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Methods for controlling two European Honey bee (Apis mellifera L.) pests:
Varroa mites (Varroa destructor, Anderson & Trueman) And Small hive beetles (Aethina tumida)
Morgan Alicia Roth
Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Master of Science In Life Sciences In Entomology Aaron D.
Gross James M. Wilson Keith R. Tignor April 18, 2019 Blacksburg, VA Keywords: Apis mellifera, Aethina tumida, Varroa destructor, Resistance, Acetylcholinesterase (AChE) Copyright © 2019, Morgan Roth
Methods for controlling two European Honey bee (Apis mellifera L.) pests:
Varroa mites (Varroa destructor, Anderson & Trueman) And Small hive beetles (Aethina tumida)
Morgan Alicia Roth SCIENTIFIC ABSTRACT Throughout the last five decades, European Honey bee (Apis mellifera) colonies have been heavily damaged by invading
Varroa mites (Varroa destructor), and, more recently, small hive beetles (Aethina tumida).
These pests infest A. mellifera colonies throughout Virginia, with V. destructor feeding upon the lipids of their hosts and spreading viruses, and A. tumida feeding extensively on hive products and brood. Because V. destructor has historically demonstrated acaricide resistance, this study examined V. destructor resistance to three common acaricides (amitraz, coumaphos, and tau-fluvalinate) throughout the three geographic regions of Virginia using glass vial contact bioassays; the results showed no resistance in the sites tested. To gain better insights into A. tumida pharmacology, several known acetylcholinesterase (AChE) inhibitors and three novel insecticides (previously shown to have low mammalian toxicity) were tested against an A. tumida laboratory colony through in vivo and in vitro bioassays. The results of these bioassays indicated that coumaphos was most selective and topically effective against A. tumida, while only one experimental compound was selective against A. tumida, with 29-fold less potency than coumaphos. These results can help apiculturists in making informed pest management choices and can lead to future studies further examining V. destructor resistance and optimizing A. tumida insecticide treatments. Methods for controlling two European Honey bee (Apis mellifera L.) pests:
Varroa mites (Varroa destructor, Anderson & Trueman) And Small hive beetles (Aethina tumida)
Morgan Alicia Roth GENERAL AUDIENCE ABSTRACT Beekeepers throughout the world have experienced great economic loss and observed a troubling decline in European Honey bee colonies over the past fifty years due to Varroa mite infestations. Varroa mites feed on the fat body of bees, depriving them of nutrients and infecting them with various diseases. Attempts made to control Varroa mites with synthetic chemicals throughout the years have led to acaricide resistance. To look at resistance in Virginia's mite populations, resistance testing was performed on Varroa mite populations throughout the three geographic regions of Virginia, and these studies showed that there was not resistance in these populations. Another significant hive pest that is increasingly prevalent in the United States is the small hive beetle (SHB), which feeds on bee brood and hive products. SHB management tactics are still being explored, and this project tested various known insecticide treatments against small hive beetles and bees, as well as three insecticide treatments that were designed for mosquito control and have low toxicity to mammals. The results of this study showed that, of the insecticides tested, coumaphos was the most selective against SHB. This information can help beekeepers in Virginia make informed choices when deciding how to treat Varroa mite infestations, and can add to the knowledge base of those fighting small hive beetle infestations. TABLE OF CONTENTS CHAPTER 1. HISTORY AND BIOLOGY OF
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CHAPTER 1. HISTORY AND BIOLOGY OF VARROA MITES (VARROA DESTRUCTOR ANDERSON & TRUMAN) AND SMALL HIVE BEETLES (AETHINA TUMIDA MURRAY): A LITERATURE REVIEW

1.1 BIOLOGY OF VARROA DESTRUCTOR

1.1.1 Lifecycle and Morphology

Introduction

The lifecycle of V. destructor is differentiable into two stages: a phoretic stage, during which the mites are freely moving in the hive through transport on their adult bee hosts, and a reproductive stage, which takes place within A. mellifera capped brood cells (Rosenkranz, Aumeier, & Ziegelmann, 2010). Morphological characteristics of each stage will be included below, along with a description of V. destructor activity during each stage.

1.1.2 Phoretic stage

Identification of V. destructor mites in the phoretic stage is relatively simple due to the fact that this stage is only comprised of females with high phenotypic consistency (Anderson & Trueman, 2000). V. destructor females are generally 1.1 mm wide and approximately 1.6 mm long, although body size can be highly variable between V. destructor populations (Maggi, et al., 2012). These mites are reddish-brown (De Jong & Morse, 1982) and are covered with small setae that occur dorsally and ventrally (Oudemans, 1904), which help them stay attached to their hosts (Kirrane et al., 2012). Phoretic stage mites have an ellipsoid, flattened 1 body shape (Rosenkranz, et al., 2010) with specialized host adherence structures called apoteles on their strong short legs (De Ruijter and Kaas, 1983). Phoretic stage mites often change hosts and are groomed off by their hosts (Delfinado-Baker, Rath, & Boecking, 1992). Because prolonged survival and reproduction apart from their host is impossible, it is thought that the phoretic stage must have physiological value (Xie, Huang, & Zeng, 2016). It has been suggested that the

phoretic stage is necessary for spermatozoa capacitation, which takes approximately five days (Ziegelmann, Ha, & Rosenkranz, 2016). The phoretic stage can last for months, since these mites can overwinter on adult bees, where they generally reside near the abdominal intersegmental plates (Boecking & Genersch, 2008). Transfer of V. destructor between colonies can also take place during this stage through robbing of nearby colonies, drifting of drones, and worker homing errors, which are especially prevalent in crowded apiaries (Seeley & Smith, 2015). The combining of hives and transfer of food stores, as well as the high bee densities, seen in large apiaries, also facilitate the transfer of V. destructor between colonies (Fries & Camazine, 2001). The preferred attachment point of V. destructor is thought to vary based upon host temperature, life stage, and cuticular chemistry changes (Kuenen & Calderone, 1997), as some studies observed that mites preferred ventral abdominal region between the sclerites (Seeley & Smith, 2015), while other studies indicated that V. destructor preferred to reside on the thorax or dorsal regions of the body (Fernandez, Eguaras, & Hernandez, 1993; Figure 1). It was observed in one study that approximately 50% of the mites on emerging worker 2 bees, and 80-90% of the mites on emerging drones, transferred to a new host bee (Kuenen & Calderone, 1997). A general distribution of 1-2 mites per bee is most often observed, with unusual cases of 3-4 mites on a single bee (Fernandez et al., 1993). Newly emerged bees are generally preferred hosts, likely because of their frequent access to brood, but pollinators are sometimes chosen, which can facilitate the spread of these mites to different colonies, especially when their current colony is in the process of dying (Kuenen & Calderone, 1997). 1.1.3 Reproductive Stage V. destructor ontogenesis takes place in approximately 5.8 days in females and 6.6 days in males (Martin, 1994), and is comprised of four life stages: egg, protonymph, deutonymph, and adult (Rosenkranz et al., 2010). The reproductive life stage of V. destructor begins when kairomones draw previously mated female mites to enter a brood cell and hide within the brood food, where they remain inactive until the cell is capped, and the bee larva enters the prepupa stage, which takes approximately 60-70 hours (Boecking & Genersch, 2008). Host signals from A. mellifera L5 larva, which are thought to be either nutritional factors or volatiles, cue the laying of an unfertilized male egg. (Garrido & Rosenkranz, 2003). The male egg is followed by a fertilized female egg, approximately 30 hours later (Rehm & Ritter, 1989), after which three other female eggs are generally laid at 20-32 hour intervals (Martin, 1994). Males and nymphal female mites do not leave the cell and only can thrive outside of capped 3 brood cells (Rosenkranz et al., 2010). Though not generally observed, it has been noted that up to three female mites could survive to adulthood in ideal circumstances (Rehm & Ritter, 1989), and some have speculated that up to four fertilized females may mature, based on bee development time (Donze & Guerin, 1994). It is thought that up to seven eggs may be laid in drone brood, which have a longer developmental time, while a maximum of six eggs may be laid in worker brood (Infantidis,1983). After emerging from their eggs, V. destructor goes through protonymph and deutonymph stages, as is seen in general mite development (Boecking & Genersch, 2008). These stages are further subdivided an initial mobile phase, followed by an immobile pharate phase that occurs before molting (Donze & Guerin, 1994), referred to as the protocrysalis and deutocrysalis, respectively (Ziegelmann et al., 2013). V. destructor mothers exhibit parental care in providing a single feeding hole in the cuticle of the bee pupae, usually on the fifth abdominal sclerite, which is located near a fecal accumulation site, facilitating easy travel between the two areas; the feeding hole is crucial to mite development, since the chelicerae of V. destructor female nymphs are too soft to penetrate cuticle, and male nymphs have chelicerae that are modified as spermodactlys, rendering them useless in cuticle penetration (Donze & Guerin, 1994). Only one feeding area and fecal accumulation area are created on bee pupa, even when multiple V. destructor females reproduce in the same cell, in order to reduce the risk of the pupa hemorrhaging and drowning the mites (Donze & Guerin, 1994). The continuously open feeding hole also presents 4 another opportunity for infection, since it is difficult to heal (Kanbar and Engels, 2003). After their second molt, deutonymphs reach adulthood and are ready to mate, immediately following ecdysis. Females can mate multiple times with a male if other females do not continue to arrive at the fecal accumulation site, where fertilization takes place (Donze et al., 1996). It has been shown that male mites have a preference for the most recently matured females, which helps ensure that the maximum number of mites can be fertilized in the time available while

