the Boulder County in areas of rural Larimer County, Broomfield , the Adams County portion of Westminster, Lakewood and Larimer county immediately adjacent to Boulder County (Colorado Department of Agriculture 2020).

Feeding by the larvae of the emerald ash borer has the ability to kill ash trees (*Fraxinus* species), even those in good physical condition (Herms and McCullough 2014). Studies projecting economic costs to communities resulting emerald ash borer are reviewed by Herms and McCullough (2014) with variable figures, but all are in the billions of dollars ($ US). In Colorado it has been estimated that the future costs of emerald ash borer in the state could exceed one billion dollars (Colorado State Forest Service 2016). These calculations largely consider only the costs of treatment and/or removal and replacement of high-value trees

(Hauer and Peterson 2017). There are many ecological and social costs associated with the emerald ash borer invasion of North America (Herms and McCullough 2014).

Treatments for emerald ash borer involve the use of insecticides (Herms et al. 2019). Four insecticides have been found effective for helping to manage emerald ash borer: azadirachtin, emamectin benzoate, dinotefuran, and imidacloprid (Herms et al. 2019, Cranshaw 2020). All are insecticides that can move systemically in the plant. For emerald ash borer control azadirachtin and emamectin benzoate must be injected into the trunk of the tree using specialized equipment. Dinotefuran and imidacloprid are applied as a soil drench/injection to be picked up by plant roots. Dinotefuran can also be used as a spray onto the bark, where it may move into through thin areas of the bark and then transported within the tree.

Treatments of imidacloprid and dinotefuran must be made annually to be reliably effective. Trunk injections of azadirachtin are recommended to be made on an annual or biannual schedule. Emamectin benzoate has proven to be the most effective of the emerald ash borer treatments, an application consistently providing control for two years and more recent evidence, and modifications in rates, suggesting three-year treatment intervals may be reliably effective.

Where emerald ash borer becomes established, treatments for its control result in an enormous increase in insecticide applications to trees in urbanized areas. Ash trees – primarily a mixture of green ash, *Fraxinus pensylvanica* and white ash *F. americana* – make up between 15 and 20 percent of the urban forest in Colorado (Colorado State Forest Service 2016), a typical figure for the composition of ash in the urban forest canopy of much the northern half of the country.

No published figures on the increased amount of insecticide use that results from emerald ash borer but these can be estimated. For example, in Denver there are an estimated to be 1.45 million ash trees and at least 98,000 in Boulder (Colorado State Forest Service 2016). If the assumption is made that decisions by the various tree owners of ash trees (municipalities, businesses, homeowners) are to save only one-third of the trees through use of insecticides, and that the treatments applied could be made every other year, that would result in nearly one quarter million additional trees being treated with insecticide in Denver each year and some sixteen thousand in Boulder. The actual percentage of trees treated with insecticide could easily be higher, as several economic models show insecticide treatment to be more cost effective than the alternative, which is to let the trees die from emerald ash borer injuries and then remove them (Sadof et al. 2017).

Shortly after emerald ash borer was found in Colorado and awareness developed about the implications of the potential amount of insecticide use that would be involved, numerous concerns were raised by the public. Particularly common were concerns regarding the potential of the emerald ash borer insecticide applications to harm pollinators, particularly honey bees, and effects of pesticides in leaves dropping from insecticide-treated trees (Whitney Cranshaw, personal communication, 2014). Major data gaps existed surrounding non-target effects of emerald ash borer insecticide applications, preventing definitive response to many of these questions.

Regarding exposure to honey bees and other pollinators, the only potential route of exposure to soil applied/trunk injected systemic insecticides used for emerald ash borer would be through pollen. North American *Fraxinus* species are dioecious, wind pollinated and do not produce nectar, but flowers on male trees do produce pollen in early spring. Some collection

of *Fraxinus* pollen by honey bees has been reported by Severson and Parry (1981) and Richardson et al. (2015a) from rural sites in Wisconsin and Ohio, respectively. Studies of *Fraxinus* use in urbanized areas, where emerald ash borer insecticides are concentrated, have not been conducted.

This study was conducted to address several questions related to the types of pollen collected by honey bees in urbanized areas of Colorado. These studies address several different issues affecting honey bee health in these sites and include five objectives:

1) identify sources of pollen used by honey bees in Colorado during the period overwintered bees have first access to pollen sources in late winter and early spring;

2) identify plants that have high value to honey bees during this period so that their use can be promoted in planned improvements of habitat for honey bees;

3) identify plants that are highly used by honey bees so that pesticide use on those plants can be avoided or modified to reduce adverse effects on honey bees;

4) determine the extent of use of *Fraxinus* pollen by honey bees to help assess the potential exposure of honey bees to pesticides that may be associated with ash pollen; and

5) identify flowering plants that bloom coincidently with *Fraxinus* in urbanized sites and produce pollen of high value to honey bees, which may be promoted to alter the amount of *Fraxinus* pollen collection by honey bees.

MATERIALS AND METHODS

Pollen Sample Collection

Pollen samples of this study were acquired with the cooperation of local beekeepers located in urbanized areas along the northern Front Range area of Colorado. During fall

2013 the Colorado Beekeepers Association was approached to help find beekeepers that would be willing to periodically collect pollen samples from their hives. In early 2014 six beekeepers had responded to this request and ultimately five of these were able to participate in the first study year. The following year local beekeepers were again approached and a total of seven were able to provide pollen samples in 2015, including four of those that participated in 2014. The hives used in the study provided by the cooperators were located in Boulder, Fort Collins, Longmont, Loveland, and Denver.

For the collection of pollen, each cooperator was provided with a Sundance Bottom Mounted Pollen Trap (Betterbee, Greenwich, New York) and instructions for its use. These pollen traps are designed so that when the trap is activated ("turned on") returning foragers must pass through narrow openings that dislodge most of the pellets of pollen being carried on the corbiculae ("pollen baskets") on the hind legs of foragers. In the pollen trap used in this study, the pollen drops to a tray at the bottom of the hive, which can be easily pulled out and the collected pollen removed.

The instructions provided the beekeeper cooperators were to make a weekly collection of pollen, by activating the pollen trap for a 24-hour period. They were asked to initiate collections as early in spring as possible, after pollen was first being brought to the hive in late winter/early spring and to continue it until peak spring bloom had passed in late May/early June. The pollen collected by the pollen trap is a mixture of most all the pollen being brought into the hive during the collection period. During each collection, the beekeepers were asked to provide a 10 gram or greater sample of the mixed pollen, providing a representative sample used for later analysis.

These samples were then refrigerated by the cooperators until they could be collected and transported to the laboratory in Fort Collins. These samples were refrigerated until later being processed for analysis of pollen type. A total of 133 samples were collected in 2014 from five location; 168 samples from seven locations were available in 2015.

Establishing a Pollen Reference Library

To assist in the identification of pollen collected by honey bees, a pollen reference library was developed. This was done by making weekly surveys to detect all species of flowering plants during late winter/spring 2014 and again in 2015, beginning with the observance of the first plants in bloom. In the field, flowers from each sampled plant were photographed and either identified at the time of collection or later. During collection flowers were picked and those from each plant were stored in a clean vial to be returned for immediate processing onto slides or were temporarily refrigerated until processed.

Pollen for the reference library were mounted on microscope slides in a stained gel media following techniques described by Kearns and Inouye (1993), Jones (2012) and Larson et al. (2014). All equipment used in the transfer and mounting of the pollen samples was rinsed ETOH in between each use and stored in pollen-free areas when not in use. Preparation of the staining gel (Basic Fuchsin Jelly) involved mixing 20 g gelatin (Knox Unflavored Gelatin®) in 70 ml distilled water. This was allowed to stand for 5 minutes, to allow the gelatin to absorb the liquid, then gently brought to a boil, stirring until the gelatin was completely dissolved. This was then allowed to cool to about 50ºC then enough Basic Fuchsin (Sigma-Aldrich) stain was added to produce a color close to port (deep red-almost purple color). Upon cooling the stain solution forms a dark pink gel and this was stored in an airtight jar in at 4°C for future use.

When an individual sample was prepared, the anthers of the collected flower was pinched over a glass microscope slide to release a small amount of pollen. A small amount of the mounting media gel was then placed on top, then heated gently with a lighter to encase the pollen. After the jelly became fluid, a cover slip was placed immediately on the glass slide to avoid contamination. This preparation stained the pollen in a way that later allowed features of the grains to be visible under a light microscope. Each prepared slide was examined to confirm that it contained only a single pollen type and were then labeled with the plant family, genus and/or species names, and date of collection.

Photographs of the slide-mounted stained pollen was then digitally photographed and ultimately allowed production of a visual library of the pollen produced by the plants, which was later used in identification of pollen collected for samples provided by beekeeper cooperators. This reference library contained samples of pollen collected from 215 species of plants within 73 plant families.

Analysis of Pollen Samples Collected from Pollen Traps

From each sample provided by cooperator-beekeepers a one-gram subsample was removed for examination. This sample was then visually inspected and individual pollen pellets were separated into groups based on grain color, following methods by Barth et al. (2010) and Richardson et al. (2015a). The weight (grams) of the various color groups were recorded to determine the percentage of each color of pollen grain for that one-gram sample.

Two pellets from each color grouping were then further processed to allow for microscopic examination. These were blended and diluted in 70% ethyl alcohol (ETOH) and shaken to form a suspension as using methods of Kendall and Solomon (1973) and Jones (2012). One drop of this homogenate was then spread on a slide which was allowed

to dry. Subsequently, a small amount of jelly of the stained mounting media was added then the slide was gently heated. This encased the pollen on the glass slide, and a cover slip was then placed on top. The prepared slides were then stored in a refrigerator (~2ºC) until they could be examined, and the pollen type identified.

All identifications of the slide mounted pollen were done by visual examination using light microscopy at 400-1000X and photomicrographs (Jones 2012). There are alternate methods of microscopic examination of pollen (Jones and Bryant 2007), including use of SEM, which have differences in the ability to better distinguish taxa and cost. The approach used in this study was to use light microscopy and to cross-check identifications with reference slides of pollen prepared during production of a pollen library. Pollen grain characteristics need for identification were normally visible using fine focus. Since the morphology and dimensions of pollen grains vary, a combination of photomicrographs and direct observation of the pollen grains through the light compound microscope was found most useful for identification.

Field Surveys of Bloom Periods of Spring Flowering Plants

Over four years (2015, 2017-2019) field surveys were conducted in Fort Collins to determine the timing of observed bloom of all flowering plants found present in the during the late winter-early spring period that was the focus of this study. These provided information on the occurrence and progression of all flowering plants during the period of pollen collections.

Field surveys involved between 3 and 5 individuals/season that collectively made weekly observations of all plants in flower. These were a combination of informal surveys, noting plants encountered during the course of normal activities within the city and by some dedicated weekly surveys of sites where multiple species of plants were present (Gardens at Spring Creek, Plant

Environmental Research Center). These weekly reports were collated to create lists of all plants found in flower during each week of the study during the years 2015, 2017-2019.

RESULTS

In 2014, there were 28 samples of pollen provided by five beekeeper cooperators during the early spring period of 16 March – 3 May 2014. In 2015, the number of samples that became available almost doubled, to 48 samples, provided by seven beekeeper cooperators during the nine-week period of March 2-May 3. During this period honey bees collected a wide range of pollen, which included both wind pollinated and insect pollinated plants (Table 1.1).

Only a single collection was provided on the first week of March (March 2-8, Site G, Fort Collins, 2015). This sample was almost entirely (94.8%) comprised of pollen from a single species, silver maple, *Acer saccharinum* L. The remaining pollen was from ash (*Fraxinus*). Although the species could not be distinguished from the pollen samples, green ash, *F. pennsylvanica* Marshall is the only species that may be in bloom this early in the season. Both of these are wind pollinated (anemophilous) plants.

In the period of the second week in March (March 9-15), five samples, all from 2015, were made available. Maple, particularly silver maple, again predominated, being found in all five and comprised an average of 56.1% of the total. Among the samples the percentage of silver maple pollen ranged five-fold, from 16.5-81.4 percent. A second *Acer* species, red maple, *Acer rubrum* L., was collected at two of the sites, at percentages of 2.0 and 9.5 percent, respectively.

Table 1.1 Incidence of pollen types in pollen samples collected from pollen traps placed on beehives located in urbanized sites in northern Colorado (Boulder, Larimer, Denver counties). Reported samples are for the nine week period (2 March – 3 May) following the onset of plants in flower visited by honey bees during 2014-2015. Results are the proportion of pollen types, by weight, identified for each collection by light microscopy.

Date	Family	Genus	Species	Site[a]											
				A		B		C		D		E	F	G	H
				2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2015	2015
March 2- 8	Oleaceae	*Fraxinus*												5.2	
	Sapindaceae	*Acer*	*saccharinum*											94.8	
March 9 - 15	Asteraceae	*Taraxacum*	*officinale*											4.0	
	Iridaceae	*Crocus*							2.6				6.3		14.5
	Liliaceae	*Tulipa*							5.6						
	Oleaceae	*Fraxinus*									3.7			21.6	23.4
		Forsythia												18.3	
	Rosaceae	*Prunus*							7.3		14.0		10.3		9.0
		Pyrus													2.1
	Salicaceae								1.0					39.6	3.8
	Sapindaceae	*Acer*	*saccharinum*						64.7		70.8		81.4	16.5	47.2
			rubrum								9.5		2.0		
	Violaceae	*Viola*							18.8		2.0				
March 16 – 23	Amaryllidaceae	*Narcissus*			12.0									4.2	
	Asteraceae	*Taraxacum*	*officinale*										5.4		
	Betulaceae										33.0				
	Fabaceae						9.1								
	Iridaceae	*Crocus*							1.5						
	Liliaceae	*Tulipa*							7.5						
	Oleaceae	*Forsythia*			11.0				1.0					3.4	
		Fraxinus			3.0		15.1						8.8		

	Rosaceae	*Prunus*			6.0			11.0	8.2				10.0	17.0	
	Salicaceae				35.1		37.3	33.5	4.5		15.0			10.8	6.6
	Sapindaceae	*Acer*	*saccharinum*		24.4		37.4	55.5	73.6		51.0		63.8	63.2	86.7
			Other										8.0		5.0
	Ulmaceae	*Ulmus*											4.0		
	Violaceae	*Viola*			7.2				3.7						
	Other				1.3		1.1				1.0			1.4	1.7
March 24- 29	Amaryllidaceae	*Narcissus*		1.2	22.4				1.1						
	Asparagaceae	*Muscari*		10.7											
	Asteraceae	*Taraxacum*	*officinale*	2.5	6.1	1.0	1.0	9.0	4.1		2.2	4.4	2.3	4.2	
	Berberidaceae	*Mahonia*	*aquifolium*				1.3								
	Brassicaceae	*Chorispora*	*tenella*	22.5		7.3		61.8	10.9					27.3	
		Other		4.0		45.1			75.4		24.4	48.2	73.2		
	Iridaceae	*Iris*		10.4	13.4	26.0	31.3		8.1			24.7	3.0	10.0	
	Liliaceae	*Tulipa*			12.0		2.1								
	Oleaceae	*Foresteriera*		12.6	3.0					7.0		4.4		13.0	
		Fraxinus					11.1						7.0		
	Onagraceae	*Primula*		6.6											
	Rosaceae	*Prunus*		5.1		1.6	13.2			65.0	25.5	5.0	11.0	33.0	
	Salicaceae					17.0	33.8	28.0		26.5	11.8	9.4			
	Sapindaceae	*Acer*	*platanoides*		3.8		4.1		1.4		34.1	2.6	3.5	10.0	
	Violaceae	*Viola*		23.1	39.3		2.1								
	Other			1.3		2.0		1.2		1.5	2.0	1.3		2.5	
March 30 - April 5	Asteraceae	*Taraxacum*	*officinale*		9.0	15.0	7.0	10.0	1.0		22.4	17.0			
	Brassicaceae				26.6	70.5	20.0	78.0	20.0	10.0	11.3				
	Iridaceae												8.0	15.2	
	Oleaceae	*Foresteriera*		12.1	6.0								12.0	8.0	
		Fraxinus											7.0		
	Rosaceae	*Chaemomeles*	*japonica*	14.0	26.0				11.6		3.7	8.0	4.0		

		Malus									9.0		13.0		
		Prunus		54.7	22.0	6.0	58.6	5.0	63.1	63.6	40.0	65.0	30.0	14.4	
	Salicaceae	*Salix*		7.5	9.0	7.0	8.6	5.0	3.3		11.0	9.0	14.4	22.4	
	Sapindaceae	*Acer*	*platanoides*	11.7	1.4		5.8	2.0	1.0	26.4	2.6	1.0	11.6	40.0	
	Other					1.5									
April 6 -12	Asteraceae	*Taraxacum*	*officinale*	11.0	1.0	9.0	4.0	18.3	4.0	3.0		8.4		5.3	
	Berberidaceae	*Mahonia*			2.8										
	Brassicaceae				7.0	47.0	1.3	23.0	24.2		16.2	5.0	4.7	14.6	
	Fabaceae	*Cercis*								27.4			6.3	12.0	
	Iridaceae						2.5				6.0		1.0		
	Lamiaceae	*Lamium*											6.0		
	Magnoliaceae				2.7										
	Oleaceae	*Foresteriera*		1.0	4.1						7.0		42.0		
		Fraxinus					10.0								
	Rosaceae	*Chaemomeles*	*japonica*	1.5	6.4										
		Prunus		9.3	14.7		12.0	10.4	27.4	22.0	48.0	84.2	17.0	4.6	
		Malus		13.7	40.6	24.6	50.6		41.4				21.0		
		Crataegus						11.0							
		Other			5.7	1.1	8.0			6.4	7.0				
	Salicaceae	*Salix*				15.8		10.3		5.0					
	Sapindaceae	*Acer*	*platanoides*	62.2	15.0		9.6	27.4	3.0	36.2	15.8		2.0	63.5	
	Other			1.3		2.5	2.0					2.4			
April 13 - 19	Asteraceae	*Taraxacum*	*officinale*	2.0	8.0	2.0	8.0	5.4	5.0				1.5	8.3	
	Brassicaceae			5.0	7.9	5.9	5.2					23.5	2.0		
	Caprifoliacea	*Lonicera*					4.0					11.3			
	Cupressaceae													1.6	
	Fabaceae	*Cercis*												26.5	
	Liliaceae	*Tulipa*					1.0						1.4		
	Oleaceae	*Syringa*				23.0							33.0		
	Rosaceae	*Malus*		38.7	20.5	35.0	22.0	22.0	8.5				40.6	4.7	
		Prunus		19.4	40.0	30.0	29.0	42.6	84.5				6.2	33.5	
		Crataegus		5.3	10.8		11.0								

		Aronia			7.0		5.3						5.0		
		Chaemomeles		5.5				30.0							
		Other										47.0			
	Salicaceae				4.6	3.1	2.0						4.3		
	Sapindaceae	*Acer*	*ginnala*	22.0			12.5					16.8	6.0	25.4	
	Other			2.1	1.3	1.0			2.0			1.4			
April 20 - 26	Amaryllidaceae	*Narcissus*		10.8											
	Asteraceae	*Taraxacum*	*officinale*	2.2	4.0		3.0	5.0	8.0		3.0	3.3	5.0	1.0	
	Brassicaceae				3.0		31.4		4.0		24.4	23.2		17.0	
	Fabaceae	*Cercis*		1.5	18.2					9.5	11.3	6.0	10.4	9.3	
	Fagaceae	*Quercus*								2.0		5.2			
	Iridaceae													3.7	
	Lamiaceae												1.7		
	Liliaceae	*Tulipa*		7.0			1.7								
	Oleaceae	*Syringa*		22.3	10.0	1.7	11.5		3.0				15.6		
	Papaveraceae	*Papaver*					19.7								
	Ranunculaceae	*Ranunculus*								6.3					
	Rosaceae	*Malus*			46.4		23.7		75.0				48.0		
		Prunus	other	45.5	9.0					29.3	17.4		19.3	34.4	
			virginiana		7.2	65.3		52.0	10.0		33.2	42.3			
		Chaenomeles	*japonica*					17.0							
	Salicaceae				2.2		2.3			15.4				6.3	
	Sapindaceae	*Acer*	*ginnala*	10.7		32.0	5.4	24.3		36.0	10.7	20.0		28.3	
	Violaceae	*Viola*					1.3								
	Other					1.0		1.7		1.5					
April 27 - May 3	Amaryllidaceae	*Narcissus*											1.4		
	Anarcadiaceae												26.0		
	Asteraceae	*Taraxacum*	*officinale*		5.3	1.7	4.5	1.8	1.4		1.8		14.5		
	Brassicaceae				18.0		22.0	28.3	10.4	28.0	11.0		18.5		
	Caprifoliaceae	*Lonicera*							47.5						

	Crassulaceae				3.0										
	Cupressaceae											5.3			
	Fabaceae	*Cercis*												22.0	
	Fagaceae	*Quercus*				1.5				12.0	8.0				
	Geraniaceae	*Erodium*	*chrysanthum*										8.1		
	Iridaceae	*Iris*			9.3										
	Lamiaceae												3.6		
	Magnoliaceae				10.7	21.8									
	Oleaceae	*Syringa*	*vulgaris*		6.4						13.4				
	Papaveroideae										9.5				
	Pinaceae												6.0		
	Ranunculaceae				5.0						6.0				
	Rosaceae	*Prunus*	*virginiana*	14.2	30.2	16.1	38.6	5.8	9.7						
			other	2.1								40.0			
		Malus			3.0					34.0				57.0	
		Crataegus									42.3				
	Rubiaceae	*Galium*	*odoratum*	3.2											
	Sapindaceae	*Acer*	*ginnala*	78.3	7.6	57.9	32.8	62.1	26.7	16.5	2.8	53.2	21.9	16.7	
	Salicaceae						2.1		3.0	7.5	2.5			2.3	
	Other			2.2	1.5	1.0		2.0	1.3	2.0	2.7	1.5		2.0	

[a] Sample sites: (A) Elizabeth Black, 4340 N 13th Street, Boulder CO 80304; (B) Jim Douglas, 918 Benson Lane, Fort Collins, CO 80525; (C) Kristina Williams 960 Ithaca Drive, Boulder, CO 80305; (D) Mark Coleman 2613 McKeag Drive, Fort Collins, CO 80526; (E) Tom Nagle 227 Lincoln Street, Longmont, CO 80501; (F) Eric Reiff, 3095 Wilson Court, Denver, CO 80205; (G) Bruce Brown N Loomis Ave Fort Collins, CO 80521; (H) Carolina Nyarady 1115 Sycamore Drive, Loveland CO 80538

The genus *Fraxinus* again provided the second greatest contribution to pollen collections, being found in samples from 3 of the 5 sites, ranging from 3.7% to 23.4% at the sites and in overall average of 9.7% among all sites during the week-long period. This would be from *F. pennsylvanica* (green ash), which was in bloom at this time.