the cell is capped (Ziegelmann et al., 2013). The reproductive stage ends when the adult bee exits the cell carrying the mother and mature daughter mites, leaving the male and immature females to die in the abandoned cell (Boecking & Genersch, 2008). V. destructor females may reproduce up to seven times and can lay 30 eggs throughout their lifetime (Ruijter, 1987). Though V. destructor males and females both have two clearly defined body sections, the idiosoma, or dorsal and ventral shields, and gnathostoma, or mouthparts, (Rosenkranz et al., 2010), they display high levels of sexual dimorphism, making sex differentiation uncomplicated (Infantidis, 1983). V. destructor adult males are pale yellow in color, with oblong, triangularly shaped bodies (De Jong & Morse, 1982), and longer legs, comparatively, than females (Infantidis, 1983). Males are also much smaller than females, with average body widths and lengths of 7.00 and 7.15 mm, respectively (De Jong & Morse, 1982). 1.1.4 Taxonomy 5 V. destructor was originally documented by A. C. Oudemans in 1904 and was classified as Varroa jacobosoni (Oudemans, 1904). V. destructor was not correctly differentiated from V. jacobsoni until 2000 by Anderson and Trueman. They classified six haplotypes of V. jacobsoni that varied in shape, were reproductively isolated, and were found infesting Apis cerana colonies in mainland Asia as V. destructor (Anderson & Trueman, 2000). Although V. jacobsoni can also be found on A. mellifera, it is considered to be transient, as it is only known to reproduce when residing in A. cerana colonies (Anderson & Sukarsih, 1996). The major phenotypic difference between V. jacobsoni and V. destructor is the larger body size observed in V. destructor specimens (Anderson & Trueman, 2000). 1.2 VARROA DESTRUCTOR HOST RANGE AND HISTORY Because A. mellifera is less adapted to V. destructor infestations than the Asian honey bee (Apis cerana), this species faces a greater threat when V. destructor is present. (Rosenkranz et al., 2010). This resistance difference is thought to stem from the fact that A. cerana was able to evolve along with V. destructor, allowing for host defenses that A. mellifera is not equipped with (Locke, 2016). A. cerana demonstrates advanced hygienic efficiency in the grooming of phoretic mites, and these bees efficiently uncap, remove, and entomb infested brood during mite reproduction (Rath, 1999). V. destructor is also highly limited in reproductive ability in A. cerana colonies, as it can only successfully reproduce on A. cerana drone brood, due to the shorter A. cerana pupation times of workers 6 and drones (Boot et al., 1999). It is suspected that the spread of A. mellifera to V. destructor took place via queen and colony transport from Asia into Europe in the early 1970s, as V. destructor damage was observed in eastern and western Europe, North Africa, and most of South America, by 1975 (De Jong & Mose, 1982); V. destructor infestations are now a near-global occurrence (Fries, Camazine, & Sneyd, 1994). 1.3 VARROA DESTRUCTOR FEEDING DAMAGE AND BEHAVIOR V. destructor infestations were first noted in the United States in 1987 (Delfinado- Baker, 1988), and have since been identified as the primary cause of colony collapse in A. mellifera (Rosenkranz et al., 2010), leading to colony death in 2-3 years in temperate climates, if left untreated (De la Rua et al., 2009). There is increasing evidence that V. destructor is causing significant damage by feeding on lipids (Ramsey et al., 2018) rather than just hemolymph, which can heavily impact nutritional physiology (Dolezal et al., 2016). However, it is thought that of the principal threats presented by V. destructor are the viruses that they transmit, with one of the most prevalent being Deformed Wing Virus or DWV (Anguiano- Baez et al., 2016), which is thought to be responsible for overwintering colony loss (Dainat et al., 2011). Heavy losses have been sustained in the U.S. since V. destructor discovery, for example, 1/3 of the A. mellifera colonies in the United States were lost over the winters between 2006-2009, and the impact of these devastating ectoparasites only continues to escalate (Van Engelsdorp et al., 2010). The economic impacts of colony loss pertain not only to bee products, but also heavily affect 90% of pollinator dependent crops (Tantillo et al., 2015), which 7 have an economic value of approximately $15 billion dollars in the United States alone (Calderone, 2012). Though V. destructor mites feed on the hemolymph of larval, pupal, and adult bees, thereby weakening the host and reducing immunity (Shen et al., 2005), the most serious concern when dealing with mite infestation is the development of parasitic mite syndrome (Tantillo et al., 2015). Parasitic mite syndrome is manifested in variable symptoms, involving the presence of diseases that are known to be transmitted by V. destructor (Tantillo et al., 2015). Of the 18 known honey bee viruses, six are of chief concern, including: Deformed Wing Virus (DWV), Sac brood virus, Black queen cell virus, Acute bee paralysis virus, Kashmir bee virus, and Chronic bee paralysis virus (Chen & Siede, 1971), with DWV now being most frequently observed (Tentcheva et al., 2004). The

prevalence of DWV has caused the identification of A. mellifera specimens with symptomatic DWV to be a key indicator of V. destructor infestations. (Bowen-Walker et al., 1998). Aside from the wing deformities that characterize this virus (Figure 2), symptoms of DWV can also include paralysis, abdominal bloating, rapid death of emerging bees (Lanzi et al., 2006), and learning deficiencies (Iqbal & Mueller, 2017). However, it has been observed that over 99% of bees with DWV are asymptomatic (Lanzi et al., 2006) with symptomatic A. mellifera showing the highest viral loads (Brettell et al., 2017). DWV is a positive-strand RNA virus with three master variants (Types-A, -B, and -C), has been identified in all A. mellifera life stages (Kevill et al., 2017), and tends to be concentrated in the head and abdominal regions of adults (Shah, 8 Evans, & Pizzorno, 2009), though it can be isolated in other body areas, such as the thorax and wings (Lanzi et al., 2006). DWV can be transferred vertically to eggs laid by infected queens, and transmitted horizontally through colonies via infected honey, pollen, and, potentially, feces (Chen, et al., 2006). 1.4 SCOUTING AND MANAGEMENT OF VARROA DESTRUCTOR As V. destructor infestation became a more prevalent global issue, numerous sampling methods and treatments were developed. Though treatment is necessary for colony collapse prevention, invasive sampling, high treatment costs, damage to bee products, and mite resistance all play a significant role in determining which sampling methods and treatments should be implemented (Rosenkranz et al., 2010). 1.4.1 Sampling Methods Four prominent V. destructor sampling methods have been developed for determining V. destructor population density. One of the most invasive methods of V. destructor collection, and the only method that directly samples A. mellifera brood (Branco, Kidd, & Pickard, 2006), consists of the uncapping and removal of A. mellifera drone and worker brood with a cappings-scratcher, or other uncapping tool (Thompson, 2013). Once the brood are removed, they can be individually examined, and the incidence of adult female V. destructor mites per larva or pupa are recorded (Branco et al., 2006). The other highly invasive, though popular and precise, method for estimating V. destructor infestation is the 9 ether roll method (Calderone & Turcotte, 1998). Although procedures differ as to the number of worker bees to collect, 100-300 bees are gathered by moving a wide mouthed glass jar along the side of a frame of brood comb (Barlow & Fell, 2006; Morse, 1999). Once the bees are collected, they are sprayed with a two second burst of diethyl ether; once the ether is added, all bees and mites die as the jar is shaken for 10 seconds, then rolled horizontally three times, leaving detached mites stuck to the sides of the jar (Calderone & Turcotte, 1998). Because the ether roll may not remove all mites (Barlow & Fell, 2006), the bees and mites are generally also soaked in ethanol and separated for counting (Calderone & Turcotte, 1998). In some cases, the ether rolling step is bypassed and bees are simply washed in ethanol and mites are counted. The least invasive method is performed by sampling mites off of a sticky board/paper. In this method, a sticky board, with a grid system to help show spatial distribution, is placed beneath the bottom hive body to catch dead or fallen mites over time, thus leading to a better knowledge of V. destructor population dynamics (Kretzschmar, 2015). The sticky board is separated from the rest of the hive by a screened bottom board, which also helps improve hive ventilation, although the screened bottom board itself is a cultural control that is not always associated with sticky board sampling (Conrad, 2008). Sticky boards can be purchased commercially, but can also be homemade using petroleum gel, or other clear adhesive substances, and are often used in conjunction with an acaricide treatment, causing the mites to fall more quickly (Calderone & Lin, 2003). Subsampling from the grid on the sticky board (based upon spatial 10 patterning) can be the most efficient method when large numbers of mites are collected, with population estimate methods depending on grid configuration (Calderone, 1999). When not using the spatial patterning style sticky board, the total number of mites found after 24 hours are counted, and treatment is recommended if over 40 mites are counted (Barlow and Fell, 2006). The final major sampling method, the powdered sugar shake, combines the collection concept of the ether roll method, but replaces the ether and shaking solutions with powdered sugar, eliminating bee death in the sampling process. During the development of this method, various inert dusts, including talcum powder, wheat flour, baking soda, corn starch, and fine sugar, were all tested; however, the greatest accuracy in mite detection was achieved with powdered sugar (Macedo, Wu, & Ellis, 2002). Powdered sugar has also been shown to have no negative effects on adult or brood bee health, does not detrimentally affect the respiratory system of the bees (Fakhimzadeh, 2001) and even stimulates grooming behaviors (Stevanovic et al., 2012). As in the ether roll, the number of bees used in sampling may vary by researcher, and depending upon the

collection device, but the concept remains the same. One recommendation for collection details the use of a ¼ c. measuring cup, which is known to hold up 200 (± 25) bees (Honey Bee Health Coalition, 2015). Once collected and deposited into a jar with a mesh-topped lid, bees are shaken in approximately 1-2 tablespoons of powdered sugar for 1 minute and allowed to rest for one minute; the jar is next inverted and dislodged mites are shaken out of the jar into a collection tray for 1-4 minutes, then mites are counted (Gregorc, 11 Knight, & Adamczyk, 2017). This method is now most highly recommended to beekeepers because of its high levels of accuracy and nondestructive sampling procedures (Honey Bee Health Coalition, 2015; Macedo et al., 2002). Although this method does not guarantee that all mites were removed during shaking, this method is the best way to gather a V. destructor population estimate without damaging the hive. 1.4.2 History of Treatments and Acaricide Resistance Chemical treatments for V. destructor can be arbitrarily divided into two categories: “hard” and “soft” acaricides. The former is comprised of synthetic chemicals such as amitraz, coumophos, and tau-fluvalinate, while the latter entails organic acids and essential oils, including oxalic, formic, and lactic acid, thymol, and several other essential oils (Rosenkranz et al., 2010). These treatments are marketed under various names, and concentrations currently approved for in-hive use by the EPA can be seen in Table 1 (Rosenkranz, et al., 2010). Historically, the synthetic acaricides amitraz, coumaphos, and tau-fluvalinate were (and continue to be) the most common V. destructor treatments used, however, overuse has predictably led to the development of acaricide resistance throughout the world (De Mattos, Soares, & Tarpy, 2017). Some acaricides are also known to detrimentally affect bees, slowing behaviors associated with learning and memory, and causing premature adult death (Berry, et al., 2013). It has also been suggested that the high buildup of acaricide 12 residues in bee hives is a contributing factor in colony collapse (Mullin et al., 2010). Historically, the most popular synthetic acaricide was the pyrethroid tau- fluvalinate, which disrupts mite voltage sensitive sodium channels (Hubert et al., 2014), and was effective through the early to mid 1990s, after which resistance was widely observed (Johnson,
et al., 2010; Lodesani, et al., 1994; Mozes-Koch et al., 1999). The
organophosphate coumophos, which acts as an anticholinesterase, was also previously used to control V. destructor in both liquid and strip formulations (Elzen, et al., 2004) but has since been shown to modestly impair bee olfaction(Williamson, et al., 2013), reduce longevity in adults that were exposed as larvae (Wu, et al., 2011), and accumulate in wax (Bajuk et al., 2017); V. destructor resistance to coumaphos has also been observed (Pettis & Jadczak, 2005). Coumaphos has also been shown to detrimentally impact sperm viability and queen health (Chaimanee et al., 2016). Amitraz targets octopamine receptors (Blenau et al., 2012) and has, similarly, become an unfavorable treatment option due to V. destructor resistance. Less popular synthetic acaricides include: flumethrin, which is generally known to block voltage sensitive calcium and chloride channels in mites (Rosenkranz et al., 2010), cymiazole, which is fed to the bees and reaches mites through bee hemolymph (Stanimirovic et al., 2005), and brompropylat, which was distributed as a smoke in hives, but is now prohibited in most countries due to its harmful residues (Adamczyk et al., 2010). Coumaphos has also been estimated to have a half-life of only 115-346 days) in beeswax (Martel et al., 2007). Since most synthetic acaricides are lipophilic (with the exception of cymiazole), they 13 build up quickly in wax, posing a threat to larval survival (Bajuk et al., 2017); additionally, mixing of these synthetic acaricides can also lead to detrimental synergistic effects, such as mortality observed upon the mixing of coumaphos and tau-fluvalinate (Johnson, et al., 2009). Soft acaricide treatments have been steadily increasing in popularity, as they rarely accumulate to harmful levels in hives and bee products (Imdorf et al., 1999). It has also been shown that organic acid and essential oil treatments lower bee stress levels, as measured by heat shock proteins (HSP 70, a molecular stress quantification) in bee brain tissues (Gunes et al., 2017). Oxalic acid can be sprayed, trickled, evaporated into hives, or used as crystals, is recommended for use in autumn and winter months (Rademacher & Arz, 2006) and is spread primarily by contact between bees (Aliano & Ellis, 2008). Lactic acid is also recommended for use in small apiaries during the autumn and winter, is administered by spray, but is a time-consuming treatment strategy (Kraus & Berg, 1994). Formic acid is administered as a fumigant, inserted into hives on saturated pads (Elzen et al., 2004) or in gel packs, and is best used during summer months, or during a period of the year when average daily temperatures reach 15°C (Satta et al., 2005). These organic acids all occur naturally in honey (Rademacher & Arz, 2006, Gunes et al., 2017, Kraus

& Berg, 1994), however, high levels of formic acid vaporization can be toxic to bees, so acid concentrations and hive temperatures should be monitored while treatments are being administered (Elzen et al., 2004). It is recommended that day temperatures range between 10-33 °C when formic acid strips are in use (Honey Bee Health 14 Coalition, 2015). Despite the risks, formic acid is also the only soft acaricide that is known to kill mites in A. mellifera capped brood cells, which makes it an attractive option (Fries et al., 1994). The modes of action for these acids are unclear, but it is suspected that oxalic and lactic acid lead to mite death via solution acidity, and formic acid is thought to eventually interfere with V. destructor metabolism and respiration (Rosenkranz et al., 2010). Many essential oils have also been tested for use in V. destructor control, however, thyme (Thymol), marjoram, sage, wintergreen, clove, and turpentine (camphor) oil, are most commonly implemented, and have been somewhat successful (Imdorf et al., 1999). These treatments may be administered as sprays, fumigants, powders, saturated absorbent materials, or gels (Mondet, Goodwin, & Mercer, 2011). These essential oils are thought to be effective due to the neurological effects that they are suspected to have on V. destructor, based on these effects in other arthropods (Blenau et al., 2012). A variety of neurological modes of action of essential oil terpenoids have been investigated, including octopamine (Price & Berry, 2006), tyramine (Enan 2005), GABA (Priestly et al., 2003), and nicotinic acetylcholine receptors (Tong, et al., 2013, as well as acetylcholinesterase inhibition (Picollo et al., 2008). Tobacco extract was also shown to be an effective acaricide, especially when used in combination with clove oil (Mahmood et al., 2014). Thymol, purchased as Apiguard® gel or powder, is the most commonly used essential oil, and has been shown to be more effective than tau-fluvalinate, which could be due to resistance (Ahmad et 15 al., 2013), though it can have different effects on bees of various ages and is still ineffective on mites in bee brood (Mondet et al., 2011). In order to avoid promoting V. destructor resistance, while most effectively using minimal treatments to maintain A. mellifera health, Integrated Pest Management (IPM) control tactics have been developed. An important distinction between IPM and other control methods is that the goal of IPM is to keep V. destructor populations below the economic threshold, acknowledging that eradication is unrealistic (Tew, 2001). Though biorational acaricides are preferred, synthetic acaricides can still be used, but are alternated to prevent resistance development (Vandervalk & Nasr, 2017). Alternative mechanical controls, such as drone brood trapping and screened bottom boards, can be included, and frequent infestation monitoring with the powdered sugar shake method is recommended (Wantuch & Tarpy, 2017); when populations are below the economic threshold (between 2-5 mites per 100 bees, depending on the season), they are left untreated (Honey Bee Health Coalition, 2015). It is thought that the heavy treatments administered to many A. mellifera colonies has hindered them from developing natural defenses by removing selective pressure, therefore, IPM methods may encourage the eventual development of these defenses (Locke, 2016). 1.5 BIOLOGY OF AETHINA TUMIDA 1.5.1 Lifecycle and Morphology 16 Although A. tumida has been somewhat overshadowed in recent years by V. destructor, these beetles are also a formidable threat to A. mellifera colonies. A. tumida adults are 5-7 mm long and 3-4.5 mm wide, are generally brown or black in color, and have strong elytra (Neumann et al., 2017; Figure 3A). These beetles are known to be attracted to a variety of volatiles emitted from A. mellifera worker bees, brood, unripe honey, pollen, beeswax, wax byproducts, and alarm pheromone (Suanzo et al., 2007). Because destruction of hive products by A. tumida larvae increases the release of these volatiles, other adults can be attracted as a result of the initial infestation (Cuthbertson et al., 2013). The yeast Kodamaea ohmeri, which is carried by A. tumida, produces a compound that is similar to alarm pheromone (isopentyl acetate) which also serves as an attractant to other beetles (James & Ellis, 2016). A. tumida adults have also been shown to have very large antennal lobes, when compared with the rest of their brain structures, which further confirms their strong olfactory senses (Kollmann et al., 2016). Once adults arrive at a hive, they often hide in cells, other small crevices, or hive debris, and tend to congregate at specific sites within the hive (Cuthbertson et al., 2013). These beetles demonstrate various evasion tactics, such as dropping, running, hiding, and assuming defense postures, which help them evade attacks by A. mellifera workers (Neumann & Elzen, 2004). Additionally, volatiles produced by A. tumida are being characterized for use in attractants for traps (Stuhl & Teal, 2017) Once A. tumida adults are sexually mature, approximately one week after emergence, hives are located, mating occurs, and

females oviposit their eggs on 17 pollen and brood comb (Cuthbertson et al., 2013), sometimes even chewing holes in capped brood for oviposition (Ellis, 2004). It is estimated that A. tumida females may lay anywhere from 1,000-2,000 eggs throughout their lifetimes (Somerville, 2003), but they generally oviposit in clusters of 10-30 eggs (Cuthbertson et al., 2013). A. tumida eggs are an opaque white color, and cylindrically shaped, measuring approximately
1.4 mm in length, and 0.26 mm width (Lundie, 1940;
Figure 3B). Egg hatching is influenced by humidity and temperature, and in ideal conditions they hatch in three days (Lundie, 1940; Somerville, 2003). Upon their emergence, A. tumida larvae are quite small, growing in size throughout their development from approximately 1.3 mm to 1 cm in their last instar (Neumann et al., 2017). Distinguishing features of A. tumida larvae are the paired rows of short dorsal spines that run along each segment, and the two larger spines on the last dorsal segment of their light-yellow bodies (Neumann et al., 2017; Figure 3C). The waste products of the larvae, in concert with their yeast symbiont, Kodamaea ohmeri, produce large quantities of fermented hive byproducts, sometimes referred to as “slime” (Hayes et al., 2015). In addition to feeding upon hive products, A. tumida larvae will also readily feed upon bee brood (Neumann et al., 2001). Once they reach their last instar, which generally takes from 10-14 days, A. tumida larvae must migrate to surrounding soil to pupate, sometimes crawling distances of over 200 meters (Stedman, 2006). The larvae of A. tumida bury themselves in the soil, generally at depths of at least 4-8 cm (Neumann et al., 2017), where they molt into free pupae (Meikle 18 & Diaz, 2012). Pupation time varies greatly depending upon soil temperature, and can last anywhere from 15-100 days (Stedman, 2006). In their early stages, the pupae have white coloration, but slowly darken as they near emergence (Lundie, 1940). Significant female biased sex ratios have been observed in emerging A. tumida adults, where 2:
1 ratio of females to males is generally seen (Neumann et al., 2001). The sex of A. tumida adults can easily be determined by gently squeezing their abdomens, which will cause the ovipositor to protrude in females and the 8th tergite to extend in males (Neumann et al., 2017). 1.5.2 Taxonomy A. tumida was originally discovered and classified by Murray in 1867 as a member of family Nitidulidae (Murray, 1867), which contains approximately 2800 species in 172 genera (Neumann & Elzen, 2004). Distinguishing features of the Nitidulidae family, as described by Haebeck in his book on American Beetles, include: “transverse procoxal cavities, grooved metacoxae, dilated tarsal segments, small forth tarsi and three segmented antennal club” (Neumann & Elzen, 2004). Though generally easily distinguishable from other members of family Nitidulidae, A. tumida is sometimes confused with its near relative Cychramus luteus; however, close examination easily elucidates the correct identification of the specimen, as they differ morphologically in many ways (Neumann et al., 2017). 1.6 AETHINA TUMIDA HOST RANGE AND HISTORY For many years A. tumida received little attention, as it is not considered to be a significant A. mellifera pest in its native range (Neumann and Elzen, 2004) of 19 sub-Saharan Africa (Murray, 1867). African A. mellifera subspecies are not generally threatened by A. tumida, as they are scavengers that only cause significant damage in weak, diseased, or abandoned hives in their native habitat, therefore, they are regarded by some to be beneficial quality control agents (Ellis & Hepburn, 2006). However, A. tumida infestations proved a serious threat when infestations were observed in Florida in 1998 (Elzen, et al., 1999). A. tumida distribution now extends across much of the country, covering the East Coast, Southeast, and Midwest by 2004 (Neuman and Elzen, 2004) and reaching the West Coast by 2008 (Neumann & Ellis, 2008). Infestations are generally more severe in Southern states, and higher A. tumida populations have been corelated with larger apiaries (Spiewok et al., 2007). 1.7 AETHINA TUMIDA FEEDING DAMAGE AND BEHAVIOR Although A. tumida is of lesser economic importance than V. destructor, it is estimated to account $3 million dollars in damages on an annual basis in the United States (Hood, 2004). The most significant damages to A. mellifera colonies are caused by A. tumida larvae as they prey upon A. mellifera brood, honey, and pollen; this feeding process leads to hive pollution with fermenting waste (Hayes et al., 2015). When damage becomes extensive, defined by one source as over 1000 adult beetles in a single colony (James & Ellis, 2016), A. mellifera colonies may abscond, leaving their hives to collapse in a mass of A. tumida larvae and waste (Hayes et al., 2015). 1.8 SCOUTING AND MANAGEMENT OF AETHINA TUMIDA 20 Though A. tumida has been a cause of concern, outside of its natural range, for over a decade, development of management tools is still in