An additional source of pollen collected at this time from the family Oleaceae was from *Forsythia*, appearing only in one sample but in high percentage (18.3). The value of forsythia to honey bees is *Forsythia* is a subject of some debate within the U.S. beekeeping community. There are some that state it is without any value as a nectar or pollen plant to honey bees (Lindtner 2014).

Pollen from willows, *Salix* spp., was also moderately important pollen sources during this period. Although only found in two of the samples, it comprised 39.7 percent of the pollen collected by honey bees at one site (Site G). *Salix caprea* L. (pussy willow) was the earliest flowering *Salix* species made in observations of flowering plants (Table 1.2).

Pollen from some woody plants in the family Rosaceae began to appear in samples during this week. Most was from early flowering *Prunus*, which was present in four of the samples (range 7.3-14.0), in overall average of 8.1% of the total for the week. Several *Prunus* species were observed to flower by mid-March including *P. americana* Marsh. (American plum), *P. besseyi* L. (sand cherry), *P. sargentii* Rehder (Sargent's cherry), and *P. tomentosa* Thund. (Nanking cherry) (Table 2). Pollen analysis could not distinguish between these species but repeated observations of *P. besseyi* over several years have never found honey bees to forage on the plant (Whitney Cranshaw, personal communication), suggesting this widely planted native species is not attractive to honey bees.

Table 1.2 Observations of plants in bloom in Fort Collins during early spring weekly (Mid-March – Early May) in 2015, 2017, 2018 and 2019. Surveys were conducted weekly and plants that were observed during this period that were in bloom are marked "X"; blank boxes indicate that the plant was not observed in bloom during the period.

			March 19-25				March 26-April 1				April 2-8				April 9-15				April 16-22				April 23- 30				May 1-5			
Family	Genus	Species:	2015	2017	2018	2019	2015	2017	2018	2019	2015	2017	2018	2019	2015	2017	2018	2019	2015	2017	2018	2019	2015	2017	2018	2019	2015	2017	2018	2019
Adoxaceae	*Sambucus*														x	x	x		x	x	x					x				x
	Viburnum	*Viburnum carlesii*														x	x		x	x	x	x	x	x		x				x
		Viburnum lantana																	x	x			x	x						x
		Viburnum 'Emerald Triumph'																	x	x	x		x	x	x	x				x
Anacardiaceae	*Rhus*	*Rhus trilobata*																					x	x	x		x	x	x	
Apiaceae	*Cymopterus*	*Cymopterus constancei*										x	x			x	x													
		Cymopterus glomeratus										x	x			x	x													
	Lomatium	*Lomatium grayi*						x	x		x	x	x					x				x								
		Lomatium parryi															x													
	Musineon	*Musineon divaricatum*											x				x													
Apocynaceae	*Vinca*	*Vinca minor*								x			x	x	x		x			x	x	x		x			x	x		
Asteraceae	*Doronicum*	*Doronicum orientale*																							x				x	x
	Tanacetum	*Tanacetum cinerariifolium*																												x
	Taraxacum	*Taraxacum officinale*	x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Tetraneuris	*Tetraneuris ivesiana*											x				x													
	Townsendi	*Townsendia incana*											x	x			x	x				x								
		Townsendia rothrockii															x	x				x		x						
		Tragopogon porrifolius																		x				x				x		
Asparagaceae	*Leucocrinum*	*Leucocrinum montanum*											x	x			x	x				x								
	Muscari	*Muscari neglectum*	x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	x		x								
	Puschkinia	*Puschkinia scilloides*			x				x				x	x				x				x								

	Scilla	*Scilla siberica*								x				x			x	x			x										
Amaryllidaceae	*Narcissus*	*Narcissus poeticus*	x	x	x		x	x	x		x	x	x		x	x	x	x	x	x		x	x	x		x					x
Boraginaceae	*Brunnera*	*Brunnera macrophylla*																											x	x		
	Lithospermum	*Lithospermum incisum*										x				x																
	Mertensia	*Mertensia fusiformis*										x				x																
		Mertensia lanceolata																	x													
	Pulmonaria																											x				
Brassicaceae	*Alyssum*	*Alyssum alyssoides*								x	x			x	x																	x
		Alyssum desertorum										x				x																
		Alyssum parviflorum															x	x	x		x	x	x	x								x
		Alyssum simplex							x			x				x	x			x	x											
		Alyssum spinosum															x	x														
		Alyssum stribryni										x				x	x			x	x											
	Arabis																	x	x	x		x	x	x								x
	Aubrieta								x			x																				
	Aurinia	*Aurinia saxatilis*																x	x	x		x	x	x		x	x					x
	Boechera	*Boechera quadrangulensis*								x	x	x		x	x	x		x	x	x												
	Capsella	*Capsella bursa-pastoris*										x				x		x	x	x	x	x	x		x	x	x		x			x
	Chorispora	*Chorispora tenella*			x				x			x		x	x	x	x	x	x		x	x	x		x	x	x		x			x
	Descurainia	*Descurainia sophia*										x				x		x	x	x		x	x	x		x	x	x	x			x
	Draba	*Draba aizoides*								x	x			x	x	x																
		Draba reptans										x				x																
	Eruca																					x				x					
	Erysimum	*Erysimum sp.*							x																							
	Iberis	*Iberis sempervirens*									x			x	x			x	x	x		x	x	x		x	x	x	x			x
	Lepidium	*Lepidium montanum*														x				x												
		Lepidium virginicum							x			x																				
	Physaria	*Physaria acutifolia*										x				x																
		Physaria bellii										x				x																
		Physaria humilis							x			x																				
Berberidaceae	*Mahonia*	*Mahonia aquifolium*					x	x	x		x	x	x		x	x	x		x	x	x		x	x	x		x	x		x			x
		Berberis fremontii																	x		x		x	x	x		x	x		x			x

Betulaceae	*Alnus*	*Alnus glutinosa*	x	x	x		x	x	x		x	x	x	x	x	x	x	x																
	Betula	*Betula spp*						x	x			x	x																						
	Corylus	*Corylus colurna*					x	x	x		x	x	x	x				x				x													
	Ostrya	*Ostrya virginiana*									x	x			x	x																			
Cactaceae	*Echinocereu*	*Echinocereus triglochidiatus*														x	x			x	x	x				x									
	Pediocactus	*Pediocactus simpsonii*																x				x													
Cannabaceae	*Celtis*	*Celtis reticulata*																			x				x										
Caprifoliaceae	*Lonicera*	*Lonicera caprifolium*																	x	x	x		x	x	x	x	x			x					
Caryophyllaceae	*Cerastium*	*Cerastium fontanum*											x				x																		
Chenopodiaceae	*Atriplex*	*Atriplex confertifolia*											x				x																		
Cornaceae	*Cornus*	*Cornus mas*			x		x	x	x	x	x	x	x	x	x	x		x				x													
		Cornus sericea																							x										
Cupressaceae	*Juniperus*	*J. scopulorum*																								x									
Elaeagnaceae	*Shepherdia*	*Shepherdia argentea*			x		x	x	x		x	x	x		x	x	x				x				x	x				x					
Ericaceae	*Arctostaphylos*	*Arctostaphylos uva-ursi*																			x				x										
		Arctostaphylos patula											x				x																		
Euphorbiaceae	*Euphorbia*	*Euphorbia brachycera*																		x	x		x	x	x		x	x	x	x					
Fabaceae	*Astragalus*	*Baptisia australis*																		x	x														
		Astragalus desperatus											x				x																		
		Astragalus crassicarpus											x				x																		
		Astragalus shortianus											x				x																		
	Caragana											x			x	x	x		x	x	x	x	x	x	x	x	x	x		x					
	Cercis	*Cercis canadensis*											x		x	x	x		x	x	x	x	x	x	x	x	x	x	x		x				
		Cercis siliquastrum																	x	x	x		x	x	x										
	Medicago	*Medicago lupulina*							x				x																						
	Oxytropis	*Oxytropis multiceps*																x				x				x									
	Robinia																									x									
Fagaceae	*Quercus*	*Quercus gambelii*																																	x
		Quercus rubra			x				x																										
Geraniaceae	*Erodium*	*Erodium cicutarium*						x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x								
	Pelargonium	*Pelargonium australe*								x				x				x																	
Grossulariaceae	*Ribes*	*Ribes alpinum*																						x											

		Ribes aureum								x	x	x			x	x	x		x	x	x		x	x	x		x	x	x
		Ribes odoratum																	x				x		x				x
		Ribes rubrum												x	x	x		x	x	x									x
Hydrangeaceae	*Fendlera*	*Fendlera rupicola*																	x	x			x	x					
Iridaceae	*Iris*	*Iris sibirica*	x		x		x		x				x				x				x								
		Iris reticulata			x				x			x	x			x	x				x								
	Crocus	*Crocus sativus*	x	x	x		x	x	x		x	x	x	x	x		x				x								
Lamiaceae	*Ajuga*	*Ajuga reptans*																				x	x	x		x	x	x	
	Anisomeles																					x				x			
	Lamium	*Lamium amplexicaule*								x	x	x	x	x		x	x	x	x	x	x								
	Lavandula	*Lavandula angustifolia*																											x
	Nepeta	*Nepeta × faassenii*																x	x	x		x	x	x					
	Teucrium	*Teucrium subspinosum*										x				x	x			x	x				x				
Liliaceae	*Fritillaria*																	x	x	x		x	x	x					x
	Lilium																	x				x							
	Tulipa albertii		x		x		x	x	x		x	x	x		x	x									x				x
	Tulipa		x	x			x	x	x		x	x	x		x	x	x		x	x				x	x	x			x
Magnoliaceae	*Magnolia*	*Magnolia liliflora*						x	x		x	x	x		x	x	x	x	x	x	x	x				x				x
Malvaceae	*Sphaeralcea*	*Sphaeralcea coccinea*																						x				x	
Montiaceae	*Claytonia*	*Claytonia rosea*															x				x								
Oleaceae	*Fraxinus*	*Fraxinus anomala*										x				x													
		Fraxinus americana													x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Fraxinus pennsylvanica	x	x			x	x	x		x	x	x		x	x	x	x	x	x	x	x				x				
	Forsythia	*Forsythia suspensa*	x	x	x		x	x	x	x	x	x	x	x	x	x	x	x			x	x			x				
	Forestiera	*Forestiera pubescens*									x	x	x	x	x	x	x	x											
		Forestiera neomexicana													x	x	x												
	Jasminum	*Jasminum officinale*																				x	x	x		x	x	x	x
	Syringa	*Syringa vulgaris*																x	x	x	x	x	x	x		x	x	x	x
Plantaginaceae	*Collinsia*	*Collinsia parviflora*										x				x													
	Penstemon	*Penstemon crandallii*																				x	x	x					x
	Veronica																	x	x	x		x	x	x					x
Poaceae	*Oryzopsis*	*Oryzopsis hymenoides*										x				x													

Polemoniaceae	*Ipomopsis*	*Ipomopsis spicata*											x				x															
	Phlox	*Phlox austromontana*								x	x	x		x	x	x			x	x	x		x	x	x		x		x
		Phlox grayi												x	x			x	x	x		x	x	x		x	x	x	x
		Phlox subulata								x	x	x				x	x	x	x	x	x	x	x	x	x	x	x	x	x
Pinaceae	*Larix*	*L.decidue*																									x					
Primulaceae	*Primula*	*Primula elatior*							x			x	x				x				x											
Ranunculaceae	*Anemone*																	x	x	x		x	x	x		x	x	x	x
	Clematis	*Clematis tenuifolia*							x	x	x	x	x	x	x	x	x	x	x		x									
		Clematis hirsutissima												x	x	x	x	x	x	x	x				x					
	Fumaria	*Fumaria officinalis*																				x				x				
	Pulsatilla	*Pulsatilla patens*							x	x	x	x	x	x	x	x	x				x									
	Ranunculus	*Ranunculus glaberrimus*										x				x				x										
Rosaceae	*Amelanchie*	*Amelanchier alnifolia*																x	x	x		x	x	x	x	x	x		x
		Amelanchier utahensis										x				x						x	x	x		x	x		x
		Amelanchier x grandiflora												x	x	x		x	x	x		x	x	x					x
	Aronia																	x				x				x				
	Cercocarpus	*Cercocarpus intricatus*																						x						
	Chaenomeles	*Chaenomeles japonica*	x				x	x			x	x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Crataegus												x				x		x	x	x		x	x	x		x	x		x
	Malus	*Malus sylvestris*					x	x			x	x			x	x	x		x	x	x	x	x	x	x	x	x	x		x
	Prunus	*Prunus americana*							x				x	x			x	x	x	x	x	x	x	x	x	x	x	x	x	x
		Prunus besseyi								x	x	x		x	x	x	x	x	x	x	x	x	x	x	x				x
		Prunus cerasus																x				x	x	x	x	x	x	x	x
		Prunus cerasifera										x				x	x			x	x			x	x				
		Prunus glandulosa												x	x	x		x	x	x	x				x				
		Prunus mahaleb																x	x	x		x	x	x	x				x
		Prunus padu																x	x	x		x	x	x		x	x	x	x
		Prunus pumila										x				x				x	x				x				x
		Prunus sargentii					x				x	x	x		x	x	x		x	x	x								
		Prunus triloba												x	x	x		x	x	x	x	x	x	x	x				x
		Prunus tomentosa					x				x	x			x	x	x				x								
		Prunus virginiana												x	x	x		x	x	x					x				x

	Purshia	*Purshia tridentata*																				x															
	Pyrus		x	x	x		x	x	x		x	x	x		x	x	x	x	x	x	x	x	x	x		x	x	x									
	Spiraea	*Spiraea × arguta*														x		x		x	x	x		x	x	x											
	Waldsteinia	*Waldsteinia fragarioides*										x			x	x	x		x	x	x			x		x											
Salicaceae	*Salix*	*Salix alba*													x	x	x		x	x	x					x											x
		Salix caprea	x	x	x		x	x	x	x	x	x	x	x	x	x	x				x																
		Salix exigua						x	x			x	x					x			x	x															
		Salix monticola														x																					
	Populus	*Populus tremuloides*						x	x																		x										
Sapindaceae	*Acer*	*Acer ginnala*																					x				x										
		Acer negundo													x				x				x														
		Acer platanoides					x	x	x			x	x																								
		Acer saccharinum	x	x	x	x	x	x	x		x	x			x	x																				
		Acer saccharum																									x										x
		Acer truncatum																				x														x	
		Acer rubrum	x		x				x																												
Thymelaeaceae	*Daphne*																			x	x			x	x											x
Ulmacea	*Ulmus*	*Ulmus pumila*	x	x	x		x	x	x	x	x			x																						
		Ulmus americana	x		x				x	x	x			x																						
Violaceae	*Viola*	*Viola odorata*	x		x		x		x		x	x																								
		Viola nuttallii					x	x	x	x	x	x	x	x	x	x	x	x			x																

A small amount (2.1%) of pollen from *Pyrus* was found in the collections from one hive during the week, and this was the only *Pyrus* collection during this study. The source was probably Callery pear (Bradford pear), *P. calleryana* Decne, an early flowering ornamental pear that is widely planted in Colorado (James Klett, personal communication 2020), including the urbanized sites of this study. The almost complete absence of *Pyrus* pollen in collections, in contrast to the widespread use of this flowering ornamental tree, suggests that this is a low-value plant for pollen by honey bees. Callery pear has developed into a highly invasive species in some areas of North America (Culley and Hardiman 2007).

Several herbaceous plants were heavily used as pollen sources during this week. Pollen from violets (*Viola* spp.), common weeds in lawns, was found in two 2015 collections and comprise 18.9 percent of the total pollen from one site. Spring flowering bulbs of Iridaceae (Iris) and Liliaceae (*Tulipa*) were represented in pollen collections from three and one site, respectively. During this week pollen from dandelion, *Taraxacum officinale* (L.) Weber ex F.H. Wigg, was first noted, in low amounts, from one 2015 sample.

In the third week of March (16-22) seven samples, all from 2015, were available and the diversity of pollen types collected increased. Silver maple continued to be the most heavily foraged plant, with it being present in all samples in percentages that ranged from 24.4-86.7% of the total at a site. Pollen from willow also was common, present in five samples in ranges of 4.5-37.3% of total. In one site (D) a third of the pollen was from Betulaceae. This was very likely from alder (*Alnus*), which, along with *Salix*, was growing in a dense stand along a small creek very near the hive and the trees were in bloom at that time. A small amount of pollen, 4.1% at one site, was also collected from elm (*Ulmus*). *Prunus* was also widely collected, present in five collections (range 6.0-27.1%).

In the Oleaceae, pollen from ash was collected from three of the seven sites in ranges of 3.0%-15.3% of total/site (average 3.8% among all sites). In addition, *Forsythia* continued to be collected and was present in three collections (range 1.0%-11.0%/site), reinforcing the observation that this plant has some value as a pollen source in the region.

Among herbaceous plants, *Viola* sp. was again represented in two collections. Pollen from dandelion was collected from only one site (5.4%). Among spring flowering bulbs pollen of three families - Amaryllidaceae (*Narcissus*), Iridaceae (*Iris*), and Liliaceae – were present, scattered among the six collection sites. In addition to Amaryllidaceae, Fabaceae was a new pollen type in collections of this week. A likely source of this was black medic, *Medicago lupulina* L. which was the earliest flowering member of the family observed in flower (Table 2) and is reported by Hilty (undated) as a source of pollen and nectar used by honey bees.