its early stages (Neumann & Elzen, 2004). 1.8.1 Sampling Methods A. tumida detection is generally accomplished by visually examining the hives for A. tumida adults or larvae. Additional detection methods include digging in surrounding soil for A. tumida pupae or performing PCR on hive debris samples with the goal of amplifying the A. tumida cytochrome oxidase I gene (Ward et al., 2007). Adult beetles can be easily collected either by hand, through use of an aspirator, or by using baited traps (Neumann et al., 2017). 1.8.2 Treating Hives for Aethina tumida Infestations Treatment options are still being explored, with little progress made since initial A. tumida discovery, especially in the area of chemical controls (James & Ellis, 2016). Permethrin (GardStar) soil drenching has also been attempted (Hood, 2004), but because of the difficulty of timing these treatments correctly this method is not thought to be highly effective (Kanga & Somorin, 2012). Alternative cultural and biological controls have been further explored since A. tumida discovery, and these methods have received more attention than chemical controls (James & Ellis, 2016). A. tumida cultural controls include the removal of 21 excess hive products, reduction of hive humidity (James & Ellis, 2016), and placement of traps and diatomaceous earth around the hives (Cuthbertson et al., 2013). The pupation of larval A. tumida in the dirt outside of A. mellifera hives has led to treatment strategies targeting this life stage, including placement of diatomaceous earth and slaked lime around hives to kill larvae and limit their access to soil (Cuthbertson et al., 2013), and the release of the entomopathogenic nematodes of genera Steinernema and Heterorhabditis, which have been found to reduce A. tumida populations (Cabanillas and Elzen 2006; Ellis et al., 2010). Another management method that is still being developed is the release of sterilized A. tumida adults for potential reduction of population dispersal (Downey, Chun, & Follett, 2015). Several synthetic insecticides have been tested, the most common and successful of which cardboard containing fipronil and strips containing the acetylcholinesterase inhibitor Coumaphos (Cuthbertson et al., 2013), although bees can also be detrimentally affected by these compounds (Kanga & Somorin, 2012). 1.8.3 Acetylcholinesterase (AChE) Inhibitors Although coumaphos is the only AChE inhibitor currently used in A. tumida control, there are other organophosphates and carbamates that could be tested for this purpose. Organophosphates are all derived from phosphoric acid and were originally developed as chemical warfare agents during World War II, while carbamates are esters of carbamic acid (Yu, 2010). Despite their structural differences, both organophosphates and carbamates work by interfering with the 22 passage of nervous system impulses throughout the insect central nervous system, and these impulses are transmitted via acetylcholine (Yu, 2010). When a nervous system impulse reaches the end of a neuron, this impulse triggers the release of acetylcholine from presynaptic vesicles into the synaps.

Acetylcholine diffuses across the synaptic cleft and binds to receptors on the post-synaptic cell, altering the ionic permeability

of the cell membrane and passing the signal onward (Shankland, 1976). The activity of acetylcholinesterase (AChE) is necessary for eliminating the continued presence of acetylcholine in the synapse (English & Webster, 2012), which can lead to repeated excitation of the postsynaptic cell (Yu, 2010). AChE is primarily found in the neuropile areas of the insect CNS (Toutant, 1989). AChE recycles Acetylcholine in a three-step process that begins with binding of the AChE anionic site to the quaternary nitrogen on acetylcholine, coupled with an interaction between the carbonyl of AChE and the serine hydroxyl group at the esteric site of AChE (Yu, 2010) After binding, the acylation step takes place, in which a hydrogen from the hydroxyl group at the esteric site is transferred to the choline moiety of acetylcholine, causing choline release. Lastly, deacylation takes place through hydrolysis of AChE, resulting in the formation and release of acetic acid and freeing the enzyme for repeated use (Yu, 2010). This process allows for reuptake of choline into the pre-synaptic cell, and recycling of acetic acid to make more Acetyl CoA, which allows recycling of acetylcholine for transmission of the next action potential that arrives (O'Brien, 1976; Oakshott, et al., 2005). 23 Both organophosphate and carbamate insecticides inhibit AChE through binding to the serine hydroxyl group on AChE and go through a process that resembles the binding, acylation, and hydrolysis steps of the reaction with acetylcholine (Oakshott et al., 2005). The primary difference, between organophosphates and carbamates is that organophosphates, aside from their structural differences, is that have a very slow dephosphorylation rate (from days to weeks) and are considered irreversible, while AChE is able to undergo decarbamylation quickly (usually within minutes), making carbamates reversible AChE inhibitors (Pang,

2014). In both events, AChE receptors are blocked by the inhibitors and are unable to bind to acetylcholine, causing a buildup of acetylcholine in the synapse and prolonged binding to postsynaptic receptors (English & Webster, 2012). The symptoms of intoxication with organophosphates and carbamates are the same, including hyperexcitability, convulsions, paralysis, and, ultimately, death (Yu, 2010). To date, higher Diptera (including Drosophila melanogaster), and potentially some lower Diptera, have one gene that codes for AChE, while all other insects studied have been shown to have two AChE genes, with ticks having three (Weil et al., 2002; Oakshott, et al., 2005). However, even in the arthropods with two AChE genes, there appears to be only one enzyme (AChE- 1) that is responsible for acetylcholine hydrolysis; when the AChE-1 gene is mutated, target site resistance to organophosphates and carbamates can result (Russell et al., 2004; Oakshott et al., 2005). The function of the second AChE gene (ace-2/AChE-2) is unknown to date but is likely involved in cell-to-cell 24 communication and adhesive properties (Grisaru, et al., 1999). Drosophila and the higher Diptera likely evolved by a loss of the AChE-1 gene (Weil, et al., 2002), subsequently causing the noncholinergic AChE-2 gene to begin functioning in its current capacity, compensating for the loss (Russel et al., 2004). The AChE gene that is paralogous to the Drosophila gene can also be termed the AP-AChE gene, and the orthologous (AO-AChE) gene (Kono and Tumita, 2006). A major concern in the use of AChE inhibitors stems from the adverse effects of these compounds on mammalian health. The crystal structure of Torpedo californica AChE was finally published in 1991, aiding advancements in the design of selective AChE inhibitors (Sussman et al., 1991), later followed by the crystal structure of Drosophila melanogaster AChE (Pang 2014). In the quest for an AChE inhibitor with high insect selectivity, the structural differences between the active-site gorge on insect and mammalian AChE have been elucidated, revealing a sulfhydryl group on the cystine residue, which is near the insect AP-AChE active site (Pang 2014). This insect specific cysteine residue has since been the focus of those attempting to synthesize insect-specific AChE inhibitors, as these compounds would be expected to have low mammalian toxicity (Pang et al., 2012). Additionally, no resistance to these compounds should be experienced, as, to date, only serine targeted insecticides have been used (Cassida and Durkin, 2013). Of the insects studied thus far, 76% used the AP-AChE enzyme in hydrolysis, the AO-AChE enzyme was used in 26%, and 7% utilized both. Interestingly, 26% of insects using the AO-AChE enzyme were 25 higher order hymenopterans (Kim and Lee, 2013). Because AO-AChE is the A. mellifera cholinergic enzyme, which is not sensitive to cysteine-targeted insecticides, these compounds could distinguish between A. tumida and A. mellifera, in addition to having low mammalian toxicity (Pang, 2014). 26 1.9 TABLES & FIGURES Figure 1. V. destructor phoretic female on A. mellifera worker thorax. 27 Figure 2. A. mellifera worker highlighting the deformed wings seen in severe cases of Deformed Wing Virus (DWV). Product Name Active Ingredient 28 Zoecon Rf-318 Apistan Strip; Apistan Anti-Varroa Mite Strips For-Mite Avachem Sucrose Octanoate Api Life Var Mite-Away Quick Strips Formic Pro Apigaurd Hopguard Ii Apivar Oxalic Acid Dihydrate Checkmite + Bee Hive Pest Control Strip Fluvalinate (10.25%) Formic acid (65.9%) Sucrose octanoate (40%) Thymol (74.09%), Oil of eucalyptus (16%), Menthol (3.73%) Formic acid (46.7%) Formic acid (42.25%) Thymol (25%) Hop beta acids resin (16%) Amitraz (3.33%) Oxalic acid (100%) Coumophos (10%) Table 1. V. destructor treatments currently approved by the EPA, obtained from (US Environmental Protection Agency, https://www.epa.gov/ pollinatorprotection/ epa-registered -pesticide-products-approved-use-against-varroa-mitesbee-hives. 2016). 29 A B C D Figure 3. The life stages of A. tumida: egg (A), larva (B), pupa (C), and adult (D). CHAPTER 2. 30 ASSESSING VARROA DESTRUCTOR ACARICIDE RESISTANCE AND THE SUCCESS OF INTEGRATED PEST MANAGEMENT (IPM) TECHNIQUES IN APIS MELLIFERA COLONIES OF VIRGINIA 2.1 ABSTRACT Survival of European honey bee (Apis mellifera L.) colonies has been a growing concern over the past few decades, as Varroa mites (Varroa destructor Anderson & Trueman) have become a significant threat to apiculture on a near-global scale. V. destructor inflicts serious damage, ranging from weakening of the colony through mite feeding, to transmission of viruses. Prominent V. destructor treatment methods have included the use of synthetic acaricides, including amitraz, coumaphos, and tau-fluvalinate. However, widespread resistance to these compounds has been noted, therefore, this study sought to test the prevalence of V. destructor resistance to these three compounds in Virginia. To obtain specimens for this study, V.