The number of collections from the last week of March (March 23-29) included samples from ten sites from both 2014 (5) and 2015 (6). The number of pollen types collected by honey bees expanded to 13 families during this period. A sharp shift to collections of Brassicaceae was observed, which coincided with the end of silver maple bloom. Blue mustard, *Chorispora tenella* (Pall.) DC and/or unidentified species were present in eight samples, in proportions ranging from 24.4%-75.4%/site. The diversity of Brassicaceae that were observed to be in flower at this time start to greatly increase (Table 2) and include plants from at least five different genera (*Alyssum*, *Aubrieta*, *Erysimum*, *Lepidium*, *Physaria*) in addition to the earliest flowering member of the family, blue mustard. Combined, Brassicaceae comprised 36.2 percent of the total pollen collected during the week.

A later blooming *Acer*, Norway maple, *A. platanoides* L., was present in samples from six sites in percentages ranging from 1.5-34.1 percent. Collections of pollen from *Prunus*

continued to increase. It was found in eight samples, in percentages ranging from 1.6-65.0 percent. Japanese flowering quince, *Chaenomeles japonica* (Thunb.) Lindl. ex Spach, a plant that is relatively uncommon in urban landscapes within the study area, was recovered in pollen from six of the sites (range 3.7%-26.0%/site). First collections from crabapple (*Malus*) appeared during this week. These are very commonly planted trees and crabapple pollen appeared in two collections, in percentages of 9.0% and 13.0%.

Spring flowering bulbs, which now included a fourth family, Asparagaceae (*Muscari*), were collectively represented from four sites, in percentages ranging from 3.0-31.3% of total. The most widely collected type of pollen was from dandelion present in all but one of the sites, in percentages ranging from 1.0-22.5%.

Collections form *Fraxinus* continued to decline, being found in two of the samples, at 7.0% and 11.1%, respectively. At this point white ash, *F. americana*. was present, as green ash was past bloom. An additional genus of Oleaceae to emerge as a pollen source was common lilac, *Syringa vulgaris* L., found in five samples (range 3.0-13.0%).

There were eleven samples entering the first week of April (March 30-April 5), five from 2014 and six from 2015. Among woody plants, pollen from Norway maple was found in the largest number of samples (9), ranging from 2.0-62.7 of the total. Ten of the eleven sites had samples from *Prunus* spp., ranging from 5.0%-65.0% of the total at the site and averaging 38.4% of all pollen brought into the hives during that week. Among other Rosaceae, pollen from crabapple (*Malus*) increased greatly over the previous week, being found in six of the collections (range 13.7%-50.6%/site), averaging 17.4% of all pollen collected during the week. Pollen from Japanese flowering quince continued to be used at one site (A), but in lower amounts than the previous week.

In Oleaceae, pollen from ash was collected in one site, comprising 7% of the total. Pollen from lilac was present in four samples and comprised a high percentage, ranging from 6.0%-12.1%/site

Among herbaceous plants, Brassicaceae predominated as a pollen source, being found in seven samples (range 10.0%-78.0%). It comprised an extremely high percentage of the pollen from two sites (B, C) in 2014, comprising 70.0% and 78.0% of the total. The latter was the third highest percentage of pollen from a single family source during any weekly collection. The fourth highest single family collection was also at this site (C) and also consisted of Brassicaceae, during the previous week period in 2015. Collections from dandelion were widespread, recovered from pollen in seven of the sites, in percentages ranging from 1.0%-22.4% per site.

During the second week of April (April 6-12) woody plants in the Rosaceae were the largest sources of pollen collected. *Prunus* predominated, found in ten of the eleven samples during this week, ranging from 4.6%-84.2%/site. The highest percentage (84.2), from Site 3 in 2014, comprised the second highest single percentage from a family and a genus among all collections, excepting the very first early March collection which was almost exclusively silver maple. Collections from *Malus* increased, coincident with peak bloom of crabapples, with it being found in six samples (range 13.7%-50.6%). Japanese flowering quince continued to be collected from Site A, but in lower percentage than the previous week period. During this week pollen was also collected from a new rosaceous host, hawthorn (*Cratageus*) at one site.
Pollen from *Acer*, provided by Norway maple, continued to be a major pollen source for the seventh week of the study. *Acer* pollen was in nine of the eleven samples, ranging from 2.0%-

63.5% /site. *Salix* also continued to be an important source, present in three samples (range 5.0%-15.8%/site).

In Oleaceae, pollen from ash was collected in one site, comprising 10% of the total. This was the latest collection of pollen from *Fraxinus* in this study. Pollen from lilac was present in four samples and comprised a high percentage (42%) in one 2015 site (F).

First collections from eastern redbud, *Cercis canadensis* L., also appeared at this time, present in three samples (range 6.3%-27.4%/site). Pollen was also collected for the first, and only, time from two families, Berberidaceae (*Mahonia*) and Magnoliaceae.

Among herbaceous plants, Brassicaceae continued to predominate as a pollen source, being found in eight sites (range 4.7%-47.0%). Collections from dandelion were even more widespread, recovered from pollen in nine of the sites, in percentages ranging from 1.0%-18.3%/site.

Nine samples were available from the seventh week of the study (April 13-19). The majority of collected pollen was from Rosaceae. *Prunus* continued to be heavily used and was found in collections from seven sites (range 6.2%-84.5%/site). At one site (Site C, 2015) an extremely high percentage of the pollen was from *Prunus* (84.5%), the highest percentage of single source pollen found in all collection since the first week of the study. Pollen from *Malus* was also high, found in all but one sample (range 4.7%-40.6%). There was an increase in collection from *Cratageus* (hawthorn), being found in pollen at one third of the sites, and pollen from a new rosaceous host, *Aronia*, was present in two samples.

Pollen from *Acer* continued to be a significant pollen source, present in five samples. The species of *Acer* present at this time of year would likely have included pollen from later blooming species, such as sugar maple, *A. saccharum* Marshall, bigtoothed maple, *A.*

grandidentatum Nutt., and boxelder, *A. negundo* L. Dandelion continued to be a widely collected pollen, found in all but one sample, in relatively low percentage (2.0%-8.3%/site).
Pollen from two families, Caprifoliaceae (*Lonicera*) and Cupressaceae were appeared for the first time in samples during this week.

During the eighth week of the study (April 20-26) there was pollen from 15 families of plant represented in the ten samples. The majority (57.5% of total) was from Rosaceae. *Prunus* was present in six samples (range 9.0%-45.0%) *Malus* in four samples (range 23.7%-75.3%) and a new, and pollen from other unidentified rosaceous plants was present in six samples (range 7.2%-65.3%). One possibility of the unidentifiable pollen was *Amelanchier*, a genus that was in bloom during this period (Table 2) and several species are widely used in landscape plantings. This is a genus that is well recognized as a source of nectar and/or pollen in North America (Krochmal 2016)

Other trees that were important as pollen sources were *Acer* (8 sites, range 5.4%-36.0%) and *Cercis* (7 sites, range 1.5%-18.2%). A new type of pollen found in samples of this week was from Fagaceae (*Quercus*), present in three samples (range 1.5%-12.0%)

Among herbaceous plants dandelion continued to be widely collected, present in samples from 9 of 10 sites, in relatively low percentage of the total (range 1.0%-8.2%). Novel types of pollen collected at this time were from Papaveraceae (*Papaver*) and Ranuculaceae (*Ranunculus*). Collection from Viola (Violaceae) was also recorded after an absence of three weeks, found in a small amount (1.3%) in a single sample.

Although there were limited collections made later in the season, this study was concluded in the ninth week (April 27-May 3). Samples from ten sites were available in this

period and these included pollen from 21 plant families, the greatest diversity of pollen types collected during any week of the study.

Acer was the only genus found in collections at all sites (range 2.8%-78.3%) and comprised 36.7% of all pollen collected during this week. This genus was found in highest percentage not only during the first week of the study (March 2-8) but also the last and was a major pollen source throughout the ten week period. Pollen from various Rosaceae were the second most heavily collected pollen, comprising 28.3 percent of the total. Pollen was most commonly collected from *Prunus*, *Malus* and *Crataegus*, in that order. Brassicaceae again emerged as a major pollen source, collected from 7 sites (range 11.0%-28.3%) and comprised 13.6 percent of the total pollen collected. Several weedy Brassicaceae were in bloom during the last two weeks of the study period (Table 2), but a particularly commonly used landscape plant that was observed to be commonly visited by honey bees was basket-of-gold, *Aurinia saxatilis* (L.). Desv.

Dandelion continued to be represented in pollen collections, found in seven samples (range 1.4%-14.5%). Pollen from dandelion was collected over a longer period than from any other species, present in all but the very first week sample.

Several families of plants were represented in samples for the first time this week. Anacardiaceae represented 26.4% of a sample from one site. The species was likely *Rhus trilobata* Nutt. If so, this would be one of the very few plants native to the area of the study from which pollen was collected by honey bees in this study and *Rhus* is mentioned specifically as an example of a native plant that is of value to honey bees by Vorndam (2018). Pollen found for the first time also included Crassulaceae, Geraniaceae, Pinaceae, and Rutaceae, each being represented in one sample.

DISCUSSION

This study focused on the period when pollen resources first become available in urban areas of the northern Front Range. During this time a wide range of plants were utilized for pollen including 28 plant families and at least 38 genera. This diversity is comparable to the "spring" collections of pollen from urbanized areas reported by Lau et al. (2019) from Michigan (27 families), Florida (30 families), Texas (36 families), and California (39 families). However, there was very little overlap of the most important types of pollen collected in Colorado during this study, compared with what was reported from these other states (Figures 1.1, 1.2).

During the earliest period of collections (March 2-22), including the very earliest flowering plants present during the year, Sapindacaeae (*Acer*) was overwhelming abundant in samples, a "predominant" species in the terminology used by Lau et al. (2019). Following this classification Salicaceae, Rosaceae, and Oleaceae may be considered "secondary" pollen sources at this time, with Iridaceae, Asteraceae, and Violaceae as "important minor" families. In the following three week period (March 23-April 12) predominant taxa collected were Rosaceae and Brassicaceae; Sapindaceae and Salicaceae were secondary pollen sources, and minor important taxa would include Asteraceae, Oleaceae, and Irididaceae. In the last, mid-spring collections (April 13-May 3 2014 and 2015), the only predominant taxa were Rosaceae, secondary taxa Sapindaceae, and important minor plant families in pollen collections included Brassicaceae, Oleaceae, Fabaceae, and Asteraceae. The most similar results to these reported by Lau et al. (2019) were collections from Michigan, where Sapindaceae (as Aceraceae) and Salicaceae were found as secondary taxa in spring collections and California, where Rosaceae and Brassicaceae were classified as secondary sources of pollen. No secondary or predominant sources of pollen

reported in spring collections from Texas and Florida were ever found as even "minor important" taxa in Colorado.

Anemophilous sources of pollen were heavily used by honey bees throughout the course of the study, but particularly in the earliest dates of collection. Among collections made between 2-22 March pollen from trees that are wind pollinated made up over 72 percent of the collected pollen. The sources of these, in order, were Sapindaceae (*Acer*), Oleaceae (*Fraxinus*, *Forsythia*), and Betulaceae (*Alnus*). The use of some anemophilous pollen by honey bees is well known. Severson and Parry (1981) in Wisconsin found high use of anemophilous pollen (45% or greater) from the earliest collections (16 April – 14 May) before plunging to near zero at the end of May. O'Neal and Waller (1984) commented that honey bees in the Tucson, Arizona area used pollen from anemophilous sources "to a far greater extent that would be expected by other bees", reporting that in one apiary it comprised as much as 71 percent of the pollen during the first half of the flowering season. The early March collections of this study appear to show higher use of anemophilous pollen in Colorado, during the onset of flowering in late winter and early spring, than has been previously reported elsewhere.

That there is a particular dependence of honey bees located in urban areas of Colorado on pollen from maple (*Acer*) during early bloom is very evident in this study. During the first three weeks it comprised 61.6 percent of all pollen being brought into hives and was found in high proportion in all eight hives sampled during this period during the study. Almost all of this was from a single species, silver maple (*A. saccharinum*). A small amount from red maple (*A. rubrum*) was identified. In later samples, later blooming Norway maple (*A. platanoides*) became important. At the end of the study ginnala maple (*A. ginnala*) was confirmed as being collected. Likely other later blooming maples that are widely planted were also used including *A.*

tartaricum (Tartarian maple), *A. saccharum* (sugar maple), *A. negundo* (boxelder), and *A. grandidentatum* (bigtoothed maple).

Maples, particularly red maple, are listed in several general references of pollen plants (Somme et al. 2016, Tew 1998, Batra1985) and unspecified maples (as Aceraceae) were listed by Lau et al. (2019) as secondarily important spring sources of pollen in Michigan and in winter in Florida and as "minor important" pollen sources in Texas (spring) and California (winter). Severson and Parry (1981) noted specifically *A. negundo* as a pollen source in Wisconsin. Published references to the specific use of silver maple as a pollen source by honey bees are rare but the species was noted by Moore (2000) as potentially important to "the biology of bees and other pollen dependent insects" because of its early pollen production.

Pollen from plants in the Oleaceae that were collected included a mixture of wind pollinated species (*Forsythia*, *Fraxinus*) early in the season and insect pollinated plants (*Forestiera*, *Syringa*) later in spring. Most all collections of pollen from *Fraxinus* (85%) occurred during the first four weeks of the study (8 March – 4 April) when only the early flowering species *F. pennsylvanica* (green ash) was in flower (Table 2). *Fraxinus* pollen collected after this time (5 April – 18 April was likely the later flowering *F. americana* (white ash).

At three sites (Sites C, F, G) in 2015 *Fraxinus* pollen was collected in two consecutive weeks, indicating a flowering period of about two weeks. In the remaining sites where *Fraxinus* pollen was collected it was only found in one collection. All collections from *Fraxinus* were in 2015. No pollen from *Fraxinus* was found in any 2014 collections, although these did not begin in that year until the week of 29 March-4 April.

Use of the anemophilous *Fraxinus* pollen in North America by honey bees has been reported. Pollen from unspecified *Fraxinus* was reported as "abundant" in spring collections (unspecified date) by Richardson et al. 2015 in Ohio. In Wisconsin *Fraxinus* pollen was common in collections from 16-30 April, then declined Severson and Parry (1981). The native range of both green ash and white ash overlap in the area of this study (LaCrosse County, Wisconsin) but the late dates of collection suggest that pollen white ash was the likely predominant species collected in this study. Tew (1998) mentions white ash, but not green ash, as a pollen source used by honey bees in Ohio.

The second wind-pollinated species of Oleaceae from which pollen was collected was *Forsythia*. The value of Forsythia as a floral resource by honey bees is a subject of debate. Most lists of plants of value to honey bees make no mention of *Forsythia* and some (Lindtner 2014) specify it as a species without any value to honey bees. But it is mentioned as an important plant in some informal beekeeper discussion forums, photos exist of honey bees visiting *Forsythia*, honey bees were seen on *Forsythia* during the course of this study (Cranshaw, personal communication) and pollen from *Forsythia* was found in pollen collected in five of seven sites in 2015 collections between 15-28 March. Collectively these findings suggest that *Forsythia* has some value as a pollen resource during the early season when few alternative sources are available in the area of this study. During this collection (Site D, 16-22 March, 2015) an unusually limited variety of pollen types were collected: Sapindaceae (*Acer*), Betulaceae (*Alnus*), and Salicaceae (*Salix*).

Later collections of pollen were from two different genera *Forestiera* and *Syringa*. The former is represented by *F. pubescens* var. *pubescens* (stretchberry, New Mexico privet) a native species to the areas of the western United States, including Colorado. It is recognized as a

source of honey in New Mexico (Grasswitz and Dreesen, undated; New Mexico Beekeeepers Association 2020) under the junior synonym *F. neomexicana*, although its use as a pollen source has not been reported. Much more commonly found in Colorado urban landscapes is *Syringa*, mostly *S. sativum* (common lilac) which produces abundant blossoms in late April and early May.

Pollen from Betulaceae was found in one collection, originating from *Alnus* growing in a wet area near the apiary. Although only a single collection was made, it comprised a high percentage of the pollen (33%) in the sample, indicating that *Alnus* may be readily collected under some conditions. *Alnus* pollen has been reported to be collected by honey bees. Girard et al. 2012 found it to be collected commonly from an area of *Vaccinium* monoculture, but also noted that *Alnus* pollen is deficient in some essential amino acids used by honey bees and was a poor nutritional source. Tew (1998) lists it as a pollen source used by honey bees in Ohio.

Salicaceae that were used by honey bees in Colorado were not identified. This family includes two commonly planted genera, *Populus* and *Salix*. The former is entirely wind pollinated, but has long been known to be collected by honey bees (Synge 1947) and produces a pollen of high nutritional value to honey bees (Liolios et al. 2015). No honey bees were ever been noted visiting aspen (*Populus tremuloides*) when it was in bloom during this the past five years (Whitney Cranshaw, personal communication) but these observations were limited to plants with visible flowers less than 4m above the ground and did not include observations of other *Populus* spp. where pollen is produced much higher above ground level. Most *Salix* are pollinated by insects (entomophilous), some are wind pollinated (anemophilous), and others use a mixture of pollination methods (ambophilous). Honey bee visitation of these was regularly observed during the course of this study.

Species identifications were not possible, but the only species flowering during the earliest period of this study, when Salicaceae was collected in highest proportion (Table 2), was *Salix caprea* (goat willow) and, possibly, aspen, the earliest flowering *Populus* spp. found in the study area. *Salix caprea* is a European species that is pollinated both by insects and wind (Vroege and Stelleman 1990, Dötterl et al. 2014). Other common *Salix* species that produce flowers later in spring that are present in regional landscapes include *S. exigua* (coyote willow), a native species that is insect pollinated (Anderson 2006) and *S. alba* (white willow), a European species that is insect pollinated (Karrenberg et al. 2002).

Willow (*Salix* spp.) is mentioned as a major sources of pollen and nectar by Tew (1998) and the species *S. discolor* (pussy willow) is also mentioned as a source of nectar and pollen. Salicaceae were listed by Lau et al. (2019) as secondarily important spring sources of pollen in urbanized areas of Michigan and a "minor important" pollen sources in Texas (spring), California (winter), and Florida (winter). Ostaff et al. (2015) did not report honey bee use of native willows in berry production areas of New Brunswick, but did find them heavily visited by a variety of native bees, mostly in the families Andrenidae and Halictidae.

Rosaceous plants became very important pollen sources shortly after commonly planted species of *Prunus* began to come into bloom in late March and early April. *Prunus* was the most important genus used by honey bees in the study area and many species are commonly used as landscape plants. These provide a sequence of bloom with earliest flowering species including American plum (*P. americana*), Nanking cherry (*P. tomentosa*), and Sargent's cherry (*P. sargenti*) (Table 2). These are then followed by bloom of the "sand cherries" *P. pumila* and *P. pumila* var. *besseyi*, myrobalan plum (*P. cerasifera*). Slightly later blooming species include the "flowering almonds" (*P. glandulosa* and *P. triloba*), chokecherry (*P. virginiana*), Mayday tree

(*P. padu*), maheleb cherry (*P. maheleb*) and tart cherry (*P. cerasus*). All of these are non-natives of North America, with the exception of American plum, the "sand cherries" and chokecherry, all of which are native to Colorado. Whether all are used by honey bees could not be determined by this study.

Malus, from flowering crabapple, was also an important pollen source and is well recognized as an important nectar and pollen plant (Tew 1998, Pernal et al. 2000). In this study, in some weeks during April, collections from *Malus* approached, but never exceeded, the amount of pollen from *Prunus*.

Pollen from Japanese flowering quince was first appeared in the fourth week (30 March-April 5) when it appeared in over half of the samples (range 3.7-26.0). It continued to be represented in samples for more weeks, indicating an unusually long period of bloom. This plant is well known as both a source of both pollen and nectar (Denisow 2002) but is quite uncommonly found in landscapes within the study area. That it was so commonly used by honey bees in proportion to its availability in landscapes suggests that *C. japonica* is an underutilized plant that has exceptionally high potential value as a pollen resource.

Conversely, pollen from *Pyrus* was collected very rarely, in a single collection, during the second weeks of the study (9-15 March). During this time of year Callery pear/Bartlett pear (P. callerya) is in flower and this is a widely planted tree in the area of the study. The low collection of pollen from callery pear, compared to the very common presence of this plant in landscapes suggests it is a plant of low value as a pollen resource to honey bees. Pollen from another rosaceous host that is widely planted, hawthorn (*Crataegus*), appeared in collections beginning in early April but was never collected in large amounts. Hawthorn was considered to be a "marginal" source of pollen for honey bees by Tew (1998).

Brassicaceae were very important pollen sources, particularly during the middle period of this study, in late March and early April. The specific types of pollen could rarely be distinguished, as there are a great many flowering plants in this family present during the period of this study; at least 22 species from this family were found in bloom during annual surveys (Table 1.2). Many of these are common winter annual or perennial weeds and flowering weeds are well recognized as potentially important pollen sources in some settings (Bretagnolle and Gaba 2015). Pollen from oilseed brassicas have been correlated with measures of improved honey bee health (DeGrandi-Hoffman et al. 2016, DiPasquale et al. 2016).

At least two species of Brassicaeae can be reliably inferred as sources of pollen collected by honey bees in this study, based on their flowering time and observation of bee visitation. Blue mustard is the earliest flowering of the Brassicaceae, and is very conspicuous and abundant species along roadsides and disturbed sites throughout the study area. It is the only species widely found in flower (Table 2) during the fourth week of this study (23-29 March) when Brassicacae was found in highest proportion and honey bee visitation of this plant was observed during the course of this study. However, there were other kinds of pollen from Brassicaceae in the samples from that first date, and the identification of these are unknown. Another increasingly common invasive weed in the study area, whitetop/hoary cress , was not tracked for flowering date (Table 2) but was observed in flower in late April in 2020. Honey bees have regularly been collected from this plant in Colorado and it is reported to be a source of nectar from which high quality honey can be produced (Texas Invasive Species Institute 2020).

Among cultivated Brassicaceae, the earliest flowering species is candytuft, *Iberis sempervirens* L. This is listed as a source of nectar only by Tew and its use as a pollen source needs to be confirmed. Later in the season basket-of-gold, *Aurinia saxatilis* (L.) Desv. comes

into bloom, and this has been regularly observed to be visited heavily by a wide variety of flies and bees, including honey bee.

Pollen from early flowering bulbs of four families (Amaryllidaceae, Asparagaceae, Iridaceae, Liliaceae) are regularly represented in collections, and are most commonly collected in March. Most important is Iridaceae, which includes two commonly planted genera (*Crocus*, *Iris*) that are represented in pollen collections. *Crocus* is found in early collections and was found in high percentage among pollen collected at the end of March and was mentioned by Tew (1998) as a pollen source. Later, pollen from *Iris* is available. Commonly, but in less abundance, pollen from *Tulipa* (Liliaceae) and *Narcissus* (Amaryllidaaceae) were found. Grape hyacinth/*Muscari* (Asparagaceae) was found in pollen from only a single collection, but represented 10.7% of the sample.

Pollen from *Viola* (Violaceae) were found in many of the March collections. These are commonly found in lawns and may invade gardens. At least two species may commonly be found in lawns in the study area, sweet violet *Viola odorata,*L., a European species, and *Viola sororia,* Willd., a North American species (Tony Koski, personal communication, April 12, 2019). Other species may be present, as this genus is difficult to identify and many hybridize readily.

Dandelion is the only plant in Asteraceae found in collections during this study. It is a widespread plant in lawns and disturbed areas within the study site and no other species had such widespread and prolonged representation in pollen collections of this study. In most collections it was in low percentage, but did make up 22.4% in one late March collection and was most heavily represented in collections during late March and early April.

Dandelion is widely reported as a plant that is used by honey bees both for pollen and nectar and is considered to be important in some areas (O'Neal and Waller 1984, Tew 1998), blooming mostly in spring but also providing secondary bloom through frost. The protein quality of dandelion is sometimes considered to be low due to deficiencies in some amino acids considered to be essential to honey bee nutrition (Loper and Cohen 1987). However, as Liolios et. al (2015) and others point out protein richness is not sufficient to classify its quality to honey bees. The honey bee is polylectic (O'Neal and Waller1984) and their collection of a wide variety of pollen avoids deficiencies in any particular nutrient. The varied diet resulting from use of diverse pollen sources has been often shown to improve performance of honey bee colonies (Bretagnolle and Gaba 2015).

This review of pollen sources used by honey bees provides guidance on plant selections that can improve habitat for honey bees in urbanized sites of Colorado during the critical period in late winter and early spring when colonies build to prepare for the spring nectar flow. Soft maples, such as silver maple, red maple or hybrids of these (e.g. "Autumn Blaze" maple) are the major pollen source used by honey bees early in the season and should be considered foundational sources of early season pollen in urbanized sites of Colorado.

As diversity of pollen types being taken in and used by a hive has such a wide variety of benefits to colony health and development plantings should also incorporate as many as possible of the other earliest season pollen sources into planting programs. Plants that were found first found to be used as pollen sources in March that may be considered in planting selections are listed in Table 1.3. These would be species that may have particularly critical importance to honey bees since pollen sources may be very limited at this time.

In addition to soft maples the early flowering *Prunus* are showy plants that provide pollen and should be considered in planned habitats to sustain honey bees. However, the relative use of the different species of *Prunus* by honey bees needs to be further defined. The non-native species *P. tomentosa* (Nanking cherry) and *P. sargentii* (Sargent's cherry) are often seen to be visited by honey bees. The use by honey bees of native Prunus is less clear. There is some documented use of the *P. americana* (American plum) as a nectar source for honey bees; documentation of *P. pumila* and *P. pumila besseyi* (sand cherry) is less clear and needs further study. The other genus of rosaceous plants in bloom in March is *Pyrus*, largely represented by *P. calleryana* (Callery/Bradford Pear). This appears to be a plant used very little as a pollen source, being detected only in a single sample, in small amounts.

Willows (*Salix*) are important sources of pollen during late winter and early spring. Goat willow (*S. caprea*) is a particularly early blooming *Salix* species that would be an available pollen source for honey bees very early in the season. Somewhat later in the month *Forestiera* species (*F. pubescens*, *F. neomexicana*) come into bloom and are available as pollen resources.

Some of the earliest flowering plants from which pollen may be collected may have marginal value or only be collected when alternatives are very limited. This includes *Forsythia*, which was used to fair extent by some hives, but is a species that often is dismissed as having value to honey bees. Alder (*Alnus*) was used heavily by one colony on one date, although it is reported to have poor nutritional value. Dandelions also begin to appear in March and this plant was found to be used more broadly over a longer period of time than any other species, although never in high percentages by any one hive. Dandelion is also considered to produce pollen of low nutritional value, based on the profile of amino acids it contains. However, given the limited

numbers of pollen sources at this time, the use of even marginal plants can have value, providing further diversity of diet.

Another plant rarely considered for use as a pollen resource by honey bees is violets (*Viola*), but in this was found to be used by honey bees in high percentage at some sites. Given that this plant presently is not commonly found in large patches in yards/gardens, *Viola* is a potentially high value pollen source during the earliest month when honey bees collect pollen. It also has some additional benefit as a host plant for variegated fritillary, *Euptoieta claudia* (Cramer), one of the most commonly seen butterflies that develop in area gardens
Several spring flowering bulbs were used as pollen sources at this time and their expanded use may provide valuable alternative sources of pollen. Most important were early flowering species of *Crocus* and *Narcissus*. Some use of pollen from early flowering tulips (*Tulipa*) was used and grape hyacinth (*Muscari*) pollen was detected in one sample. Collectively these types of spring bulbs provide the most easily adopted source of earliest season pollen.

Several of the plants that provide the earliest pollen used by honey bees are low growing and one place where they could be incorporated into plantings are within lawn areas. Held and Potter (2012) showed that certain plants growing within lawns, specifically dandelions and white clover, are used by a wide variety of pollinator species, including honey bee. Dandelions are the most obvious of flowering plants used as a pollen source that is already widely present in Colorado lawns. It is often considered an undesirable weed but its presence in limited populations may be considered acceptable, when balanced with the benefits provided to honey bees and other species that utilize it as a pollen/nectar source. Less visible in lawns when in flower, and potentially of much higher value to honey bees early in the season, are violets (*Viola*). Although violets can spread and move into garden beds, where it can become more

weed-like, its further use as a planting to be tolerated and perhaps encouraged in lawns has substantial potential to improve critical earliest season pollen resources for honey bees in the region.

Some of the spring flowering bulbs that are heavily used as pollen sources can also be planted within lawn areas. Most important are crocus, which begin to flower often before lawns even begin to green in spring. Later mowing can cut foliage but smaller, earlier flowering crocus can often thrive in mowed lawns. Larger crocus and grape hyacinth may also be able to establish and spread in lawns, particularly if mowing over areas of these plants is delayed a few weeks.

Table 1.3 Earliest flowering plants used as pollen sources by honey bees in urbanized areas of the northern Front Range of Colorado.[a] Plants included were found in pollen samples collected during March, 2014-2015.

Acer rubrum
Acer saccharinum
Acer saccharinum X *rubrum* hybrids
Alnus
Brassicaceae
Crocus (early flowering species)
Forsythia
Narcissus (early flowering types)
Prunus (earliest flowering)[c]
Salix caprea
Tulipa (early flowering species types)
Viola

[a] *Fraxinus* is collected early in the season, but was not included in this table because its future use is compromised by the effects of the invasive species emerald ash borer.
[b] Species of Brassicaceae that are used as pollen sources by honey bees at this time need to be determined.
[c] Earliest flowering *Prunus* include *P. americana*, *P. pumila* x *besseyi*, *P. tomentosa*, and *P. sargentii*. Some species produce flowers that are known to often be visited by honey bees, particularly *P. tomentosa* and *P. sargentii*) the use of others, particularly *P. pumila*, is less clear and needs further study.

Later, during the early and through to midApril, there is a shift in the kinds of plants that provide pollen used by honey bees (Table 1.4). Maples are still an important source of pollen,

but the species shift with bloom times. Norway maples are a common pollen source in early April. Later in the month ginnala maple comes into bloom, followed by several other *Acer* species towards the end of the month. Use of *Salix* as a pollen source declines, but remains significant. Later flowering *Salix* are present at this time, such as *S. alba* and *S. exigua*.

Several genera of roseaceous plants predominate as pollen sources during this time. Most commonly used are *Prunus*, and several species often start to come into bloom by midApril, including *P. cerasifera*, *P. glandulosa*, and *P. virginiana*. Crabapple (*Malus*) is an important pollen source, and perhaps the most visible of the flowering plants within urban landscapes that is heavily used by honey bees. A plant that is uncommonly planted but present in some older landscapes is Japanese flowering quince. This plant was broadly represented in samples and appeared in collections for over five weeks. As this plant is presently rarely used, but appears to have very high potential value as a floral resource for honey bees, expanded use of this species in urban landscape would benefit honey bees. There is also some use of hawthorn (*Cratageus*) as pollen sources. Several *Cratageus* species and cultivars are found in landscapes with different bloom times and floral structures, including double-bloomed cultivars that may prevent access to pollen. The species/cultivars of *Crataegus* that are of value to honey bees needs further investigation.

Eastern redbud was a pollen source that was found in high percentage in some collections during this period. When in full-bloom this plant produces a very large number of blossoms and honey bee visitation is very evident. Use of this tree in the region as a pollen source may be a bit unreliable, as severe freeze events in spring, such as happened in 2020, can destroy developing flowers. Other woody plants that can be producing pollen used by honey bees at this time include *Forestiera* (New Mexico privet) and early flowering species of *Lonicera* (honeysuckle).

Among herbaceous plants Brassicaceae begin to be used by honey bees in late March but significant use continues through April. Several Brassicaceae are in bloom during this time but species used by honey bees cannot be determined by microscopic examinations used in this study. Later blooming *Iris*, *Lamium*, and dandelion are also pollen sources found to be collected by honey bees.

Table 1.4 Important flowering plants being used as pollen sources by honey bees in early to – mid-April in urbanized areas of the northern Front Range of Colorado.

Acer platanoides
Brassicaceae[a]
Cercis canadensis
Chaenomeles japonica
Crataegus (early flowering species)
Forestiera
Iris (early flowering types)
Lamium
Lonicera (earliest flowering)[b]
Malus (early flowering crabapples)
Prunus (early flowering)[c]
Taraxacum officinale
Salix[d]
Viola

[a] Species of Brassicaceae in flower at this time that are collected for pollen at this time have not been determined. Certain earliest flowering weedy mustards, such as Chorispora tenella, likely are most important at this time. Other brassicaceous plants flowering at this time that may be visited by honey bees are found among some species in the genera *Alyssum*, *Draba*, and *Physaria*. Their actual value as pollen sources needs confirmation.

[b] *Lonicera fragrantissima* (winter honeysuckle, sweet-breath-of-spring) is a particularly early flowering *Lonicera* that can be grown in Colorado.

[c] Earliest flowering *Prunus* include *P. americana*, *P. pumila* x *besseyi*, *P. tomentosa*, and *P. sargentii*. Species that bloom slightly later and are first present during this time period include *P. cerasifera*, *P. glandulosa*, and *P. virginiana*. Some species produce flowers that are known to often be visited by honey bees; honey bee use of others, such as *P. pumila*, is less clear and needs further study.

[d] *Salix* species known to be visited by honey bees that bloom at this time include *S. caprea*, *S. exigua* and *S. alba*. All are insect pollinated, but their relative use by honey bees has not been investigated.

Identifying plants adapted to regional conditions that provide pollen during early season, and then expanding their use in landscape plantings, can improve habitat for honey bees in urbanized sites (Lepczyk et al. 2017). This study was able to identify many plants that can provide these needs, but further studies are needed to refine lists of high value pollen sources. One need is to provide better identification, to the species or cultivar level, of several kinds of pollen. This is particularly important for distinguishing the different Brassicaceae that are collected by honey bees, which could not be reliably separated beyond the family level using the light microscopy methods of this, and most all similar, studies. Species level identifications for pollen from *Prunus*, *Salix*, and *Crateagus* would also be particularly valuable. Molecular methods of identification are being developed that could refine pollen identifications to apply to this problem (Keller et al. 2015, Richardson et al. 2015b, Bell et al. 2016). There are limitations to these methods, and a combination of both molecular techniques and microscopy may be the best way to resolve questions of pollen identification (Lau et al. 2019).

Another approach would be to expand field observations of plants that bloom early to determine if honey bees visit the plants. Large collections of plantings, such as exist at regional botanical gardens, could be used for intensive observation of the earliest flowering plants to provide clues for additional species that may be locally adapted pollen sources used by honey bees.

Almost all of the species of plants that were demonstrated to be of high value to honey bees as pollen sources are species that are not native to Colorado or the region. Among very earliest sources of pollen, collected during March, very little and likely none originated from plants that are native to Colorado. Possible exceptions would be certain Brassicaceae, American plum (*Prunus americana*), sand cherries (*P. pumila*, *P. pumila* var. *besseyi*) and *Forestiera*

pubescens var. pubescens). Some native plants that flower later are known as sources of pollen collected by honey bees, including chokecherry (*P. virginiana*) and boxelder (*A. negundo*). Presently there is often an emphasis in designs of "pollinator gardens" to use native plants (Kremen et al. 2011, Matteson et al. 2013). While these can be well designed to provide floral resources used by native bees, they may provide relatively little support for the non-native honey bee.

Identification of high value plants used by honey bees can also be used to identify the plants on which insecticide use would pose greatest hazard to visiting honey bees. Woody plants in the genera *Acer*, *Chaenomeles*, *Cercis*, *Cratageus*, *Fraxinus*, *Malus*, *Prunus*, and *Salix* contain important species that are used as pollen sources by Colorado honey bees in urbanized sites. Use of insecticides harmful to honey bees (Hooven et al. 2016) on these plants must be done with extra care to avoid residues of insecticides that produce hazard to bees from contaminating flowers, pollen, and nectar. Fortunately, there are very few problems with insects on these plants in Colorado where insecticide use would provide benefit.

The notable exception is *Fraxinus*, a shade tree that is extensively planted in urban forests of Colorado. Pollen from green ash (*F. pennsylvanica*) is among the earliest produced pollen and was present on the earliest collection date of this study. Later there is some use of pollen from the later flowering white ash (*F. americana*).

Historically, ash grown in Colorado rarely has received insecticide applications. Most of these have been used to control the lilac/ash borer, *Podosesia syringae* Harris (Lepidoptera: Sesiidae). This is a native insect of eastern North America and is most damaging to ash growing under marginal conditions, such as when grown in parking lots or medians, or when it has been recently transplanted (Cranshaw 2014). Treatment for this involves sprays of the trunk and lower

limbs in May, long after ash flowering has concluded. Even less frequently ash trees in the study area may receive applications of a soil applied systemic insecticide to control leafcurl ash aphid, *Prociphilus fraxinifolii* Riley (Hemiptera: Aphididae), an insect that produces only cosmetic injury.

This situation changes dramatically when emerald ash borer establishes in a new area. Because of the ability of this insect to cause serious injury to North American *Fraxinus*, typically progressing to tree death, insecticides are used to treat trees that are considered of high enough value to justify treatment costs. If only one third of the ash are treated along the Colorado Front Range, this will result in many hundreds of thousands, if not millions, of additional ash trees annually receiving applications of systemic insecticides in these urban forests. The potential route of exposure from these insecticide applications to honey bees would be from residues present in pollen.

Determining the use of *Fraxinus* pollen by honey bees is one factor required to calculate the potential exposure honey bees to insecticides applied for emerald ash borer. This study clearly shows there is some use of pollen by honey bees from *Fraxinus* in the study area. Highest use was in the first two weeks of the study (2-15 March) when it appeared in 4 of 6 samples (range 3.7%-23.4%) and averaged 9% of all pollen among samples from that period. Use declined during the next two week period (16-29 March) when *Fraxinus* pollen was found in 6 of 19 samples (range 3.0%-15.1) and averaged 2.4 percent. In the following two weeks (30 March-12 April) *Fraxinus* collections further declined, present in only 2 of 22 samples (range 7.0%-10%) and averaged 0.8 percent. Overall collections of pollen from *Fraxinus* during the six week period when it was present in any sample averaged 2.5 percent.

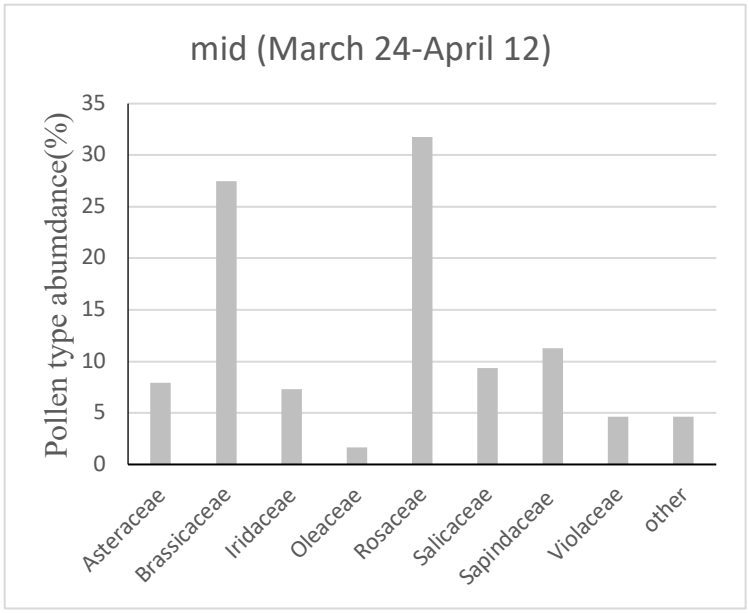
The amount of pollen collected by honey bees also varied by species of ash. While pollen from the different species of *Fraxinus* could not be determined, green ash (*F. pennsylvanica* blooms at least three weeks earlier than white ash (*F. americana*) (Table 1.2), although there can be some overlap in flowering. Based on observations of flowering during the four weeks that included all March collections (2-29 March), all can be safely assumed to have come from green ash. During this time 10 of 25 samples included *Fraxinus* and the overall percentage of pollen from *Fraxinus* was 4.0%. *Fraxinus* pollen was present in much lower incidence (0.8% of total) during the next two weeks when at least some pollen could have come from white ash, and did not appear in later collections, after 16 April, when white ash continued to be in flower.

These help further refine the collection of ash pollen by honey bees in these urbanized sites. Essentially all is from green ash, and it is most heavily collected early in the season when few alternative pollen sources, aside from silver maple, are available. During periods when *Fraxinus* pollen was collected, pollen from green ash represented an average of 9.9 percent of total pollen and was never collected for more than a two week period at any one site. Much less pollen is collected from white ash, and all of it is collected during the earliest period when white ash is in flower.