destructor infestation levels in apiaries located within the three geographic regions of the state were monitored using the powdered sugar shake method throughout the 2018 field season. Mite populations increased in almost all apiaries as the season progressed, and, by the end of the season, all apiaries had mite populations above the recommended treatment threshold. These mites were then utilized in resistance screening, which was carried out through glass surface contact bioassays. The results of these bioassays indicated no resistance to amitraz, coumaphos, or tau- fluvalinate. These results have important implications in future control strategies employed by Virginia apiculturists, as use of these acaricides, within an 31 Integrated Pest Management (IPM) approach, may offer an additional option to those battling these aggressive ectoparasites. 2.2 INTRODUCTION Fifty years ago, the name 'Varroa mite' would not have elicited alarm in the majority of the world's beekeepers, however, this name now brings to mind collapsing colonies, diseased bees, immense economic losses, and the constant struggle for pest control faced by the modern apiculturist. When living among their native hosts, Asian honey bees (Apis cerana), V. destructor populations are kept in check through host defenses (Rath 1999) and reproductive limitations (Boot et al., 1999). The pupation time of A. cerana workers is too short to accommodate V. destructor reproduction, allowing mite reproduction in drone brood only (Boot et al., 1999). In A. mellifera colonies, the story is unfortunately different. Through causes unknown, but presumed to be transport of queens, V. destructor made its way from Asia to Europe in the early 1970s (De Jong and Morse, 1982), finally reaching the United States by 1987 (Fries et al., 1994). V. destructor populations flourished in A. mellifera colonies, with lethality mainly stemming from their ability to reproduce in A. mellifera drone and worker brood, dramatically increasing mite loads (Boot et al., 1999). V. destructor adult females are the only mites that live on adult bees as part of a phoretic stage, while males can only be found throughout the reproductive stage that occurs in the brood cells (Anderson & Truman, 2000; Figure 1). V. destructor females can lay approximately 30 eggs at seven intervals 32 throughout their lives (Ruijter, 1987), and on average 1.45 daughter mites survive and exit the cell with the foundress (Martin, 1994). Although it was thought for many years that V. destructor mites feed upon hemolymph, current research has shown that they feed primarily upon lipids in the fat body (Dolezal, et al., 2016; Ramsey et al., 2018). This feeding on the fat body is integral to maximum egg production and mite survival and is likely one of the reasons that overwintering colony losses tend to be so severe, accompanied by mite-vectored bacteria that could result in sepsis (Ramsey et al., 2018). Mite feeding also transmits numerous viruses (Hung, 1996), and
Deformed Wing Virus (DWV) is one of the most common
(De Miranda & Genersch, 2010). In the early days of infestation mite control was mainly carried out through use of synthetic acaricide treatments, however, widespread mite resistance began to threaten these treatment options (De Mattos et al., 2017). The three most popular treatments to which resistance has been noted include the formamidine insecticide amitraz, an octopamine receptor agonist (Blenau et al., 2012), the organophosphate coumaphos, which inhibits acetylcholinesterase (Elzen et al., 2004), and the pyrethroid tau-fluvalinate, a voltage-gated sodium channel modulator (Baxter et al., 1998, Yu, 2008). It is also thought that these acaricides have adverse effects upon honey bees (Rosenkranz, et al., 2010). To combat these issues, traditional treatment methods are now transitioning to an Integrated Pest Management (IPM) approach (Rosenkranz et al., 2010; Honey Bee Health Coalition, 2015). IPM strategies first implement cultural and mechanical controls, such as screened bottom boards, followed by biological 33 controls, and then chemical controls (Honey Bee Health Coalition, 2015; Figure 2). IPM also incorporates treatment rotation to help prevent resistance development (Vandervalk & Nasr, 2017), and focuses on keeping the mite populations below economic thresholds rather than eradicating the population completely (Tew, 2001). Acaricide resistance can be developed and manifest itself in a variety of ways. It has been suggested that since haplodiploid and diploid arthropods could develop resistance differently (Carrière 2003), which could be advantageous to the haplodiploid (highly inbred) V. destructor, causing swift acaricide resistance development (Sammataro et al., 2005). The development of a resistant phenotype in a population occurs through natural selection, as susceptible mites are killed, a resistant reproductive majority is left behind (Le Conte et al., 2010). There are several mechanisms by which acaricide resistance can be developed. Behavioral changes are a simple way that acaricides can be avoided, and cuticle thickening can reduce

acaricide penetration (Sammataro et al., 2005). Another is through activity of general esterases, which help to hydrolyze and detoxify ester- containing compounds (Sammataro et al., 2005) and cytochrome P450 monooxygenases that aid in detoxification by enhancing oxidative metabolism (Yu, 2008). Modification of Voltage gated sodium channels through target site mutations also can lead to resistant mite populations (Wang et al., 2003). In order to advance the knowledge of acaricide resistance in the Commonwealth of Virginia, a study with two major objectives was designed. The first objective was to explore the population dynamics of V. destructor within the 34 Commonwealth by monitoring infestation rates throughout the sampling season (May-October 2018). Monitoring primarily took place in three local apiaries (Mountain region), in addition to an apiary in the Piedmont and Coastal regions of Virginia. The second objective was to assess resistance to the three historically popular synthetic acaricides used in V. destructor treatment: amitraz, coumaphos, and tau-fluvalinate (Figure 3). This evaluation was performed through resistance monitoring of V. destructor samples collected throughout the three geographic regions of Virginia (Figure 4). To further corroborate the findings of the resistance bioassays, general esterase and cytochrome P450 monooxygenase activity bioassays were carried out using mites collected within the sampling season.

2.3 MATERIALS & METHODS 2.3.1 Chemicals Acetonitrile (99%), amitraz (>98.0%), coumaphos (99.0%, PestanalTM analytical standard), 7-ethoxycoumarin, tau-fluvalinate (98.7%), 1-naphthol (>98.0%), 1- naphthyl acetate (>98.0%), sodium dodecyl sulfate (~99%), Trizma base primary standard and buffer (>99%), and fast blue RR salt (95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone (HPLC grade), sodium phosphate dibasic anhydrous (98.0%), sodium phosphate monobasic monohydrate (99.5%), Triton X-100, and hydrochloric acid (12.1N), and sodium chloride (≥99.0%) were purchased from Fisher Scientific (Hampton, NH, USA). Coomassie Brilliant Blue G (electrophoresis grade) was purchased from MP Biomedical Supplies LLC 35 (Solon, OH, USA). 7-hydroxycoumarin (99%) was purchased from Acros Organics (Pittsburg and Westchester, PA, USA). 2.3.2 Varroa Mite Sampling Mite sampling was performed using the Powdered Sugar Shake Method, as previously described in the Honey Bee Health Coalition handbook (2015). Briefly, ¼ cup of nurse bees (approximately 200 + 25 bees) were collected by shaking a frame of primarily capped brood into a plastic container (Figure 5A-B). These bees were shaken with a heaping tablespoon of powdered sugar for one minute, rest for one minute, and then the jar is inverted and shaken into a collection tray for one minute (Figure 5C-D). The number of mites counted is divided by two, giving the number of mites present per every 100 bees, which is referred to as percent infestation. To ascertain the precision of this method, two scoops of bees from the several hives were collected in individual jars, one was shaken in powdered sugar, and the other in 70% isopropyl alcohol for the same time intervals. All of the bees that were not already dead were killed post-sampling in 70% isopropyl alcohol, returned to lab, counted, and individually examined for mites that may have been missed in order to investigate potential error and scooping accuracy in the powdered sugar shake method. 2.3.3 Bees used in Varroa Mite Sampling V. destructor sampling took place consistently at three apiaries in Blacksburg, VA. Two of these apiaries, Moore Farm and Prices Fork, are associated with 36 Virginia Tech, and the third apiary is located in the Blacksburg Community Garden. Three or fewer samples came from the third Virginia Tech Apiary, Kentland Farm, where sampling ceased due to bear attacks, and two additional apiaries owned by local Blacksburg citizens. An apiary outside of Richmond, VA, and an apiary near Virginia Beach, VA, were consistently sampled throughout the season as well, providing representative samples from each geographic region of the state. The number of hives sampled in each apiary varied throughout the summer, as some hives were added, some died, and some underwent splits. If colonies were particularly weak or lacked brood, sampling was generally forgone. None of the apiaries were treated with amitraz, coumaphos, or tau-fluvalinate during the sampling season. Although an IPM approach was advocated, all treatments were controlled by the individuals who ran each apiary. 2.3.4 Acaricide Resistance Bioassays Monitoring V. destructor resistance to amitraz, coumaphos, and tau-fluvalinate was performed using glass surface contact bioassays, as previously described (Sammataro et al., 2005). Mites were collected as described above, were kept in a Styrofoam cooler with an ice pack (no-direct contact) until they returned to the lab, and excess powdered sugar was removed by rinsing the mites with 1X phosphate buffered saline (PBS), after which they were allowed to dry on filter paper. Vials

were treated with amitraz, coumaphos and tau-fluvalinate to give a concentration of 248 μg/vial, 106 μg/vial, and 4.2 μg/vial, respectively; this is 37 twice the LD90 previously reported (Sammataro et al., 2005). Vials were treated with 500 μL of test compounds diluted in acetone, and a vial treated with acetone (alone) served as the negative control. Acetone was evaporated from the vials by placing them on a hotdog roller without heat (Rollerdog Big 18); acetone dried after 15-20 minutes leaving a transparent residue of the acaricide treatment. Vials were typically stored at 4°C overnight, in the dark, prior to the collection of mites. Mite mortality was determined at 3, 6, 12, and 24 hours. Mortality was defined as the lack of movement when probed, moribundity was defined was slow minimal leg movements and inability to walk. At the end of the sampling season, results were analyzed by running
one-way ANOVA and Tukey's multiple comparisons tests using GraphPad Prism
(San Diego, CA, USA). 2.3.5 General Esterase Activity Bioassays The general esterase activity was determined in untreated mites collected from the apiaries in three geographic regions, and the assay was performed as previously described. Briefly, mites were homogenized in a sodium phosphate buffer (pH 7.8), made by mixing dibasic and monobasic sodium phosphate in quantities determined using an online buffer calculator
and centrifuged at 10,000 x g for 10 minutes 4 °C. The supernatant was used as the
enzyme source and then the supernatant was removed and 15 μL were pipetted into wells of a flat- bottomed 96-well microplate reader. Next, 135 μL of 0.3 mM 1-naphthyl acetate were added to these wells, then the plate was incubated for 30 minutes at 37°C, 38 causing the esterase present in the samples to hydrolyze 1-naphthyl acetate to 1-naphthol and acetic acid. A mixture (50 μL) of fast-blue (6.3 mM) in 5% SDS was added and the plate was incubated at room temperature for 15 -minutes. 1- Naphthol reacts with
fast blue and SDS, producing a dark blue color that was read on the endpoint setting on SpectraMax microplate reader (Molecular Devices, San Jose, CA, United States)
at 600 nm (van Asperen, 1962). Wells containing various concentrations (0, 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, and 2.6 μg) of 1- naphthol were run along with the mite samples to establish a standard curve. Absorbances could be
plotted in this standard curve to determine the amount of esterase in each sample. Protein quantification was performed using the above homogenates, which were pipetted into another 96 well flat-bottomed microplate at 20x dilution of enzyme (5 μL) with a 0.15M sodium chloride solution (95 μL). Bradford Reagent, made from Coomassie Brilliant Blue (10 mg) dissolved in an ethanol (5 mL) and 10% hydrochloric acid (8mL) mixture, diluted to 50 mL with DI water, was pipetted into the wells directly prior to plate reading. Bradford reagent binds to the proteins present in the samples, causing a brown to blue color change (Bradford, 1976). A series of bovine serum albumen (BSA)
concentrations (100, 50, 25, 12.5, 6.25, and 3.125 ?g/mL)
were read alongside the samples on the endpoint setting at 595 nm in the SpectraMax microplate reader, establishing a standard curve, which was used to determine protein quantity in each sample. The molecular weight of 1-Naphthol (141.16 g/mol) was multiplied by the volume of sample used (15 μL) and divided by time (30 min), yielding a value of 15.415, 39 which was multiplied by volume of 1-Naphthol and protein quantity to determine general esterase activity for each sample in nmol/min/mg, using the below equation (Equation 1). Equation 1: ( )
nmol/min/mg = 15.415 x ( ) μg ?-naphthol / ( ) mg/mL protein
Three replicates from each location were analyzed and then esterase activity results were compared using a one-way analysis of variance (ANOVA) test (α= 0.05), followed by Tukey's multiple
comparisons test in GraphPad Prism. 2.3.6 Cytochrome P450 Monooxygenase Activity Bioassays Monooxygenase activity was performed using methods modified from Anderson and Zhu (2004) and Vu (2016). Briefly,
0.1 M sodium phosphate buffer (pH 7.8) and 50 mM
sodium phosphate buffer (pH 7.2) were mixed (all buffers made in DI water). Then, two to three mites were homogenized in 200 μL of buffer pH 7.8 and
centrifuged at 21 ,100 x g for 10-minutes at 4°C. Supernatant was
removed and pipetted into clean microcentrifuge tubes (Genesee Scientific, San Diego, CA, USA), and then

40 μL of enzyme were pipetted into three wells per replicate in a 96-well clear-bottomed microplate with black sides, then 7-ethoxycoumarin (0.4 mM, 80 μL per well)
was added. The plate was then incubated for 4 hours at 37°C.
Because 7-ethoxycoumarin is metabolized to 7-hydroxycoumarin in this biochemical assay, a standard curve for the unknown enzyme obtained from the mites was established using various concentrations of 7-hydroxycoumarin (0.06, 0.03, 0.015, 0.008, 0.004, 0.002, 0.001, & 0.005 ppm) made in buffer pH 7.8 and added to wells containing the 0.4 mM 7-ethoxycoumarin. The control contained 40 7-ethoxycoumarin and the pH 7.8 buffer. After the 4 hour incubation was complete, 120 μL of a 50% (v/v) mix of acetonitrile and 50 mM
Trizma base buffer were added to each well, which stopped the
reaction. Metabolism of 7- ethoxycoumarin was monitored fluorometrically (excitation 380 nm and emission 480 nm) using an M2 SpectraMax plate reader (Molecular Devices).
Protein content in each sample was quantified using the Bradford protein assay described above in
2.3.5. Relative fluorescent units (RFU) of each sample were then normalized per miligram of protein in each sample and three replicates of this bioassay were performed and results were analyzed
using a one-way ANOVA (α= 0.05) and Tukey's multiple comparisons test
using GraphPad Prism. 2.4 RESULTS 2.4.1 Varroa Mite Sampling Accuracy Of fifteen hives sampled at Moore Farm and the community garden using both the sugar shaking method and the alcohol wash method, 3 mites total were missed during sampling using the powdered sugar shake method. The average number of bees scooped per sugar shake was 189.2 bees, which is consistant with the 25-bee margin of error expected when using the ¼ measuring cup (Honey Bee Health Coalition, 2015). 2.4.2 Mite Population Dynamics As expected, mite infestation levels generally increased as the season progressed (Figure 6). The Moore Farm (Figure 6A) and Prices Fork (Figure 6B) apiaries received partial treatments with formic acid Mite-Away Quick Strips 41 (MAQS), and the community garden (Figure 6C) received partial treatments with both MAQS and Formic Pro, in addition to spot treatment with thymol formulations (no synthetic acaricides), which occurred late in the season (primarily in late August through September). The Richmond (Figure 6D) Virginia Beach (Figure 6E) apiaries were not treated with organic acids or synthetic acaricides throughout the sampling season. Average apiary infestations throughout the season revealed the highest mite population increase in the Virginia Beach (Figure 6E) apiary at the end of the sampling season. It should be noted that in all apiaries, an IPM strategy would recommend treatment, since treatment is recommended when one or more hives exceeds the seasonal mite threshold level, which varies between 2 and 5% infestation levels (Figures 7; Honey Bee Health Coalition, 2015). 2.4.3 Acaricide Resistance Bioassays Due to lack a lack of mites from the VA Beach throughout much of the sampling season, only tau-fluvalinate bioassays were performed using these mites. For each compound tested, there was a significant difference between treatments and control ($P<0.0001$), while
there were no significant differences ($P> 0.9$) between any of
the tested acaricides (Figures 8 and 9). There did not appear to be knockdown resistance, as mite death progressed steadily over the time intervals that mortality was observed (Figure 8). By the time 24 hours was reached, treatment mortality ranged between 80 and 100 percent. 42 2.4.4 General Esterase Activity Bioassays Mites were collected from apiaries in the three regions of Virginia to determine the level of general esterase activity in these populations. As expected,
there was not a significant difference ($P=0.9132$) in the general esterase activity of mites collected from the three
regions of Virginia (Figure 10). The average general esterase activities for Blacksburg, Richmond, and Virginia Beach were 0.400 mmol/min/mg, 0.394 mmol/min/mg, 0.459 mmol/min/mg, respectively (Figure 10). 2.4.5 Cytochrome P450 Monooxygenase Activity Bioassays V. destructor samples from the three regions of the state collected toward the end of the sampling season were also used to determine cytochrome P450 monooxygenase activity in these mite populations.
No significant difference ($P=0.9054$) in cytochrome P450 monooxygenase Activity was seen in the
any of the three mite populations tested using this bioassay (Figure 11). The general esterase activity for

Blacksburg, Richmond, and Virginia Beach was 0.528 RFU/mg, 0.553 RFU/mg, 0.290 RFU/mg, respectively. 2.5 DISCUSSION High mite counts throughout the sampling season, with the population increase in late fall, followed the trends observed in V. destructor populations in the past (Beaurepaire et al., 2017) and reinforce the importance of hive treatment when populations rise above the seasonal thresholds (Deplane, et al., 2005). Despite the history of mite resistance to amitraz (Kamler et al., 2016), coumaphos (Pettis, 43 2004), and tau-fluvalinate (Hillesheim et al., 1996), the significant difference ($P<0.05$) in mite mortality and moribundity between the control and treated mites indicate that the V. destructor populations in these areas do not appear to be resistant to amitraz, coumaphos, and tau-Fluvalinate. The lack of significance ($P>0.05$) in general esterase activity and in cytochrome P450 monooxygenase activity ($P>0.05$) between these mite populations further corroborates the resistance bioassays by demonstrating that the general esterase and cytochrome P450 monooxygenase activities of these populations do not significantly differ, which would not be expected if one of the sites had a resistant population and the other sites did not. A past study indicated a significant difference in esterase activity between a control and resistant population at one of the Blacksburg sites used in this study, however, reported esterase and P450 monooxygenase activities differed from those reported in the current study, likely due to the use of more mites used in the bioassays performed (Vu, 2016). Future directions for this study include expansion of this study to encompass more apiaries, especially some that frequently administer synthetic acaricides, which would be useful in ascertaining a more thorough picture of acaricide resistance in Virginia. It would also be useful to test how long it takes these acaricide susceptible populations to develop resistance to amitraz, coumaphos, and tau-fluvalinate. The glass vial contact bioassays developed here could easily be deployed to collaborating apiarists throughout the state, which would yield an even more thorough picture of statewide resistance and provide higher sample numbers. However, the results yielded from this study indicate a 44 hopeful future for the use of IPM techniques in V. destructor control and provide useful insights into the biology of V. destructor populations in Virginia. 6 1 5 2 5 4 3 4 Figure 1. V. destructor life cycle: mite transferred brood cell through nurse bee (1), hides in brood food (2) until the prepupa stage when feeding begins (3). Laying of the male egg takes place after ~60 hr (4), followed by female eggs every 30 hr. Mites undergo two developmental stages (protonymph and deutonymph), reach maturity, and mate (5), then foundress and mature females exit on adult bee (6; Rosenkranz et al., 2010). 45 Intervention tau-fluvlinate, coumaphos, and amitraz To Conventional xi Pesticides cit formic acid, oxalic acid, essential oils, y Biorational including menthol and thymol Pesticides drone brood removal, screen bottom Mechanical boards, powdered sugar, Mite Zapper R Prevention comb culling, resistant stock, small- Cultural sized comb, break in brood cycle Figure 2. Integrated Pest Management (IPM) Strategy for V. destructor control, which begins with least invasive control methods, focusing on prevention, and then implements methods of increasing toxicity. The focus of IPM is management of the pest population beneath set thresholds and relies on treatment rotation to prevent resistance. Figure modified from the Honey Bee Health Coalition, 2015. Field Code Changed 46 Figure 3. Chemical structures of the three acaricides used in this study:
amitraz; N,N'- [(Methylimino)dimethylidyne]di-2,4-xylidine (I),
coumaphos, O,O-Diethyl O-3-chloro-4-methyl-2- oxo-2H- chromen -7-yl phosphorothioate (II), and tau-fluvalinate; [Cyano-(3- phenoxyphenyl)methyl] 2-[2-chloro-4-(trifluoromethyl) anilino]-3-methylbutanoate
(III). 47 Figure 4. The three geographic regions of Virginia, with stars indicating the locations of the sampling sites used in this study. Map made by and used with permission of Seth Dorman, Virginia Tech. 48 A B C D D Figure 5. Bees are removed from a frame of capped brood (A), approximately 200 bees are collected in a ¼ c. measuring cup (B), 1-2 tablespoons of powdered sugar are added (C), bees are shaken for 1 minute, rest for 1 minute, then mites are shaken out of mesh- topped jar for 1 minute (D). Photos C and D by James Wilson, Virginia Tech. 49 A B C D E Figure 6. Mite infestation levels at each sampling time throughout the season at Moore Farm (A), Prices Fork (B), Community Garden (C), Richmond (D), VA Beach (C). Individual data points represent each hive, with mean and SEM indicated by error bars. 50 Figure 7. Average Apiary infestation levels throughout the sampling season (May 14-Oct 10). It is critical to not that the same number of hives were not sampled during each day of data collection, Figure 6 addresses the percent infestations