These changes in collection of *Fraxinus* pollen appear to be related to the availability of alternate pollen sources. As alternative pollen sources become more available use of ash pollen will decline. The availability of these alternate pollen sources can be increased by promoting the use of non-*Fraxinus* plants used by honey bees. These plants would be the same types of plants identified earlier (Table 1.3) that serve are used during the earliest dates when pollen is being brought into hives.

One other consideration in assessing the potential of ash pollen as a route of exposure to pesticides by honey bees will be the percentage of trees that receive insecticide applications. During early stages of invasion by emerald ash borer only a fraction of trees was treated for this insect; a majority of trees in Colorado likely will not be treated for EAB. This occurs in situations where the high costs of sustained insecticide treatment do not outweigh the tree owner's perception of the value of the tree. Therefore, as an example, if ash pollen comprises nine percent of the total pollen being brought in to a colony during a week, likely less than half may be expected to originate from a tree that received any insecticide application, somewhere in the range of 3-5 percent.

This report solely focused on determining the collection of different kinds of pollen, particularly from *Fraxinus*, by honey bees in urbanized areas of Colorado in spring. When assessing potential collection of that pollen as a significant source of pesticide exposure other details are needed, notably the amount of pesticide residue appearing in pollen. This is a subject addressed in Chapter 3.



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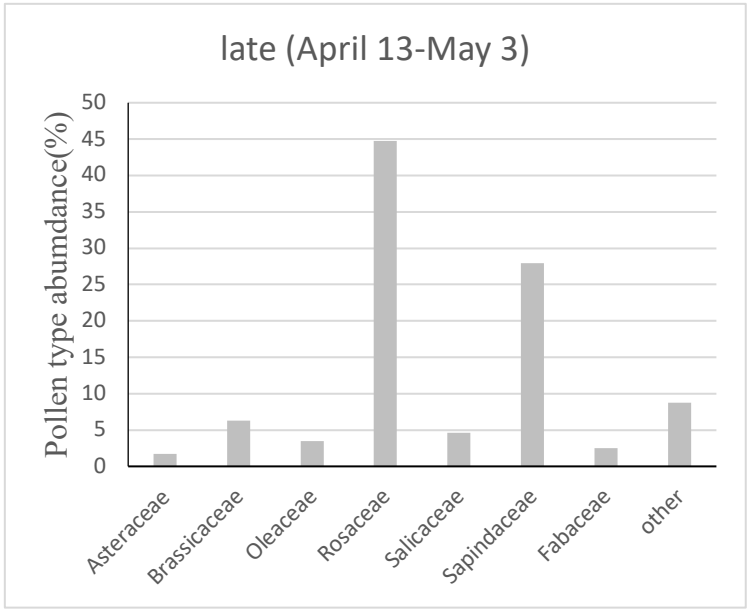

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Figure 1.1 Summary of the relative abundance of pollen, by plant family, collected at colonies of honey bees located in urbanized areas of northeastern Colorado during the early spring (A), mid spring (B), and late spring (C), 2014.

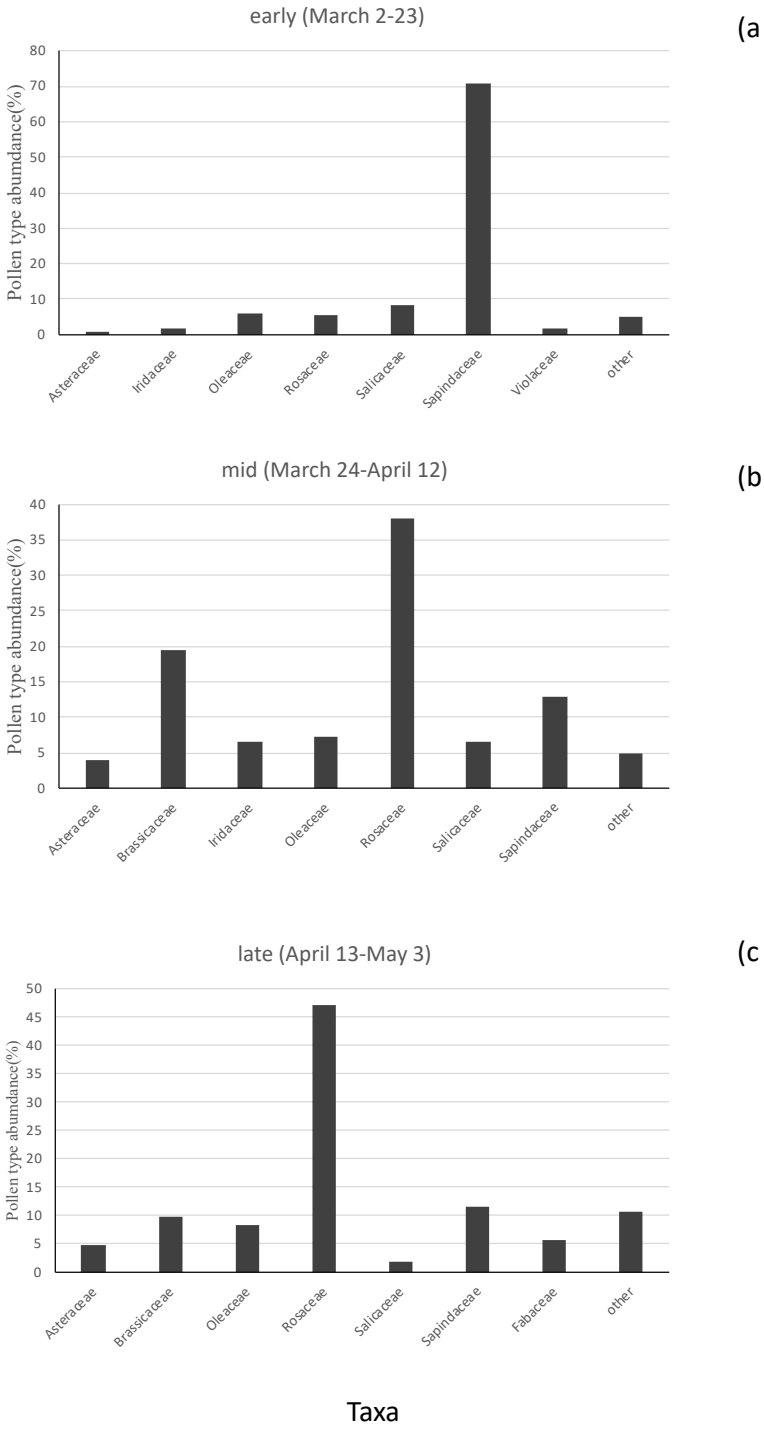

Figure 1.2 Summary of the relative abundance of pollen, by plant family, collected at colonies of honey bees located in urbanized areas of northeastern Colorado during the early spring (A), mid spring (B), and late spring (C), 2015.

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Chapter 2 Investigation of Leaf Decomposition Following Use of Trunk Injected Insecticides to Control Emerald Ash Borer

SUMMARY

Studies were conducted over a four year period (2015-2018) to determine if there were measurable differences in decomposition of green ash (*Fraxinus pennsylvanica* Marshall) leaves collected from trees that had earlier received trunk injection applications of the insecticides azadirachtin or emamectin benzoate used for control of emerald ash borer, *Agrilus planipennis* Fairmaire. In trials initiated in autumn 2015 and 2016, when leaves were placed shallowly underground within litterbags that allowed ready access by earthworm (5mm mesh), all leaves were completely decomposed when examined the following spring, regardless of insecticide history (emamectin benzoate, azadirachtin, untreated check). Subsequent trials (2017, 2018) used litterbags with a small diameter mesh (2mm) that would exclude earthworms but allow access by microarthropods involved in litter decomposition. These later trials also included different treatments that involved placement of litterbags (soil surface, shallow burial) and time of exposure in the field before collection (90 days, 150 days), and in each year leaves were collected from two different sites. There were significant reductions (compared to the untreated control) in leaf area loss from litterbags in at least one treatment involving leaves collected from emamectin benzoate (TREE-äge) treated trees in three of the four studies initiated in 2017 and 2018. There were significant reductions (compared to the untreated control) in leaf area loss from litterbags in at least one treatment involving leaves collected from azadirachtin (Tree-Azin), with reductions in leaf area loss that were comparable to those from leaves of trees treated with emamectin benzoate. The proposed reason for reduction in leaf decomposition is that it is due to effects on microarthropods involved in decomposition, such as Collembola and Oribatida.

INTRODUCTION

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a species native to eastern Asia that was accidentally introduced into and later established in North America (Poland and McCullough 2006, Kovacs et al. 2010). Since its original 2002 North American detection, in Michigan, EAB has become both highly invasive and destructive, causing extensive losses to North American species of *Fraxinus* (ash) (Siegert et al. 2007, Herms and McCullogh 2013). In the 18 years following the initial detection emerald ash borer has been found in 35 states and five Canadian provinces (Emerald Ash Borer Information Network 2020). In Colorado, the first detection of this insect was in September 2013, in the city of Boulder. Presently, EAB is known from five Colorado counties (Boulder, Adams, Broomfield, Larimer, and Jefferson) with all detections outside Boulder County occurring in 2019-2020 (Colorado Department of Agriculture 2020).

Tunneling by larvae of EAB, primarily in the cambium, can cause a progressive decline of trees that predictably will ultimately lead to tree death. Once EAB becomes established at a site essentially only two options are available regarding the future of susceptible ash at the site: 1) either treat with insecticides which have proven effective for control of emerald ash borer (Herms et al. 2019), or 2) remove the tree, either before or after it is killed by EAB. The only effective controls that have been identified are certain systemic insecticides, which are applied on an annual or biannual basis.

Where EAB becomes established, treatments for its control result in a huge increase in insecticide applications to trees in urbanized areas. Ash trees – primarily a mixture of green ash (*Fraxinus pennsylvanica* Marshall) and white ash (*F. americana* L.) – comprise between 15 and 20 percent of the urban forests in Colorado (Colorado State Forest Service 2016), a typical figure

for the composition of ash in urban forests of much the northern half of the country (Nowak 2010).

Several public concerns have been raised in Colorado regarding the potential effects resulting from the greatly expanded use of insecticides used to manage EAB. Most of these involve potential effects on non-target organism (e.g., pollinators, wildlife). Included among these concerns are what effects may result from leaves from trees that have been treated with insecticides for control of EAB and then drop to the ground with autumn leaf fall. As noted by Hahn et al. (2011) this is a subject that has received little study.

Because of where most ash trees are located in Colorado, and because of the expense and difficulty in control of EAB, essentially all ash trees that will potentially be treated with insecticides for control of EAB are high value trees that will have been planted by humans and are located on residential or commercial properties, and in public areas maintained by municipalities. The leaves that fall from these trees may have several different fates. Many, perhaps most, are raked and removed from the site, ultimately disposed either at landfills or in composting facilities. A small amount may find their way into open waters, either directly or through storm sewers.

Many of the leaves may remain on site or be blown onto adjacent properties. Some may ultimately be chopped with lawn mowers or crushed, but the leaves remaining at terrestrial sites will ultimately be decomposed. The processes involved in decomposition of leaf litter is reviewed by Krishna and Mohan (2017). Physical and chemical processes are involved, but also decomposition has a large biological component that include bacteria, fungi, and various invertebrates. Among the invertebrates involved in the processes of plant matter decomposition, earthworms are generally considered to be most important (Seeber et al. 2006, Krishna and

Mohan 2017), but microarthropods can have a moderate but significant impact on decomposition (Kampichler and Bruckner 2009). Many of the fallen leaves remaining on or near the site of an ash tree in Colorado will be on or near turfgrass Potter et al. (1990a) showed that earthworms are the most important macrodecomposer of turfgrass thatch, with Collembola (springtails) and Oribatida mites being of lesser importance. Earthworms usually occur in the vicinity of ash trees growing in Colorado, although all are introduced species, mostly of European origin (Reynolds and Damoff 2011).

Several studies have assessed the effects of pesticides in plant residues on the subsequent degradation of plant matter (Weary and Merriam 1978, McCauley 1979, Andrews and Kenerley 1979, Rai and Srivastata 1983) and a majority of these involve fungicides. A few studies have specifically examined the effects of insecticide residues occurring in plant residues on terrestrial invertebrate populations involved in plant matter decomposition (Kreutzweiser et al. 2008, Kreutzweiser et al. 2009, Kreutzweiser et al. 2011), and some other studies have looked at effects pesticide applications on activity of invertebrate macrodecomposers (earthworms, certain arthropods) (Perry et al. 1997, Dureja and Tanwar 2012), including several involving insecticides applied to turfgrass (Potter et al. 1990b, Potter 1994, Peck 2009, Larson et al. 2012).

Four insecticides have been identified to be useful for managing EAB: imidacloprid, dinotefuran, emamectin benzoate, and azadirachtin (Herms et al. 2019). Two of these are neonicotinoids (imidacloprid, dinotefuran) and were banned by city and county governments for use where use where EAB was first established (Boulder) the use of these insecticides has not been allowed by city and county governments due to concerns about potential hazards to pollinators. This has prevented the use of these insecticides on public properties, including rights-of-way alongside roads. As a result, the overwhelming majority of EAB treatments in

Colorado, to date, have involved the use of the non-neonicotinoid insecticides, emamectin benzoate or azadirachtin, both of which are applied as trunk injections. The focus of this study is to determine if there are effects in the rate of terrestrial decomposition of fallen ash leaves from trees that have been treated with these two insecticides.

MATERIALS AND METHODS

All leaves used in this study were collected from green ash trees located within Boulder, Colorado in areas of known insecticide history. Two sites were sampled, one at the University of Colorado, Boulder the other near downtown Boulder. At these sites trials were initiated in spring 2014 to evaluate performance of two EAB insecticide treatments, emamectin benzoate (TREE-äge) or azadirachtin (Tree-Azin) injected into the lower trunk of the trees. Injections were made by representatives of the company producing the product (TREE-äge, Arborjet, Inc., Woburn, Massachusetts and TreeAzin (BioForest Technologies, Inc., Sault Ste. Marie, Ontario). Rates used for all injections were based on the listed medium rate of product on the label. At each study site there were six replications of each treatment, including untreated checks.

In each year of this study, leaves were collected at both Boulder sites at the end of the season timed for a period when leaves were yellowing and beginning to fall from the trees. Leaves from individual trees were separately bagged and samples were taken from a minimum of three trees at each site, from each treatment history, which provided three replications. Upon return to the laboratory the leaves were immediately refrigerated until prepared to place in field sites.

In preparation for placement in the fields, the leaves were briefly removed from refrigeration to allow processing, prior to their placement in the field. Processing involved

removal of ten leaves from each collected sample, then placing them in a plant press to dry and flatten. These were then scanned with a photocopier and the leaf area of the sample was determined using ImageJ, an image processing software program (Rasband 1997-2016) used to obtain the area of each leaf.

The dried leaves were then placed in individual litterbags. The use of litterbags is a common method used in many studies to measure decomposition rates of leaf litter (Karberg et al. 2008) including those that have evaluated effects of microarthropods in terrestrial decomposition (Kampichler and Bruckner 2009). In all studies, nylon mesh litterbags (15 cm x 19.5 cm) were used to hold samples, although mesh size openings varied (5 mm, 2 mm) between trials as indicated. The litterbags containing the leaves were then placed at field sites. Leaves collected from the different sites (University of Colorado, downtown Boulder) were placed in parallel arrangements, with the litterbags from each site spaced in a complete randomized design.

At the end of the field exposure period the litterbags were then removed from each field site and returned to the laboratory. The leaf material remaining in the bags was removed and leaf area of the remaining material rescanned.

A total of four trials were conducted during this study, beginning in autumn of the years 2015-2018:

Trial 1, 2015. A preliminary trial was initiated on 23 October 2015 using fall green ash leaves collected from trees treated with either emamectin benzoate (2014), azadirachtin (2014, 2015), or that had never exposaed to any insecticides. Leaves were placed in nylon mesh bags with 5 mm opening and taken to a residential yard of irrigated turfgrass in Fort Collins, Colorado. Each litterbag was inserted into the upper root of the turfgrass, approximately 2-7 cm deep. The turfgrass was lifted using a flat shovel, the litterbag inserted underneath the flap of

turfgrass, then immediately covered (Potter 1990). The litterbags were recovered the following spring, 240 days after placement in the yard.

Trial 2, 2016. Since complete decomposition of all leaf samples occurred in the first study, the location of the second trial was moved to the Colorado State Forest Service Nursery in Fort Collins, Colorado. The area of the study was in a previously cultivated field used for plant production, but there was no plant cover on the site during the study and the site was not irrigated. On November 5, litterbags with a 5mm opening were inserted just beneath the soil surface, approximately 2-6 cm below ground, with a 60cm separation between each bag. The litterbags were removed for examination from the field site the following spring, 240 days after placement in the field.

Trial 3, 2017. The third trial was conducted at the same site as the previous year but included several modifications. One was a change in the litterbag mesh opening size, which was reduced to 2mm. This was done to largely exclude earthworms but to still allow the leaf litter to be accessible to various microarthropods (e.g., collembolans, oribatid mites) involved in decomposition (Karberg et al. 2008). A second modification was to include a second set of litterbags placed on the soil surface, which were staked, and lightly covered with leaf litter. A parallel set of litterbags was buried shallowly in soil in a manner similar to the 2016 study. A third modification was to collect the litterbags after shorter periods of field exposure, either 90 days or 150 days. These litterbags were placed in the field trial December 20, with a 60cm separation between bags.

Trial 4, 2018. The fourth study was a replication of the 2017 trial. This field trial was initiated on December 11.

Statistical Analysis. Data collected in this study was the leaf area of the green ash leaves initially placed at the field compared with the leaf area of the leaves at the end of the trial period. Data from both sites and years were analyzed using ANOVA models produced in opensource R statistical software area using means generated with the emmeans and dpplyr packages in R version 3.5.3 statistical software (R Core Team 2019; Ott and Longnecker, 2010). Pairwise comparisons between treatments, depth and time were considered using a Tukey's comparison method. Once means were calculated, they were grouped by levels of significance ($p < 0.05$) by creating a cld display groups of pairwise comparisons.

RESULTS

Trial 1, 2015. In all litterbags removed at the end of the exposure period, all leaf litter was completely decomposed and apparently incorporated into the soil in the decomposition process. Only small pieces of the larger veins remained in litterbags at the end (240 DAT) of this trial. No differences in the amount of leaf mass loss area were observed among the green ash leaves regardless of the history of insecticide use of the tree from which they were collected. The litterbags used in this trial had a mesh size (5mm) that easily allowed access to earthworms, and the site was an irrigated residential turfgrass area that supported abundant numbers of earthworms.

Trial 2, 2016. In 2016 the trial was repeated but the site for the trial was moved to a bare soil area not irrigated. Again, the leaf material placed in all litterbags was found to be completely decomposed and incorporated into the soil in the decomposition process when the bags were removed after 240 days. No differences in the amount of leaf area loss were observed among the green ash leaves regardless of the history of insecticide use of the tree from which they were

collected. The litterbags used in this trial had a mesh size (5mm) that would allow access to earthworms.

Trial 3, 2017. Several modifications in experimental design were made in 2017, including reduction of mesh size of litterbags from 5mm to 2mm, placement of the litterbags in two different locations (shallowly buried, placed on soil surface), and recovery of the litterbags at 90 or at 150 days after field placement.

Among experimental variables, the most consistent differences were related to placement of the litterbags. Leaves from collected from trees at the University of Colorado site (Table 2.1, Figure 2.1) and placed beneath the soil surface averaged a 40.9 percent (range 17.8-65.2) loss of leaf area, which was about six times that of leaves placed on the soil surface (average 6.6, range 4.5-8.3). Leaves collected from street trees at the Downtown location (Table 2.3, Figure 2.2) showed a similar percentage of leaf loss, averaging 39.8 percent (range 12.0-71.4) from litterbags placed below ground, about four times the leaf loss of leaves placed on the soil surface (average 10.6, range 4.8-27.3).

Consistent differences were also noted based on the length of time litterbags were maintained at the field site. Leaves from collected from trees at the University of Colorado site (Table 2.1, Figure 2.1) and exposed for 150 days averaged a 34.2 percent (range 7.6-65.2) loss of leaf area, which was about 2.5 times that of leaves collected after 90 days (average 13.4, range 4.5-26.1). Leaves from collected from trees at the Downtown location (Table 2.3, Figure 2.2) and exposed for 150 days averaged a 38.7 percent (range 8.9-71.4) loss of leaf area, over three times the average leaf area loss of 11.6 percent (range 4.8-23.4) of leaves collected after 90 days. Very few differences were observed in the amount of leaf area loss of leaves collected from trees with different insecticide history. None of the differences were significant from leaves collected

at the University of Boulder site (Table 2.2), regardless of litterbag placement or length of field exposure. Similarly, there were no significant differences among leaves collected from the Downtown site after 90 days (Table 2.4). However, significant differences did emerge at 150 days. Leaves collected from emamectin benzoate-treated trees that were shallowly buried showed a lower amount of leaf loss (53.0 percent) than leaves from trees that did not have insecticide history (61.8 percent). Among leaves placed on the soil surface, there was an approximate 3-fold lower percentage leaf area loss from leaves collected from trees treated with emamectin benzoate (9.6%) and azadirachtin (8.9%) compared to leaves from untreated trees (27.3%).

Table 2.1 Percentage loss of leaf area (mass) of green ash leaves following field exposure within litterbags. Leaves were collected at senescence in October 2017 from trees on the University of Colorado campus (Boulder, Colorado) with known history of insecticides used to control emerald ash borer. Leaves were placed in litterbags with 2 mm mesh opening and either buried shallowly or placed on the soil surface and lightly covered with leaves. Percentage loss of leaf mass was determined by comparing the original leaf area of leaves with the leaf area remaining after a field exposure of either 90 days or 150 days. All litterbags were placed in a non-irrigated area of bare soil at the Colorado State Forest Service Nursery, Fort Collins, Colorado.

Insecticide History[a]	Litterbag Placement[b]	Exposure Period[c]	% Loss of Leaf Mass ± SE[d]
Untreated	Buried	90	26.1 ±3.1
Emamectin benzoate	Buried	90	17.8 ± 3.1
Azadirachtin	Buried	90	20.9 ± 5.0
Untreated	Buried	150	57.8 ± 2.8
Emamectin benzoate	Buried	150	58.1 ± 4.0
Azadirachtin	Buried	150	65.2 ± 4.0
Untreated	Surface	90	4.5 ± 1.0
Emamectin benzoate	Surface	90	5.0 ± 1.0
Azadirachtin	Surface	90	6.2 ± 0.8
Untreated	Surface	150	8.0 ± 1.1
Emamectin benzoate	Surface	150	8.3 ± 0.9
Azadirachtin	Surface	150	7.6 ± 0.6

[a] Emamectin benzoate history involved trunk injections with the formulation TREE-äge applied June 2014 and 2016; Azadirachtin history involved annual trunk injections with the formulation Tree-Azin in 2014-2017; Untreated trees have no history of any insecticide use.
[b] Litterbags were either buried shallowly (ca 3 cm) or placed on the surface and covered with a thin layer of leaves.
[c] Exposure of the litterbags were for either 90 days or 150 days following their placement at the field site on December 20, 2017.
[d] Mean percentage leaf area loss and Standard Error based on six replications.

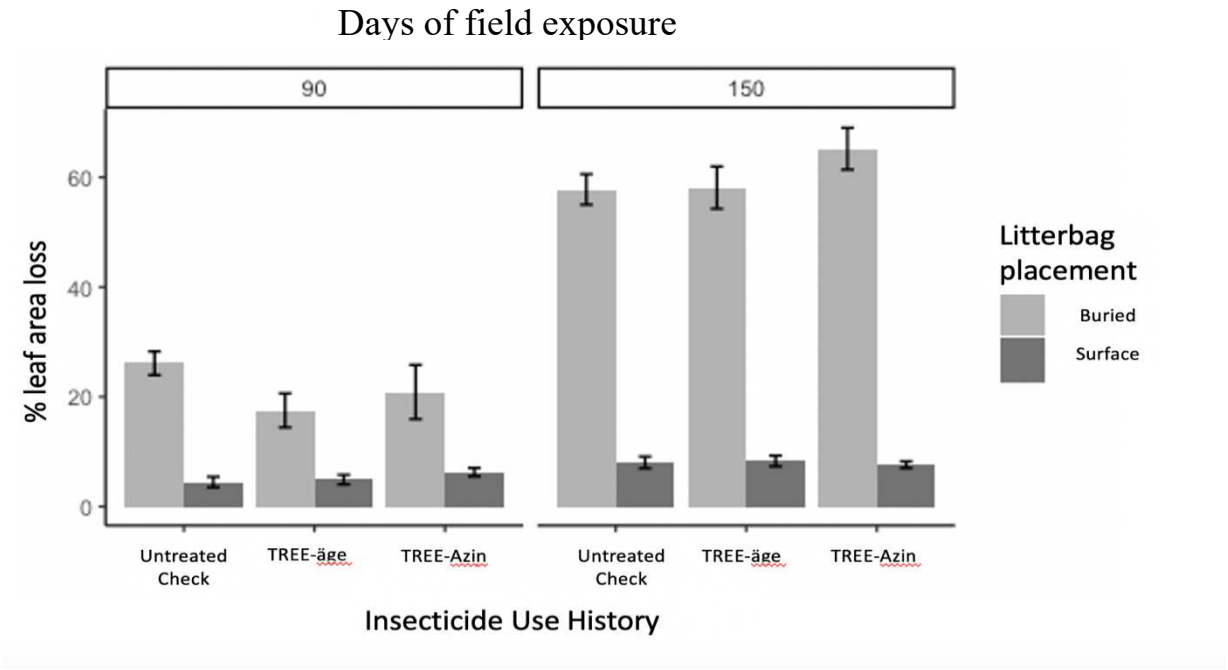

Figure 2.1 Percentage of leaf area loss of green ash leaves within litterbags following field exposure during late autumn 2017-early spring 2018. Leaves used in the study were collected at leaf fall in October 2017 from trees located at the University of Colorado that had a known history of insecticide treatment for emerald ash borer and had received trunk injections of either emamectin benzoate (TREE-äge), azadirachtin (Tree-Azin) or had never been treated with insecticides. Litterbags were either buried shallowly or placed on the soil surface and were exposed at the field site for either 90 days or 150 days. Data are mean ± SE (n = 6).

Table 2.2 Pairwise comparisons of percentage leaf loss from litterbags of green ash leaves collected October 2017 at senescence from trees located on the campus of the University of Colorado with varying history of insecticide use applied to control emerald ash borer. Tukey method used to compare families of 3 estimates, based on insecticide use history, placement of the litterbags at the field site, and length of exposure at the field site. Groups were significantly different (alpha = 0.05).

Insecticide History[a]	emmean	lower.CL	upper.CL	Group
90 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	20.89	14.71	27.1	1
TREE-äge	17.53	11.35	23.7	1
Untreated Check	26.12	19.94	32.3	1
90 Days Field Exposure, Surface Placement[b]				
Tree-Azin	6.26	-3.51	16.0	1
TREE-äge	4.91	-4.86	14.7	1
Untreated Check	4.47	-5.30	14.2	1
150 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	65.21	59.03	71.4	1
TREE-äge	58.12	51.94	64.3	1
Untreated Check	57.80	51.62	64.0	1
150 Days Field Exposure, Surface Placement[b]				
Tree-Azin	7.63	-3.08	18.3	1
TREE-äge	8.30	-2.40	19.0	1
Untreated Check	8.04	-3.92	20.0	1

[a] TREE-äge trees received applications of emamectin benzoate in 2014 and 2016. Tree-Azin trees received applications of azadirachtin annually, 2014-2017.

[b] Leaves were placed in nylon mesh litterbags with 2mm openings. These were placed at a field site at the Colorado State Forest Service Nursery (Fort Collins, Colorado) either on the soil surface or shallowly buried and recovered after placement for either 90 or 150 days.

Table 2.3 Percentage loss of leaf area (mass) of green ash leaves following field exposure within litterbags. Leaves were collected at senescence in October 2017 from street trees near Downtown Boulder (Colorado) with known history of insecticides used to control emerald ash borer. Leaves were placed in litterbags with 2 mm mesh opening and either buried shallowly or placed on the soil surface and lightly covered with leaves. Percentage loss of leaf mass was determined by comparing the original leaf area of leaves with the leaf area remaining after a field exposure of either 90 days or 150 days. All litterbags were placed in a non-irrigated area of bare soil at the Colorado State Forest Service Nursery, Fort Collins, Colorado.

Insecticide History[a]	Litterbag Placement[b]	Exposure Period[c]	% Loss of Leaf Mass $\pm$ SE[d]
Untreated	Buried	90	23.4 $\pm$ 2.6
Emamectin benzoate	Buried	90	12.0 $\pm$ 0.8
Azadirachtin	Buried	90	16.9 $\pm$ 2.1
Untreated	Buried	150	71.4 $\pm$ 3.5
Emamectin benzoate	Buried	150	53.0 $\pm$ 14.0
Azadirachtin	Buried	150	61.8 $\pm$ 9.5
Untreated	Surface	90	7.2 $\pm$ 0.8
Emamectin benzoate	Surface	90	5.5 $\pm$ 1.0
Azadirachtin	Surface	90	4.8 $\pm$ 0.9
Untreated	Surface	150	27.3 $\pm$ 2.8
Emamectin benzoate	Surface	150	9.6 $\pm$ 1.7
Azadirachtin	Surface	150	8.9 $\pm$ 1.4

[a] Emamectin benzoate history involved trunk injections with the formulation TREE-äge applied June 2014 and 2016; Azadirachtin history involved annual trunk injections with the formulation Tree-Azin in 2014-2017; Untreated trees have no history of any insecticide use.
[b] Litterbags were either buried shallowly (ca 3 cm) or placed on the surface and covered with a thin layer of leaves.
[c] Exposure of the litterbags were for either 90 days or 150 days following their placement at the field site on December 20, 2017.
[d] Mean percentage leaf area loss and Standard Error based on six replications.

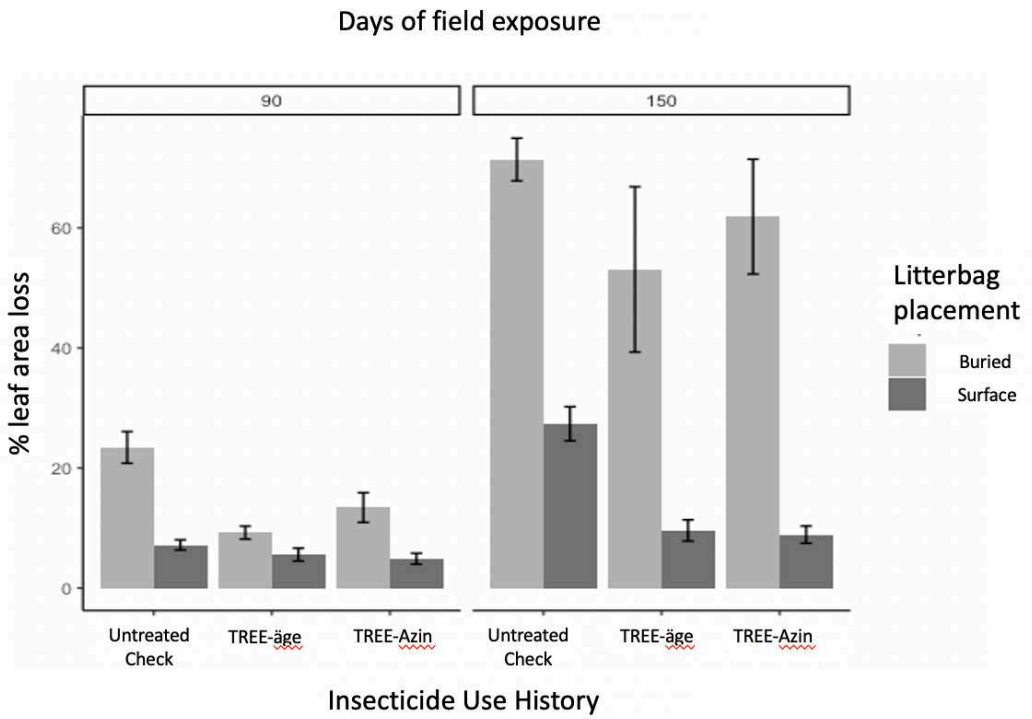

Figure 2.2 Percentage of leaf area loss of green ash leaves within litterbags following field exposure during autumn and winter 2017-2018. Leaves used in the study were collected at leaf fall in October 2017 from street trees located near downtown Boulder, Colorado that had a known history of insecticide treatment for emerald ash borer and had received trunk injections of either emamectin benzoate (TREE-äge), azadirachtin (Tree-Azin) or had never been treated with insecticides. Litterbags were either buried shallowly or placed on the soil surface and were exposed at the field site for either 90 days or 150 days. Data are mean ± SE (n = 6).

Table 2.4 Pairwise comparisons of percentage leaf loss from litterbags of green ash leaves collected October 2017 at senescence from street trees located near downtown Boulder with varying history of insecticide use applied to control emerald ash borer. Tukey method used to compare families of 3 estimates, based on insecticide use history, placement of the litterbags at the field site, and length of exposure at the field site. Groups were significantly different (alpha = 0.05).

Insecticide History[a]	emmean	lower.CL	upper.CL	Group
90 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	13.42	3.02	23.8	1
TREE-äge	9.24	-1.16	19.6	1
Untreated Check	23.44	13.05	33.8	1
90 Days Field Exposure, Surface Placement[b]				
Tree-Azin	4.90	-5.50	15.3	1
TREE-äge	5.57	-4.82	16.0	1
Untreated Check	7.20	-3.20	17.6	1
150 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	61.88	51.48	72.3	12
TREE-äge	53.09	42.70	63.5	1
Untreated Check	71.40	61.01	81.8	2
150 Days Field Exposure, Surface Placement[b]				
Tree-Azin	8.91	-1.49	19.3	1
TREE-äge	9.60	-0.79	20.0	1
Untreated Check	27.38	16.98	37.8	2

[a] TREE-äge trees received applications of emamectin benzoate in 2014 and 2016. Tree-Azin trees received applications of azadirachtin annually, 2014-2017.

[b] Leaves were placed in nylon mesh litterbags with 2mm openings. These were placed at a field site at the Colorado State Forest Service Nursery (Fort Collins, Colorado) either on the soil surface or shallowly buried and recovered after placement for either 90 or 150 days.

Trial 4, 2018. The 2018 studies repeated those of the previous year. Again, consistent differences were correlated to placement of the litterbags. Leaves collected from trees at the University of Colorado site (Table 2.5, Figure 2.3) and placed beneath the soil surface averaged a 35.5 percent (range 3.4-69.0) loss of leaf area, and those placed on the soil surface averaged 7.4 percent (range 1.0-14.2), which was nearly similar to that of the previous season study. Leaves collected from street trees at the Downtown location (Table 2.7, Figure 2.4) again indicated similar percentage of leaf loss, averaging 40.1 percent (range 13.0-66.3) from litterbags placed below ground and 24.6 percent (range 8.8-45.8) among leaves placed on the soil surface. Consistent differences were also noted based on the length of time litterbags were maintained at the field site. Leaves from collected from trees at the University of Colorado site (Table 2.5, Figure 2.3) and exposed for 150 days averaged a 38.2 percent (range 9.5-69.0) loss of leaf area, which was over 6X that of leaves collected after 90 days (average 5.8, range 1.0-14.6). Leaves from collected from trees at the Downtown location (Table 2.7, Figure 2.4) and exposed for 150 days averaged a 44.4 percent (range 26.9-66.3) loss of leaf area, over twice the average leaf area loss of 20.0 percent (range 8.8-48.1) of leaves collected after 90 days.

Greater differences were observed in the amount of leaf area loss of leaves collected from trees with different insecticide history during 2018. Among leaves collected at the University of Boulder site (Table 2.6), the only significant differences observed were at 90 days, when there was a greater measured area of leaf loss from leaves without insecticide treatments (14.6 percent) compared with those from trees treated with emamectin benzoate (3.8 percent) (Table 2.6; P= 0.002). No differences were observed among litterbags left at this site for 150 days. However, among leaves collected from trees at the downtown Boulder location, there was a consistent significantly higher leaf area loss of leaves without a history of insecticide use

(untreated check) compared to those treated with emamectin benzoate or azadirachtin on both collection dates (90, 150 days) and with both litterbag placements (shallow burial, surface) (Table 2.8 ; P <0.0001).There were no differences in the amount of leaf area loss between leaves collected from trees with insecticide use history (azadirachtin, emamectin benzoate). Across all treatments (litterbag placement, length of field exposure) the average percent leaf area loss in the leaves of the untreated check (46.7) was almost twice that of leaves collected from trees treated with emamectin benzoate (25.8) or azadirachtin (24.2).

Table 2.5 Percentage loss of leaf area (mass) of green ash leaves following field exposure within litterbags. Leaves were collected at senescence in October 2018 from trees on the University of Colorado campus (Boulder, Colorado) with known history of insecticides used to control emerald ash borer. Leaves were placed in litterbags with 2 mm mesh opening and either buried shallowly or placed on the soil surface and lightly covered with leaves. Percentage loss of leaf mass was determined by comparing the original leaf area of leaves with the leaf area remaining after a field exposure of either 90 days or 150 days. All litterbags were placed in a non-irrigated area of bare soil at the Colorado State Forest Service Nursery, Fort Collins, Colorado.

Insecticide History[a]	Litterbag Placement[b]	Exposure Period[c]	% Loss of Leaf Mass ± SE[d]
Untreated	Buried	90	14.6 ± 0.9
Emamectin benzoate	Buried	90	3.4 ± 0.7
Azadirachtin	Buried	90	6.4 ± 1.0
Untreated	Buried	150	69.0 ± 4.8
Emamectin benzoate	Buried	150	60.5 ± 3.6
Azadirachtin	Buried	150	65.2 ± 4.0
Untreated	Surface	90	2.9 ± 0.6
Emamectin benzoate	Surface	90	1.0 ± 0.6
Azadirachtin	Surface	90	6.2 ± 0.8
Untreated	Surface	150	14.2 ± 1.7
Emamectin benzoate	Surface	150	9.5 ± 0.8
Azadirachtin	Surface	150	10.5 ± 0.8

[a] Emamectin benzoate history involved trunk injections with the formulation TREE-äge applied June 2014, 2016 and 2018; Azadirachtin history involved annual trunk injections with the formulation Tree-Azin in 2014-2018; Untreated trees have no history of any insecticide use.
[b] Litterbags were either buried shallowly (ca 3 cm) or placed on the surface and covered with a thin layer of leaves.
[c] Exposure of the litterbags were for either 90 days or 150 days following their placement at the field site on December 11, 2018.
[d] Mean percentage leaf area loss and Standard Error based on six replications.