encountered in the individual hives. 51 A B C Figure 8. Mite survivorship in resistance bioassays at 3, 6, 12, and 24 hr checks from Blacksburg (A), Richmond (B), VA Beach (C). Survivorship between the controls and all acaricide treatments was significantly different by 24 hr ($P<0.05$). 52 A B C Figure 9. Mite mortality after 24 hours in mite resistance bioassays. Acaricides tested include amitraz (A), coumaphos (B), and tau-fluvalinate (C). The control differed significantly from each treatment type ($P<0.05$) and the treatments did not differ significantly from one another ($P>0.05$). Because mite counts were low at VA beach thorughout the season, only tau-fluvalinate testing took place in this location. 53 Figure 10. General esterase activity bioassay results, using untreated V. destructor specimens from each sampling location. No significant difference ($P>0.05$) was seen between sampling location. 54 A A A Figure 11. Cytochrome P450 monooxygenase activity bioassay results using untreated V. destructor specimens from each geographic region of Virginia. This bioassay demonstrated no significant difference ($P>0.05$) between mite populations from these three sampling locations. 55 CHAPTER 3. INVESTIGATING THE CONTROL OF AETHINA TUMIDA THROUGH PHARMACOLOGICAL CHARACTERIZATION OF KNOWN AND NOVEL ACETYLCHOLINESTERASE (ACHE) INHIBITORS 3.1 ABSTRACT Small hive beetles (Aethina tumida Murray) pose a serious economic threat to European
honey bee (Apis mellifera L.) colonies in the United States,
but control methods for these pests are somewhat ineffective and still undergoing development. In this study, various acetylcholinesterase (AChE) inhibitors were tested against locally collected, laboratory reared A. tumida larvae and locally collected A. mellifera specimens. Additionally, three novel AChE inhibitors designed for use in mosquito control (with low mammalian toxicity) were also explored. In vitro toxicity was examined using the Ellman Assay, and the results indicated that the only experimental compound that had a selectivity ratio (SR) greater than 1 was PRC 421 (SR=1.11), which was still 29-fold less potent than coumaphos-oxon (SR=1.14). In vivo studies, carried out through topical and injection bioassays, yielded LD50 values indicating that coumaphos was still the most selective compound topically against A. tumida (LD50=17ng/mg), while all three novel inhibitors were ineffective to A. tumida topically at doses greater than 43 ng/mg. The results of this study indicate that pharmacodynamic barriers are hindering the penetration of many of these compounds into the A. tumida nervous system and suggest the need for future studies to optimize the chemistry of these treatments for cuticle passage to increase potency. Despite the 56 ineffectiveness of these novel AChE inhibitors, this study provides useful information regarding the use of AChE inhibitors as A. tumida treatments, and this knowledge can now be applied to future attempts to develop effective A. tumida controls. 3.2 INTRODUCTION Two decades ago, Aethina tumida spread to the United States where they were first noted in Florida in 1998 (Elzen, et al., 1999). They quickly spread geographically, and now significantly threaten Apis mellifera colonies (Neumann & Ellis, 2008). Though innocuous scavengers in their native habitats of sub- Saharan Africa (Ellis & Hepburn, 2006), they inflict significant damage to A. mellifera hives through consumption of eggs, brood, and hive products, including honey and pollen stores (Hayes et al., 2015). This extensive feeding generates the buildup of fermenting waste, which can eventually instigate hive abandonment (Hayes et al., 2015). Despite the millions of dollars in damage already incurred annually through A. tumida infestations in the United States (Hood, 2004), treatment methods are still largely exploratory (James & Ellis, 2016). A wide range of treatment methods have been attempted in efforts to control A. tumida to date, and these treatments target different life stages based upon the unique lifecycle of A. tumida. This lifecycle begins when adults mate and begin egg laying in the hive, which is followed by the development of three A. tumida larval instars inside of the hive, after which third-instar larvae migrate 57 to the surrounding soil to pupate (Stedman, 2006). Several control methods seek to control these wandering third-instar larvae, including the spreading of lime or diatomaceous earth around the hives to desiccate or damage the cuticles of wandering larvae (Cuthbertson et al., 2013), pyrethroid soil drenches, or treatment of the surrounding soil with entomopathogenic nematodes (Cabanillas and Elzen 2006) are also practices used to manage A. tumida larvae and pupae outside of the hive. Additionally, trapping of adult beetles has been explored, and various styles of traps can now be procured for in-hive placement (Hood and Miller, 2005). These traps are generally baited or filled with a viscous substance (such as oil) to trap the beetles that enter them (Hood and Miller, 2003). Although the above treatments have

variable success, it is difficult to time soil treatments and coordinate treatments with larval migration (Kanga & Somorin, 2012), and trapping success is difficult to quantify, with recommendations for trap placement differing between studies (Nolan and Hood, 2010). The use of several in-hive chemical insecticides has been set forward as a viable control method. The most common in-hive chemical treatments, targeting larvae and adults, are cardboard strips saturated with fipronil (Levot, 2008) and strips impregnated with the acetylcholinesterase (AChE) inhibitor coumaphos (Elzen, et al., 1999). Although the AChE inhibitor coumaphos has been a successful treatment option, coumaphos can have detrimental effects on A. mellifera health (Gregore et al., 2018), and A. tumida resistance to coumaphos is unknown, although they are likely exposed to this treatment often, as it is 58 frequently present in hives as a Varroa mite (Varroa destructor) control (Cuthbertson et al., 2013). Proper function of cholinergic signaling in the insect nervous system is predicated upon the availability of unimpeded AChE. The neurotransmitter molecule acetylcholine (ACh) is involved in excitatory neurotransmission where it is released from a presynaptic cell, through the synapse, and binds to receptors both pre- and post-synaptically (Thany & Tricoire-Leignel, 2011). Once this signal is received, AChE binds to the quaternary nitrogen of acetylcholine (Toutant, 1989) and interacts with the carbonyl of acetylcholine (Yu, 2010). Next, a hydrogen from the esteric site of AChE transfers to the choline group, releasing choline. Finally, hydrolysis of AChE takes place, which releases acetic acid and frees AChE to bind to another molecule of acetylcholine (Toutant, 1989). The pre-synaptic cell then reuptakes and recycles the choline, allowing the process to be repeated, however, if AChE is inhibited, acetylcholine can continue to activate receptors within the synaptic cleft resulting in repetitive firing and nervous system excitation (English and Webster, 2012), which will ultimately culminate in devastating physiological effects and death (Yu, 2008). Two popular groups of insecticides that act as AChE inhibitors are the organophosphates and carbamates. Inhibition of AChE takes place through a process that is analogous to the interaction of acetylcholine with the binding pocket of AChE, which blocks acetylcholine from entering (Oakshott et al., 2005). Although they are functionally similar, organophosphates and carbamates differ in chemical structure and duration of effect. As their names suggest, 59 organophosphates are phosphoric (or thiophosphoric) acid esters (Costa, 1988), while carbamates are N-methyl carbamic acid esters (Kuhr et al., 1980). Additionally, organophosphates dephosphorylate slowly and are irreversible in function, while decarbamylation usually takes place in a matter of minutes, making inhibition via carbamates reversible (Pang 2014). Because organophosphate and carbamates are also toxic to mammals, development of selective AChE inhibitors is essential. This process was aided through discovery and publishing of the crystal structure of AChE (Sussman et al., 1991) and detection of a key structural difference between the active site- gorge in mammalian and insect AChE (Pang, 2007). In insects, there is a sulfhydryl susceptible cysteine residue near this active-site gorge, while mammalian AChE has a phenylalanine residue that interacts with catatonic ligands (Pang 2014). This residue has aided in synthesis of insect-specific AChE inhibitors (Pang 2014), and, since only serine targeted insecticides have been used historically, compounds with this target should not be subject to resistance (Cassida and Durkin, 2013). Thus far, two genes that code for AChE (ace-1 and ace-2) have been found in insects, and they may have one or both of these genes (Pang 2014). As Drosophila melanogaster only possesses the ace-2 gene (Weill, et al., 2002), the genes that are active in the other insects that have been studied can be described as AP-AChE (paralogous to D. melanogaster) or AO-AChE (orthologous) for ease of comparison (Pang 2014). Interestingly, of the insects characterized thus far, 76% used the AP-AChE (cysteine sensitive) enzyme in 60 hydrolysis, the AO-AChE enzyme was used in 26% of the insects studied, and 7% of these insects utilized both (Kim and Lee, 2013). It is important to note that the 26% of insects using the AO-AChE enzyme were all higher order hymenopterans, including A. mellifera (Kim and Lee, 2013). The transcriptome of A. tumida has revealed that they possess the genes encoding both AChEs, but the ace-1 gene (AP-AChE) produced the most enzyme, and no resistance- associated mutations have been observed in this gene (Kim, et al., 2018). Because the AO-AChE cholinergic enzyme is found in A. mellifera, this means that their enzyme lacks sensitivity to cysteine-targeted insecticides, so these compounds could distinguish between A. tumida and A. mellifera, in addition to having low mammalian toxicity (Pang, 2014). This study will use an insecticide-naïve A. tumida colony established in 2018 in the laboratory, to allow for characterization of the enzyme kinetics of known AChE inhibitors (Figure 1), leading

to better understanding of the pharmacological differences between A. tumida and A. mellifera. Although several organophosphates and carbamates have been tested against A. tumida at the organismal level in a previous study, classification of the pharmacology of the carbamates used in this study has not taken place (Kanga and Somorin, 2012). Additionally, three novel AChE inhibitors designed for mosquito AChE inhibition and known to have low mammalian toxicity (Swale et al., 2014; Figure 2) were tested against both A. tumida and A. mellifera in this study. Discovery of in vitro enzyme kinetics through a colorimetric bioassay (Ellman assay) and in vivo toxicity through topical and injection bioassays, was carried out using both 61 A. tumida and A. mellifera, and toxicological findings will be presented and compared. 3.3 MATERIALS & METHODS 3.3.1 Chemicals