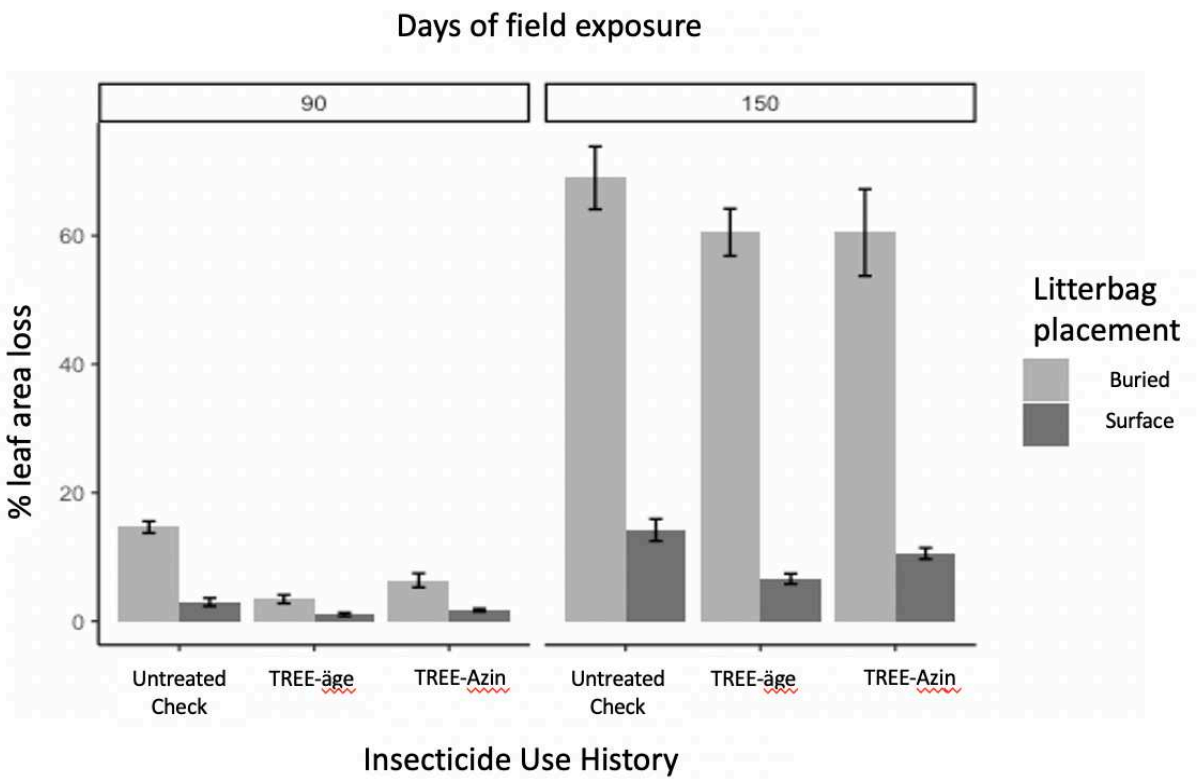

Figure 2.3 Percentage of leaf area loss of green ash leaves within litterbags following field exposure during autumn and winter 2018-2019. Leaves used in the study were collected at leaf fall in October 2018 from trees located at the University of Colorado that had a known history of insecticide treatment for emerald ash borer and had received trunk injections of either emamectin benzoate (TREE-äge), azadirachtin (Tree-Azin) or had never been treated with insecticides. Litterbags were either buried shallowly or placed on the soil surface and were exposed at the field site for either 90 days or 150 days. Data are mean ± SE (n = 6).

Table 2.6 Pairwise comparisons of percentage leaf loss from litterbags of green ash leaves collected October 2018 at senescence from trees located on the campus of the University of Colorado with varying history of insecticide use applied to control emerald ash borer. Tukey method used to compare families of 3 estimates, based on insecticide use history, placement of the litterbags at the field site, and length of exposure at the field site. Groups were significantly different (alpha = 0.05).

Insecticide History[a]	emmean	lower.CL	upper.CL	Group
90 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	6.36	0.82	8.97	12
TREE-äge	3.43	-2.10	11.90	1
Untreated Check	14.64	9.10	20.17	2
90 Days Field Exposure, Surface Placement[b]				
Tree-Azin	1.06	-3.80	16.0	1
TREE-äge	1.06	-4.48	6.59	1
Untreated Check	4.47	-2.57	8.51	1
150 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	60.49	54.96	66.03	1
TREE-äge	60.53	54.99	66.07	1
Untreated Check	69.00	63.46	74.53	1
150 Days Field Exposure, Surface Placement[b]				
Tree-Azin	10.54	4.47	16.60	1
TREE-äge	6.60	1.06	12.14	1
Untreated Check	14.20	8.66	19.73	1

[a] TREE-äge trees received applications of emamectin benzoate in 2014, 2016 and 2018. Tree-Azin trees received applications of azadirachtin annually, 2014-2018.
[b] Leaves were placed in nylon mesh litterbags with 2mm openings. These were placed at a field site at the Colorado State Forest Service Nursery (Fort Collins, Colorado) either on the soil surface or shallowly buried and recovered after placement for either 90 or 150 days.

Table 2.7 Percentage loss of leaf area (mass) of green ash leaves following field exposure within litterbags. Leaves were collected at senescence in October 2018 from street trees near Downtown Boulder (Colorado) with known history of insecticides used to control emerald ash borer. Leaves were placed in litterbags with 2 mm mesh opening and either buried shallowly or placed on the soil surface and lightly covered with leaves. Percentage loss of leaf mass was determined by comparing the original leaf area of leaves with the leaf area remaining after a field exposure of either 90 days or 150 days. All litterbags were placed in a non-irrigated area of bare soil at the Colorado State Forest Service Nursery, Fort Collins, Colorado.

Insecticide History[a]	Litterbag Placement[b]	Exposure Period[c]	% Loss of Leaf Mass ± SE[d]
Untreated	Buried	90	48.1 ± 1.7
Emamectin benzoate	Buried	90	13.3 ± 0.9
Azadirachtin	Buried	90	13.0 ± 3.6
Untreated	Buried	150	66.3 ± 7.5
Emamectin benzoate	Buried	150	51.7 ± 4.1
Azadirachtin	Buried	150	48.1 ± 4.4
Untreated	Surface	90	26.5 ± 3.0
Emamectin benzoate	Surface	90	10.3 ± 1.9
Azadirachtin	Surface	90	8.8 ± 1.0
Untreated	Surface	150	45.8 ± 3.8
Emamectin benzoate	Surface	150	27.8 ± 4.5
Azadirachtin	Surface	150	26.9 ± 6.6

[a] Emamectin benzoate history involved trunk injections with the formulation TREE-äge applied June 2014, 2016, and 2018; Azdirachtin history involved annual trunk injections with the formulation Tree-Azin in 2014-2018; Untreated trees have no history of any insecticide use.
[b] Litterbags were either buried shallowly (ca 3 cm) or placed on the surface and covered with a thin layer of leaves.
[c] Exposure of the litterbags were for either 90 days or 150 days following their placement at the field site December 11, 2018.
[d] Mean percentage leaf area loss and Standard Error based on six replications.

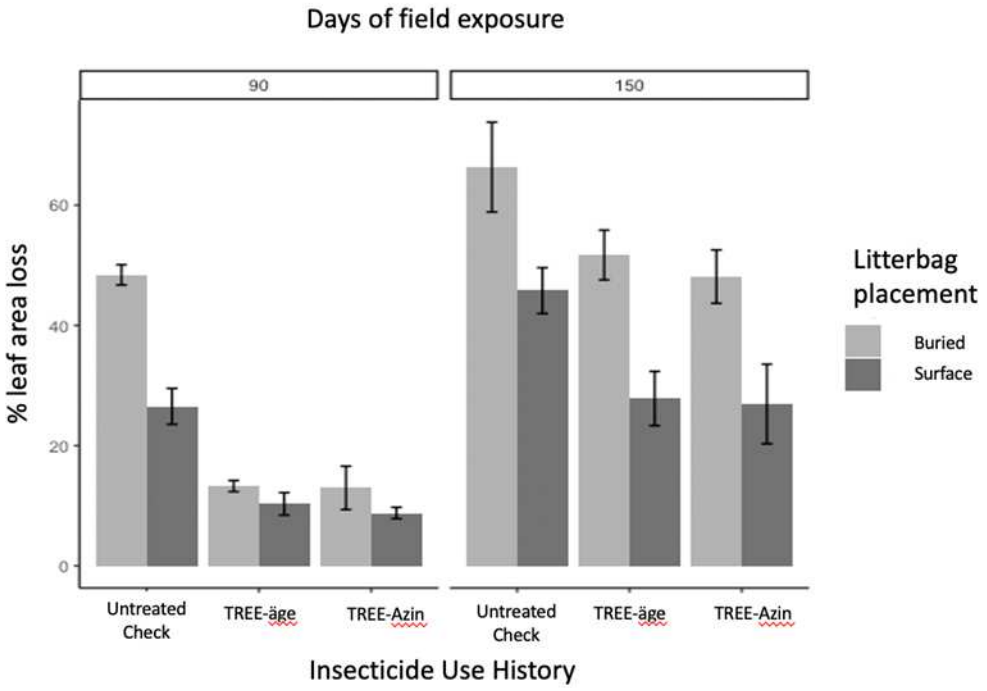

Figure 2.4 Percentage of leaf area loss of green ash leaves within litterbags following field exposure during autumn and winter 2018-2019. Leaves used in the study were collected at leaf fall in October 2018 from street trees located near downtown Boulder, Colorado that had a known history of insecticide treatment for emerald ash borer and had received trunk injections of either emamectin benzoate (TREE-äge), azadirachtin (Tree-Azin) or had never been treated with insecticides. Litterbags were either buried shallowly or placed on the soil surface and were exposed at the field site for either 90 days or 150 days. Data are mean ± SE (n = 6).

Table 2.8 Pairwise comparisons of percentage leaf loss from litterbags of green ash leaves collected October 2018 at senescence from street trees located near downtown Boulder with varying history of insecticide use applied to control emerald ash borer. Tukey method used to compare families of 3 estimates, based on insecticide use history, placement of the litterbags at the field site, and length of exposure at the field site. Groups were significantly different (alpha = 0.05).

Insecticide History[y]	emmean	lower.CL	upper.CL	Group
90 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	12.97	4.78	21.2	1
TREE-äge	13.28	5.08	21.5	1
Untreated Check	48.41	40.22	56.6	2
90 Days Field Exposure, Surface Placement[b]				
Tree-Azin	8.79	0.60	17.0	1
TREE-äge	10.32	2.12	18.5	1
Untreated Check	26.53	18.34	34.7	2
150 Days Field Exposure, Shallowly Buried[b]				
Tree-Azin	48.12	39.93	56.3	1
TREE-äge	51.72	43.53	59.9	1
Untreated Check	66.43	58.15	74.5	2
150 Days Field Exposure, Surface Placement[b]				
Tree-Azin	26.93	18.73	35.1	1
TREE-äge	27.83	19.64	36.0	1
Untreated Check	45.79	37.60	54.0	2

[a] TREE-äge trees received applications of emamectin benzoate in 2014 and 2016. Tree-Azin trees received applications of azadirachtin annually, 2014-2017.
[b] Leaves were placed in nylon mesh litterbags with 2mm openings. These were placed at a field site at the Colorado State Forest Service Nursery (Fort Collins, Colorado) either on the soil surface or shallowly buried and recovered after placement for either 90 or 150 days.

DISCUSSION

This study did document occurrences when green ash leaves collected from trees on fall that earlier had been treated by trunk injection with the systemic insecticides emamectin benzoate and azadirachtin showed reduced leaf area loss from decomposition when placed in field settings, compared to leaves from trees that did not have a history of exposure to insecticides. The studies using of litterbags in 2017-2019 did not determine the cause of the differences in leaf loss. However, the design of the experiments where it occurred involved use of a mesh size (2mm) that excluded earthworms but allowed access by microarthropods known to be involved in decomposition of leaf litter (Kampichler and Bruckner 2009), such as collembolans and oribatid mites.

Hahn et al. (2011) noted that the effect of pesticide residues in senesced leaves that drop from trees treated with emerald ash borer insecticides had not been thoroughly researched. They cited only a few studies involving use of the insecticide imidacloprid, and the study by Kreutweizer et al. (2009) that documented inhibition of leaf litter breakdown associated with adverse effects on decomposer invertebrates by the insecticides. Hahn et al. (2011) specifically mentioned that similar studies had not been conducted with emamectin benzoate. Effects of emamectin benzoate residues in senesced leaves that drop to the soil and are acted upon by aspects of terrestrial decomposition do not appear in the final report of an ecological assessment submitted to the USDA-FS prior to the registration of emamectin benzoate as a trunk injection treatment (Durkin 2010). Data gaps regarding potential non-target effects on terrestrial invertebrates, although not specifically mentioning decomposer species, were among many identified in an ecological risk assessment by the US Environmental Protection Agency while considering registration of emamectin benzoate for trunk injection use (Anderson et al. 2009).

In the present study there were significant reductions in leaf area loss from litterbags in at least one treatment involving leaves collected from emamectin benzoate (TREE-äge) treated trees in three of the four studies conducted during 2017-2018 (downtown Boulder site) and 2018-2019 (downtown Boulder, University of Colorado sites). There were no differences in loss of leaf mass in the studies conducted during 2015-2016 and 2016-2017, with litterbags with mesh size that may have allowed earthworms to enter, when all leaf material regardless of insecticide history completely degraded, regardless of insecticide use history. These results may suggest that there may be adverse effects to microarthropods involved in decomposition of leaf litter, if not earthworms.

Kreutweizer et al. (2011) conducted microcosm studies involving decomposition of ash leaves collected from trees treated by trunk injection with azadirachtin. They found no significant effects on earthworms and on leaf shredding aquatic organisms and concluded azadirachtin in senesced leaf material posed little harm to decomposer invertebrates. In this present study there were significant reductions in leaf area loss from litterbags in at least one treatment involving leaves collected from azadirachtin (Tree-Azin) treated trees at the downtown Boulder site in both 2018 and 2019 years, with reductions in leaf area loss that were comparable to those from leaves of trees treated with emamectin benzoate.

Together these studies do indicate that decomposition of leaves from trees treated with the insecticides azadirachtin and emamectin benzoate can sometimes be reduced significantly under field conditions. The proposed reason for this reduction is due to sublethal or lethal effects on microarthropods involved in decomposition. The ecological impact of this is likely modest, as these organisms often have minor roles in leaf litter decomposition, particularly when compared to earthworms (Kampichler and Bruckner 2009). Furthermore, as stated by Hahn et

al. (2011) effects would be further modified by the mixture of leaves containing insecticide residues with leaves from other sources without residues, which may further reduce the effects on leaf litter decomposition. Leaves collected from trees at leaf fall that were treated with the insecticides azadirachtin and emamectin benzoate did show reduced decomposition in some studies when placed in litterbags that excluded earthworms. The proposed reason for this reduction is due to effects on microarthropods involved in decomposition, such as Collembola and Oribatida.

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Chapter 3 Assessment of Emamectin Benzoate Residues Present in Senescent Foliage, Flowers, and Pollen Following Trunk Injection Treatment of Ash Trees

SUMMARY

During 2017 and 2018 samples were taken of various types of plant tissue (senescent foliage, flowers, pollen) collected from green ash (*Fraxinus pennsylvanica* Marshall) that had been treated with emamectin benzoate by trunk injection, used to control emerald ash borer, *Agrilus planipennis* Fairmaire. All samples of senescent foliage collected in October 2018, approximately four months after emamectin benzoate application, had detectable residues of emamectin benzoate but at levels never exceeding 1.1ppb. Sample collections of the trees made the previous season (October 2017), when a period of 16 months had passed since the insecticide application, detected far lower levels of residues. Levels of emamectin benzoate levels in 2018 foliage averaged slightly higher in trees that were treated with the TREE-äge formulation (0.425ppb) than those treated with a different formulation (Boxer) and injection method (0.362ppb) but the amount of amount of active ingredient introduced into the tree by the latter formulation/method (Boxer) was about 15% of that introduced by the former (TREE-äge). Levels of emamectin benzoate appearing in flowers was lower than that appearing in foliage and was detected only in 8 of 22 samples at the levels of quantification allowed in this analysis (0.28ppb). Among the 60 samples of pollen collected from trees that had been treated with emamectin benzoate, emamectin fragment ions were not detected at the Limit of Detection (LOD) attained with this analysis (0.1ppb). In one collection series of pollen made in 2018, 7 of the 16 samples were classed as >LOD, indicating that residues may have been present but at levels below the limit of the quantification of the analysis to be detected consistently. The very low levels of emamectin benzoate detected in pollen suggests that pesticide exposure is

negligible for species that collect pollen from green ash trees treated by trunk injection with emamectin benzoate.

INTRODUCTION

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), an insect of Asian origin that was accidentally introduced into North America. First discovered in 2002 within a six-county area in southeastern Michigan (Cappaert et al., 2005) this insect has since spread and is currently known from 35 states and five Canadian provinces (Emerald Ash Borer Network, 2020). The first detection of the beetle in Colorado was confirmed in September 2013 in Boulder.

Emerald ash borer quickly established itself in North America as an invasive species with enormous destructive potential to threaten essentially all of the estimated 8.7 billion ash (*Fraxinus* spp.) trees present in North America (Emerald Ash Borer Network 2020). Damage is caused by larval tunneling of the cambium, typical of buprestid larvae (flatheaded borers). Newly infested trees with low larval densities initially show no external symptoms (Poland and McCullough 2006) but over time the cumulative effects of larval galleries progressively damage the trees, causing disruption of water and nutrient movement that typically results in tree death (Cappaert et al. 2005).

In the years following its North American discovery there has been an intense effort to identify methods to effectively manage this invasive pest, well summarized by Herms and McCullogh (2014). Early efforts identified the systemic insecticide imidacloprid, applied as a soil treatment for root uptake or trunk injection, as the first promising control. Later work expanded control options and presently there are four insecticides, all with systemic activity in plants, that are being used in the United States to control this insect: imidacloprid, applied as a

soil treatment or trunk injection; dinotefuran, primarily applied as a spray drenching the lower trunk; azadirachtin, injected into the trunk; and emamectin benzoate, injected into the trunk (Hermes et al. 2019).

Of the above insecticides, the most common treatment used in Colorado to date has been the trunk injections with emamectin benzoate. Emamectin benzoate is a synthetic compound within the avermectin family of insecticides, compounds that were originally discovered and are produced through fermentation process by the soil actinomycete, *Streptomyces avermitilis* (Dybas et al. 1989). Development of emamectin benzoate as an insecticide used in tree care began with studies that identified is as useful in control of nematodes (Takai 2004). It was later tested for the control of various bark beetles and wood-boring beetles (Grosman and Upton 2006; Grosman et al. 2009) and its potential value in the control of EAB was recognized soon thereafter (Smitley et al. 2010, McCullough et al. 2011).

The first U.S. registration of emamectin benzoate for use as a trunk injection was provided for the formulation TREE-äge (Arborjet, Inc., Woburn, Massachusetts). Prior to this registration numerous major data gaps were identified in the US Environmental Protection Agency review while considering registration of emamectin benzoate for trunk injection use (Anderson et al. 2009), mostly involving potential impacts on non-target organisms. Many of these remained unanswered in the final report of an ecological assessment submitted to the USDA-FS prior to the registration of emamectin benzoate as a trunk injection treatment (Durkin 2010). As a result, following its widespread use many questions remained about the environmental fate of emamectin benzoate following trunk injection applications to trees and their potential effects on non-target organisms, including pollinators and organisms involved in leaf litter decomposition. Hahn et al. (2011) reviewed many of the potential side effects of

insecticides used to manage emerald ash borer. Much of this review involves research with the neonicotinoid insecticide imidacloprid which has been extensively studied (Goulson 2013, Blacquie`re 2012), including the levels of residues that appear in various plant parts that cause significant colony level effects to the honey bee (*Apis mellifera* L.) and methods for evaluating hazard (Stoner and Eitzer 2013). A recent summary of the potential exposure hazards of imidacloprid to honey bees is by Cowles and Eitzer (2017).

The two primary studies to date that have examined the environmental fate of emamectin benzoate following trunk injection of trees are those of Burkhard et al. (2015), who conducted studied the effects from fallen leaves from horsechestnut (*Aesculus hippocastanum*, L.) and Coslor et al. (2019), who measured levels of emamectin benzoate residues in different plant parts, including pollen, following trunk injection of apple (*Malus* sp.). To better understand the potential hazards of emamectin benzoate trunk injections used to manage EAB, additional studies are needed. The objective of this study was to provide basic information on the levels of emamectin benzoate that appear in specific plant tissues – fallen leaves, flowers, and pollen – following field applications through trunk injection of ash trees treated for the control of EAB.

MATERIALS AND METHODS

Sample Sites and Insecticide Treatment History

All leaf samples were collected from green ash trees located in Boulder, Colorado. Most collections were from two locations that were established in 2014 to evaluate insecticides used to control EAB.

CU Site. The site located on University of Colorado (CU) campus (40.008822, -105.243514) involved trees that were both located immediately adjacent to the street, between a

sidewalk and the street, and trees growing in a parallel row that were located beyond the sidewalk. Trunk diameters (DBH) of the trees varied, with those immediately adjacent to the street being smaller (229-279mm/9-11 inch) than those behind the sidewalk (279-330mm/11-13 inch). Insecticide treatments of these trees were initiated in June 2014 and samples taken for residue analysis used from this site involved two different insecticide use histories, in addition to the untreated controls. One treatment involved trunk injection of the insecticide emamectin benzoate, applied as the formulation TREE-äge (Arborjet, Inc., Woburn, Massachusetts). This formulation is injected into trees through a series of small diameter holes (5/8-inch/16mm diameter) drilled into the lower trunk (2-inch/51mm depth). The insecticide is then injected into the tree under low pressure using the QUIK-jet AIR injection system. Insecticides were applied at the medium rate (55ml in a tree of 254-305mm/10-12inch diameter), per label use instructions (Anonymous 2020a) by representatives of the company. Prior to residue sampling for this study trees were treated in June 2014 and 2016.

A second treatment involved a different method of application and insecticide formulation that were produced by ArborSystems (Omaha, Nebraska). The method of application, known as the Wedgle Direct-Inject™, does not involve pre-drilling and instead uses a specialized tip that can be pushed through the outer bark. The insecticide is then injected directly into the cambium, under low pressure produced by a hand pump of the injector. The formulation of emamectin benzoate used in the trees at this site, Boxer, was only applied in 2016 at this site, since it was not registered for use on trees in 2014. Boxer is a 4% concentration of emamectin benzoate. It was applied a representative of the company, at the labeled rate of 1 ml/injection hole, with injections spaced every 102mm (4 inches) into the lower trunk. Trees from this site were sampled in 2017 and 2018 for residue analysis of leaves, flowers, and pollen.

Spruce/Pearl Site. The second primary site for this study involved street trees growing between the sidewalk and street, located near downtown Boulder (40.022462, - 105.259654). Trees were relatively uniform in size, with trunk diameters (DBH) ranging between 330-381mm/13-15 inches. Insecticide treatments of these trees began in June 2014 and samples taken for residue analysis used from this site involved only one insecticide, emamectin benzoate (TREE-äge), along with the untreated controls that had no history of insecticide application. The insecticides were trunk injected in the same manner as the trees at the CU site, by the same representatives of the manufacturer of the insecticide and application equipment (Arborjet), and were made the day following the 2014 and 2016 applications at the CU site. Trees from this site were sampled in 2017 and 2018 for residue analysis of leaves, flowers, and pollen.

City Trees for Pollen Sampling. To provide a larger sample size for the detection of emamectin benzoate in pollen, additional trees were included from four different locations on City of Boulder property that were treated with TREE-äge by a city contractor in 2017. Pollen from these trees was collected in 2018 for residue analysis.

Sample Collection and Handling

In 2017 and 2018 samples of ash foliage, flowers and pollen were collected from the described study sites. In all collections individual samples of plant material were from a single tree, and all collections involved either 5 or 6 trees. This provided either 5 or 6 separate samples from each collection that could be used for residue analysis. Samples of foliage were collected at senescence to determine residue levels present in leaves that subsequently drop from the trees (Chapter 2). Collection dates were October 6, 2017 and October 18, 2018, at which time and samples were taken at both sites (CU, Pearl/Spruce). Foliage from each tree was placed in a

plastic Ziplock bag, transferred to a cooler, then returned immediately to the laboratory. The samples were then frozen at -20°C until processed for preparation of residue analysis.

Samples of pollen and flowers were collected from the CU and Spruce/Pearl sites on April 3, 2017 and April 5, 2018. Samples of additional City of Boulder trees used for collection of pollen were made on April 10, 2018. During collections flowers from each tree were placed in individual paper bags, transferred to a cooler and returned immediately to the laboratory. Pollen was then immediately extracted from the flowers using a 114mm/4.5-inch tall Aluminum Pollen Shaker (trade name Pollen Stash), which was used to shake the pollen onto a clean piece of filter paper. This was then transferred to a 5.0mL Eppendorf Tubes™.

Only extracted pollen was retained for residue testing from the additional City of Boulder trees sampled in 2018; extracted pollen and flowers were separately retained from the flowers collected at the other sites in 2017 and 2018. After extraction, the pollen and the intact flowers were frozen at -20°C until processed for preparation of residue analysis.

Sample Preparation

Samples were prepared for HPLC/MS/MS using the method of a QuEChERS Q-sep® Packets (cat. # 26235 and 26236) (Burkhard et al. 2015). First, leaf and flower tissue were homogenized in liquid nitrogen with a mortar and pestle, and 500 mg of the homogenized samples were weighed into a clean 50 mL tube (cat. # 26239 QuEChERS Q-sep®) and assayed by HPLC/MS/MS. Pollen samples were not homogenized and 100mg were weighed into 50mL tubes. Extraction solution (1 µg of deuterated (d_4) of the internal standards 31732-100mg Abamectin PESTANAL® internal standard was added to acetonitrile + 1% acetic acid) was added to each sample, 15 mL (flowers, leaves) and 3mL to the pollen of added was vortexed for 1 minute. Abamectin, also known as avermectin B1, was used as an internal standard since it

should have similar properties to emamectin, but is not present in the samples as well as having a different ion fragment that is detected, so we can compare the response of the internal standard (peak size) to the emamectin peak (Durden 2007).

Magnesium sulfate and sodium acetate were added before centrifugation for 5 minutes at 5,000 rpm to separate the solid material. The supernatant was collected and further purified using a dispersive solid-phase extraction cartridge proceeded by dSPE sample cleanup (Agilent Technologies, # 5982-5022). Samples were then transferred to 2 ml glass auto-sampler vials and were stored in the freezer until they were aliquoted in X mL volumes into a 96 well-plate and analyzed by HPLC/MS/MS.

Calibrants were created from a stock solution of 101ng/mL of emamectin benzoate dissolved in 50:50 Acetonitrile: H_2O with 0.1% formic acid by volume and diluted with supernatant from untreated samples to simulate the matrix of the samples. The calibrants ranged from 10ng/mL to 0.1ng/mL of emamectin benzoate with 0.4µg/mL of abamectin added as an internal standard (IS). The double blank contained only sample matrix, while the matrix blank contained only the IS without any emamectin benzoate.

Instruments and Analytical Determinations

The analytical determination was by tandem mass spectrometry (LC–MS/MS) performed with a Waters Acquity H-Class UPLC coupled to a Waters Xevo TQD tandem mass spectrometer system set to positive ESI. LC saperation was performed on a Waters Aquity UPLC BEH C18 column, dimensions 2.1X50 mm and a pore size of 1.7 µm (part # 186002350). The X software (version X) was used to acquire and process all data (Durden 2007, Inoue et al. 2009).

LC Method. The column temperature was set to 20 °C along with the probe temperature, and the flow rate was held at 0.4mL/min. Injection volume was 1µL. At the start of the run, the

mobile phase was a mix of 78% water, 20% acetonitrile, 2% 10 mM ammonium acetate, and increased at a linear gradient over 2.5 minutes to 0% water, 98% acetonitrile, and 2% ammonium acetate. This was held for an additional 0.5 minutes, then dropped back to 78% water, 20% acetonitrile, 2% 10 mM ammonium acetate, which was maintained for another minute (4-minute total run time).

MS Method. MS/MS was performed using an SIR of mass 896.00 for the internal standard abamectin, with a dwell time 0.005s, and a cone voltage of 20V. An MRM of 2 mass pairs for emamectin was also performed (Table 3.1). Lastly, a MS Scan from 150 to 1000.0m/z was performed with a cone voltage of 30V and a scan duration of 0.5sec. Conditions were optimized using direct injection in ES+ mode.

Table 3.1 Mass spectrometric parameters of the characteristic compound: parent ion, daughter ions conditions and collision energies of charestic compound (emamectin benzoate) MS/MS.

Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collison (V)
emamectin	886.0000	158.0000	0.005	20	35
emamectin	886.0000	302.0000	0.005	20	30

Data collection and verification. For a plate to be considered viable for further analysis, at least 6 non-zero calibrators should be ± 15% of theoretical concentrations, with the LLOQ being 20% of the theoretical concentration. For quality control at least 3 QCs: a LQC, MQC, and HQC, were run in duplicate for each batch. At least 67% of QCs total should be ±15% of theoretical concentration and at least 50% of QCs per level (L, M, H) should be ±15% of theoretical concentration. Study Pool QC (SPQC) sample was created by mixing an equal volume from each unknown sample.

RESULTS

Samples of Senescent Foliage. Among the 40 samples of senescent foliage collected from trees (Table 3.2) that had been treated with emamectin benzoate, emamectin fragment ions were detected in 26 samples, at the Limit of Detection (LOD) attained with this analysis (0.06 ng/mL = 0.06 ppb). Emamectin was not detected in any of the samples collected from untreated control trees (n=24), which had no history of insecticide application.

Between the two seasons, levels of residue were lower in the leaves collected in October 2017, which had been treated most recently treated with emamectin benzoate in June 2016. Residue levels were detected in 5 of the 6 trees treated with TREE-äge at the CU site, and 4 of the 6 trees at the Pearl/Spruce site, with average residues present occurring at levels near the LOD. Emamectin benzoate was not detected in any of the samples collected from trees treated with the emamectin benzoate formulation Boxer in 2016 at the CU site.

Residues were detected in all 2018 samples of senescent leaves collected in October from trees that had received emamectin benzoate applications approximately four months earlier in the year. Average levels of residues in the leaves treated with TREE-äge at the CU site were about 7 times higher (0.06 ppb vs. 0.425 ppb) and those collected from the Pearl/Spruce site were about 6 times higher (0.0614 vs. 0.372 ppb) than present in samples from the trees collected the previous year.

During 2018 emamectin benzoate were detected in senescent leaves of trees that had been treated with the Boxer formulation in June 2018 at the CU. Residue levels (0.372 ppb, range 0.12-0.91 ppb) were approximately 85 percent those from trees treated with TREE-äge (0.425 ppb, range 0.25-1.146 ppb).

Table 3.2 Levels of emamectin benzoate residues detected in senescent foliage of green ash trees with varying insecticide history. The LOD (Limit of Detection) for emamectin benzoate was 0.06 ng/mL (= 0.06 ppb). ND indicates samples where no evidence of residues of emamectin benzoate were detected. ND+S indicates that the levels present were below the limit of the analysis to be detected consistently, but there were indications that a metabolite may have been present in minute amounts. All trees from which samples were collected were located in Boulder, Colorado.

Insecticide Treatment[a] (Application Years)	Collection Site	Sampling Date[b]	No. samples	No. positive samples	Min (ng/mL, ppb)	Max (ng/mL, ppb)	Avg concn (ng/mL, ppb)
TREE-äge	CU	2017	6	5	ND+S	0.06	0.06
Boxer			6	0	ND	ND	ND
Untreated Control			6	0	ND	ND	ND
TREE-äge	Pearl/Spruce		6	4	ND+S	0.093	0.0614
Untreated Control			6	0	ND	ND	ND
TREE-äge	CU	2018	6	6	0.25	1.146	0.425
Boxer			6	6	0.12	0.91	0.362
Untreated Control			6	0	ND	ND	ND
TREE-äge	Pearl/Spruce		6	6	0.13	0.63	0.372
Untreated Control			6	0	ND	ND	ND

[a] Trees were treated by trunk injection. The emamectin benzoate formulation TREE-äge was applied in June 2014, 2016 and 2018, using the Arborjet QUIK-jet injector. Trees treated with the emamectin benzoate formulation Boxer were treated in June 2016 and 2018 using the Wedgle Direct-Inject system. Untreated control trees had no history of any insecticide use.
[b] Samples of senescent foliage were collected October 6, 2017 and October 18, 2018.

Samples of flowers. Among the 22 samples of flowers collected from trees (Table 3.3) that were treated with emamectin benzoate, emamectin fragment ions were detected in 8 samples, at the Limit of Detection (LOD) attained with this analysis (0.28 ng/mL = 0.28 ppb). All were from trees sampled in 2018; no residues were detected in flowers from 2017 collections. The maximum concentration detected was 0.32 ng/mL (0.32 ppb). Emamectin was not detected in any of the samples of flowers collected from untreated control trees (n=22), which had no history of insecticide application.

Table 3.3 Levels of emamectin benzoate residues detected in flowers of green ash trees with varying insecticide history. The LOD (Limit of Detection) for emamectin benzoate was 0.28 ng/mL (= 0.28 ppb). ND indicates samples where no evidence of residues of emamectin benzoate were detected. All trees from which samples were collected were located in Boulder, Colorado.

Insecticide Treatment[a]	Collection Site	Sampling Date[b]	No. samples	No. positive samples	Min (ng/mL, ppb)	Max (ng/mL, ppb)	Avg concn (ng/mL, ppb)
TREE-äge	CU	2017	6	0	ND	ND	ND
Untreated Control			6	0	ND	ND	ND
TREE-äge	Pearl/Spruce		5	0	ND	ND	ND
Untreated Control			5	0	ND	ND	ND
TREE-äge	CU	2018	6	6	0.28	0.32	0.29
Untreated Control			6	0	ND	ND	ND
TREE-äge	Pearl/Spruce		5	2	ND	0.28	0.11
Untreated Control			5	0	ND	ND	ND

[a] Trees were treated by trunk injection. The emamectin benzoate formulation TREE-äge was applied in June 2014 and 2016, using the Arborjet QUIK-jet injector.
[b] Samples of flowers were collected April 3, 2017 and April 5, 2018.

Samples of pollen. Among the 60 samples of pollen collected from trees (Table 3.4) that had been treated with emamectin benzoate, emamectin fragment ions were not detected at the Limit of Detection (LOD) attained with this analysis (0.1 ng/mL = 0.1 ppb). In 7 of the 16 samples collected from City of Boulder trees sampled in 2018 samples were recorded as >LOD, indicating that residues may have been present but at levels below the limit of the quantification of the analysis to be detected consistently. Emamectin was not detected in any of the samples of flowers collected from untreated control trees (n = 24), which had no history of insecticide application.

Table 3.4 Levels of emamectin benzoate residues detected in pollen extracted from flowers of green ash trees with varying insecticide history. The LOD (Limit of Detection) for emamectin benzoate was 0.1 ng/mL (= 0.1 ppb). ND indicates samples where no evidence of residues of emamectin benzoate were detected. ND+S indicates that the levels present were below the limit of the analysis to be detected consistently, but there were indications that a metabolite may have been present in minute amounts. All trees from which samples were collected were located in Boulder, Colorado.

Insecticide Treatment History[a]	Collection Site	Sampling Date[b]	No. samples	No. positive samples	Min (ng/mL, ppb)	Max (ng/mL ppb)	Avg conc[d] (ng/mL, ppb)
TREE-äge	CU	2017	5	0	ND	ND	ND
Untreated Control			5	0	ND	ND	ND
TREE-äge	Pearl/Spruce		5	0	ND	ND	ND
Untreated Control			5	0	ND	ND	ND

TREE-äge (2014, 2016)	CU	2018	5	0	ND	ND	ND
Untreated Control			5	0	ND	ND	ND
TREE-äge (2014, 2016, 2018)	Pearl/Spruce		5	0	ND	ND	ND
Untreated Control			5	0	ND	ND	ND
TREE-äge (2017)	City of Boulder (Colorado Avenue Site)	2018	4	0	ND	ND	ND
TREE-äge (2017)	City of Boulder (Martin Park Site)	2018	4	0	ND	ND	ND
TREE-äge (2017)	City of Boulder (YMCA)	2018	4	0	ND	ND+S	ND
TREE-äge	City of Boulder (Howard Heuston Park)	2018	4	0	ND	ND+S	ND
Untreated Control	City of Boulder	2018	4	0	ND	ND	ND

[a] Trees were treated with the emamectin benzoate formulation TREE-äge applied by trunk injection using the Arborjet QUIK-jet injector. Trees at the CU and Pearl/Spruce sites were treated in 2014 and 2016. The City of Boulder trees sampled in 2018 were treated in 2017.
[b] Samples of flowers from which pollen was extracted were collected on April 3, 2017 and April 5, 2018 at the CU and Spruce/Pearl sites. Collections from the City of Boulder trees in 2018 were made April 10.

DISCUSSION

Among the ash tree tissues examined in this study residues of emamectin benzoate were most consistently detected in foliage collected around the period of autumn leaf fall. All samples collected in October 2018, approximately four months after application, had detectable residues of emamectin benzoate. However, levels were very low, never exceeding 1.1 ppb. Furthermore,

similar sampling of the same trees the previous season (October 2017), when a period of 16 months had passed since the insecticide application, detected far lower levels of residues. Emamectin benzoate levels in ash trees decline following trunk injection applications and their effective use for EAB ash borer control is for two years (Herms et al. 2019)

The levels of emamectin benzoate found in senescent leaves of this study were also much lower than were found by McCullough et al. (2011) in leaves collected during summer within two months of application. This suggests that emamectin benzoate may largely be reabsorbed by the trees late in the season, reducing the amount or residues appearing in leaves that fall from the trees. Few, if any, impacts have been observed on decomposition of leaves dropped from trees that were treated with trunk injections of emamectin benzoate (Burkhard et al. 2015, Al-Akeel Chapter 2).

During 2018 a comparison of residue levels could also be made between two different methods of emamectin benzoate application. The most widespread and standard method of emamectin benzoate application to ash trees (Herms et al. 2019) is with injection systems that pre-drill holes in the lower trunk and infuse the insecticide into the tree under pressure, such as the QUIK-jet system of Arborjet, Inc. used in most of the studies reported here. A second method of trunk injection Wedgle Direct-Inject, developed by ArborSystems, does not involve drilling but instead with specialized needle is inserted through the bark and a small amount (1 ml) of material is introduced into the cambium and outer xylem rings. This latter method application has been controversial for controlling EAB (Harrell 2006), following an early trial using the insecticide imidacloprid which was found to be ineffective. A primary concern about the applications of the Wedgle Direct-Inject is that the rates of insecticide used are much lower than when introduced through the standard trunk injections into pre-drilled holes. For example,

in a 25.4cm/12-inch diameter tree the amount of formulated insecticide (TREE-äge, 4% emamectin benzoate) introduced through the QUIK-jet system is 55 ml/tree (Anonymous 2020b); a tree of similar size using the amount of formulated insecticide (Boxer, 4% emamectin benzoate) would apply approximately 8 ml of product through the Wedgle Direct-Inject system, approximately 15 percent the amount of active ingredient applied to the tree.

Emamectin benzoate residues collected at the CU site in 2018 were found in all leaf samples regardless of application method. Although residue levels were lower (0.362 ppb) in those treated with Boxer compared to those treated with TREE-äge (0.425 ppb), the difference in residues appearing in fallen leaves was far smaller than the difference amount introduced into the trees earlier in the season at application. This suggests that the introduction of the insecticide into the cambium and outer xylem by the Wedgle Direct-Inject may be more efficiently taken into the leaves than methods that introduce the insecticide more deeply into the trunk, at least in the initial year of application.

Levels of emamectin benzoate appearing in flowers was lower than that appearing in fallen foliage and was detected only in 8 of 22 samples (36%). These differences may be due several factors, including differences in the timing of tissue collection, but may also indicate that emamectin benzoate does not move as readily into flower tissue as it does into foliage of green ash. The appearance of insecticide residues in flowers has sometimes been used to suggest risk to pollinators and other flower visitors (Krischik et al. 2015) although the Johnson (2012) and Cowles and Eitzer (2017) argue the actual degree of exposure to insecticides through pollen cannot be equated from analysis of whole flowers.

In this study, pollen was collected from green ash with a known history of insecticide use from trunk injections of emamectin benzoate and in no samples was the insecticide detected at

the levels of quantification allowed in this analysis (0.1 ppb). Only one other study has reported on the appearance of emamectin benzoate in pollen from trunk injected trees. Cosler et al. (2019) trunk injected apple trees and were able to recover a small amount of emamectin benzoate (1.15 ppb) in trees injected in spring shortly before flowering. However, these authors did not detect any residues in pollen from trees that were injected the previous fall. Combined with this study it suggests that emamectin benzoate introduced as a trunk injection moves slightly into pollen and that the amount that may appear diminishes as the length of time between application and flowering increases. Trunk injections of emamectin benzoate for control of emerald ash borer are typically made in mid-late spring or early summer when leaves are present and after bloom. This would typically allow a lapse of 8-10 months or more between trunk injections and flowering.

The study by Al-Akeel (Chapter 1) did demonstrate that ash tree pollen is collected by honey bees from ash trees in an urbanized area of Colorado where insecticide applications for EAB were made. This may indicate a potential route of pesticide exposure to honey bees that collect pollen from ash trees treated with systemic insecticides. However, this present study shows that the levels of emamectin benzoate residues in green ash pollen are extremely low, at least below 0.1ppb. Combined with the limited use of ash pollen by honey bees, this may indicate that the risks to honey bees are negligible from the collection of pollen from ash trees treated with trunk injection treatments of emamectin benzoate.

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