Sodium phosphate monobasic monohydrate (99.5%) and sodium phosphate dibasic anhydrous (98%), and acetone (99.7%), and ethanol (200 proof) were purchased from Fisher Scientific (Hampton, NH, USA). Acetylthiocholine iodide (98%), 5,5-dithio-bis-(2-nitrobenzoic) acid (99%), and coumaphos (99.5%), and thiodicarb (99. 2%) were purchased from Sigma- Aldrich (St. Louis, MO, USA). Aldicarb (99%), carbaryl (99.8%), carbofuran (99.5%) coumaphos-oxon (98.8%) were purchased from Chem Service (West Chester, PA, USA). Bendiocarb (99.5%) was purchased from NORAM Chemical Company (Vancouver, BC, CA). Propoxur (99%) was purchased from Mobay Chemical Corporation (Pittsburg, PA, USA). PRC 331(>95%), PRC 408 (>95%), and PRC 421 (>95%) were a kind gift from Dr. Paul Carlier, Department of Chemistry, Virginia Tech (Blacksburg, VA, USA). 3.3.2 Insects A. tumida adults were collected from the Prices Fork apiary in Blacksburg, VA, USA. These adults were returned to the lab and placed in plastic containers with ventilation (Rearing Container A), a pollen patty, and paper towel, and a 10 mL test tube filled with water and capped with a piece of cotton ball (Figure 3A). Once larvae reached their second and third instars, they were moved to a 62 ventilated shallow plastic container (Rearing Container B) containing a 1:1 sand and soil mix (sterilized). Larval escape was reduced by applying a thin layer of Vaseline around the edge of the Rearing container B. Larvae were provided a pollen patty for sustenance (Figure 3B). Deionized water was sprayed on the sand/soil mix every 1-2 days. Once larvae travelled from the pollen patty to the sand/soil, they were transferred to 50 mL conical centrifuge tubes that were filled with the sand/soil mixture (moisten). Approximately 15 third-instar larvae were added to each conical tube, and the top was covered with parafilm with small holes for ventilation (Figure 3C). The 50 mL conical tubes allowed observation of larval burrowing and pupation in the substrate. After visible pupae emerged as adults, the conical tubes were left for several days to allow the cuticles of the newly emerged adults to sclerotize (and ensure that the majority of adults had emerged), then the conical tubes were emptied, and adults were added to the Rearing Container A (Figure 3A) for the appropriate generation. The colony is kept in a dark environmental chamber (Thermo Scientific, Waltham, MA, USA) maintained at 28°C and greater than 70% relative humidity. 3.3.3 Ellman Assays for Cholinesterase Activity The Ellman Assay (Ellman et al., 1961), a colorimetric assay, was used to measure the inhibition of acetylcholinesterase. The tissue to perform this assay was composed of the heads of frozen A. mellifera adults or the heads and thoraces of third-instar A. tumida larvae (Figure 4A). Insect tissue was homogenized in 0.1 M sodium phosphate buffer pH 7.8 buffer on ice (Figure 4B) followed by centrifugation for 10 minutes at 10,000 x g at 4°C (Figure 4C), the 63 supernatant was used as the enzyme source. Ellman assays were performed in a clear 96-well flat bottom plate (Fisher Scientific) and the reaction composed of 20 μL of enzyme source (homogenate), 140 μL of 0.1 M sodium phosphate buffer pH 7.0, and 20 μL of AChE inhibitor or vehicle control. A minimum of three replicates containing two determinants of each inhibitor concentration used per replicate (Figure 4D). Contents of the plate were vortexed for 10 minutes at room temperature prior to the initiation of the reaction through the addition of acetylthiocholine iodide (10 μL; various concentrations) and 6.4 mM 5,5-dithio- bis-(2-nitrobenzoic) acid (DTNB; 10 μL) to each well (blank contained buffer, acetylthiocholine iodide, and DTNB). A SpectraMax M2 (Molecular Devices, San Jose, CA, USA) was used to monitor the hydrolysis of acetylthiocholine where the

sulfhydryl group reacts with DTNB to produce the yellow product 5-thio-2- nitrobenzoic acid (Figure 4E; Figure 5). Absorbance was measured at 405 nm for 20 minutes and velocity using
Beer's law, also known as Beer-Lambert law, was used (Beer,
1952; Equation 1) using the extinction coefficient for 5-thio-2- nitrobenzoic acid, which is 14,150 L mol-1cm-1 (Eyer, et al., 2003). Equation 1
(Beer's Law): A= εbc Where A=absorbance (no units), ε= molar absorptivity
(14150 Lmol-1cm
-1) b=Path length of sample (1 cm), and c= concentration of compound in solution (mol L-1).
Enzyme velocities were plotted in GraphPad Prism (San Diego, CA, USA), which calculated the inhibitory constant (Ki) for each compound by plotting the 64 substrate velocity curves for each compound and best-fitting one inhibitory constant for all of these substrate velocity curves while preforming competitive inhibition analyses. Three replicates using each inhibitor were performed, using new insects each time. Once Ki values were used to calculate the selectivity ratio (SR), which is defined as the A. mellifera Ki value is divided by the A. tumida Ki value. A SR greater than 1 indicates that the compound is more selective against A. tumida. The experimental compounds, which are not commercially available, were analyzed in vitro using the Ellman assay, but instead of a Ki an
inhibitory concentration of 50% of the enzyme activity in
the sample, or the
half-maximal inhibitory concentration (IC50) was determined in GraphPad Prism.
The IC50 was generated using 8 mM of acetylthiocholine iodide with various concentration of the experimental inhibitor. In addition to the experimental compounds, the IC50 values for coumaphos-oxon, propoxur, and thiodicarb were also obtained, to enable comparison to PRC 331 (I), PRC 408 (II), and PRC 421(III; Figure 2). When determining the IC50 of each compound, all treatments were normalized to the control, which contained buffer instead of inhibitor, and results were reported as a "percent of control." 3.3.4 In Vivo Toxicity Bioassays In vivo toxicity of test compounds were performed through topical application, if low activity was observed topically, compounds (Figures 1 and 2) were injected (Figure 6). Test compounds were initially dissolved in 200 proof ethanol and 65 ethanol alone served as the negative control. Treatments were delivered using
gas-tight syringe equipped with a repeating dispenser (Hamilton Company, Reno, NV, USA).
A 25 μL syringe, again equipped with a repeating dispenser
(delivers 0.5 μL increments) was used for topical treatment applications, and a 10 μL repeating dispenser with syringe (delivers 0.2 μL) was used for all A. mellifera injections. After treatment, all insects were housed in an environmental chamber (Thermo Scientific) at 28°C and >60% relative humidity for 24 hours in the dark. Moribundity was monitored for 24 hr, and if control mortality was greater than 20% (topical) or greater than 30% (injected) for the control data was not used. Otherwise
control mortality was corrected using Abbott's modification (Abbott, 1987). The LD50 values
for each treatment were
calculated using the PROC PROBIT procedure in SAS (SAS Institute, Carey, NC, USA). For toxicity tests, a minimum of
three replicates containing 10-21 individuals per treatment concentration were performed for each insecticide tested. A. mellifera were collected from apiaries in the Blacksburg VA geographic area and were primarily nurse bees. Bees collected at different times or from different colonies were used for each replicate, and at least three replicates for each bioassay were performed. Topicals were carried out by anesthetizing bees with carbon dioxide and then dividing them into groups of 10-21 bees in paper deli cups (32 oz) with tulle. The bees were then anesthetized and treated on ice, where treatments were delivered to the dorsal thoracic area of the bee. If compounds proved to be ineffective topically, they were injected into treatment concentration into the ventral abdominal area of the bee. 66 Compounds were applied topically to A. tumida larvae on ice using the 25 μL repeating dispenser syringe, which delivered 0.5 μL per application. Several seconds on ice was sufficient to anesthetize larvae to allow for easy insecticide application. A nano-injector (World Precision Instruments, Sarasota, FL, USA) was used to deliver 200 nL of test compounds via intrathoracic injection.

Injection was performed with pulled-glass capillary tubes (P1000 Micropipette Puller (Sutter Instrument Company, Novato, CA, USA); the tips of the micropipette were slight broken. A. tumida third-instar larvae were injected and topically treated with the highest dose of each treatment available (10,000 ppm), which delivered 2 μg of each treatment to the insects in question, and the same volume was topically delivered. Two hours post-treatment, Ellman Assays were performed, with topically treated insects washed in ethanol prior to use. The resulting enzyme activities were then reported as a percentage of untreated control insects after 20 minutes. An ANOVA was then performed on all results, comparing ethanol treated controls to the insecticide treatments using GraphPad Prism. 3.4 RESULTS 3.4.1 In Vitro Bioassays The only compound to yield a selectivity ratio greater than one, when examining the Ki values from the Ellman Assays, proved to be coumaphos (SR =1.69), coumaphos-oxon (SR=1.61) and thiodicarb (SR=2.18; Table 1). Additionally, the high Ki value seen for coumaphos here further confirms that coumaphos is not easily metabolized to coumaphos-oxon in vitro, therefore, coumaphos functioned 67 somewhat like a control for coumaphos-oxon. The IC50 results were comparable to the above data, in that coumaphos-oxon (SR=1.14) and thiodicarb (SR=1.05) again had selectivity ratios greater than one (Table 2). PRC 331 (0.41) and PRC 408 (0.53) were both more selective against A. mellifera, but PRC 421 (SR=1.11) was more selective against A. tumida, although less so than coumaphos-oxon (Table 2). PRC 331 was slightly more potent than coumaphos-oxon, PRC 408 and PRC 421 were both much less potent, with PRC 421 showing 27-fold less potency toward A. mellifera and 29-fold less potency toward A. tumida (Table 2) 3.4.2 In Vivo Bioassays The average weight of the A. mellifera specimens used in these bioassays was 0.117 g, which is a characteristic nurse bee average weight in the late summer/autumn, as seen in previous work (Pettis et al., 2012), while the average A. tumida larval weight was 0.0181 g. Aldicarb, bendiocarb, carbaryl, carbofuran, propoxur, and PRC 331 where the only compounds that yielded a topical LD50 in A. mellifera, while aldicarb, bendiocarb, carbofuran, and coumaphos were the only compounds that generated a topical LD50 for A. tumida. The A. mellifera injections showed that thiodicarb had the lowest LD50 level (0.7 ng/mg), while coumaphos had an LD50 of 6.3 ng/mg, and PRC 408 and 421 had LD50 values of 18.5 ng/mg and 32.6 ng/mg, respectively. When comparing coumaphos topical treatments, the LD50 for A. mellifera was in excess of 43 ng/mg, while this compound was topically much more potent to A. tumida (17 ng/mg). Neither PRC 68 331, PRC 408, nor PRC 421 were topically effective to A. tumida at doses of 276 ng/mg (Table 3). The results of the injection bioassays that were hybridized with the Ellman Assay revealed a very perplexing trend. there appears to be some factor, potentially a pharmacokinetic barrier present that is allowing these compounds to enter the cuticle but keeping them from interacting with the nervous system. As can be seen in Figure 7, there was no significant difference (P>0.05) between any of the topically treated or injected insects, while the topical and injection controls were both significantly different (P<0.05) from all of the treatments. 3.5 DISCUSSION This study set out to examine the in vitro and in vivo effects of known and novel AChE inhibitors, on A. tumida and A. mellifera. While the in vivo and in vitro bioassays performed in this study largely corroborate one another, the three experimental compounds, PRC 331, PRC 408, and PRC 421, indicate that further optimization is needed. While PRC 421 displays selectivity for A. tumida AChE, and coumaphos-oxon (activation product of coumaphos) displays similar selectivity against A. tumida in vitro but coumaphos is more potent in vivo against A. tumida indicating that this is still a more effective insecticide against this pest. However, the increasing concerns regarding the effects of coumaphos on honey bees (Chaimanee et al., 2016; Tihelka, 2018) highlight the need for more selective and potent A. tumida controls. The topical bioassays further demonstrated that the experiment compounds were ineffective against the A. tumida cuticle (LD50>276 ng/mg), which poses a difficulty in applying these 69 treatments, while coumaphos was toxic topically against A. tumida (17 ng/mg). The results of the injection and topical bioassays comparing enzyme inhibition in A. tumida and A. mellifera demonstrate that while PRC 408 and PRC 421 were much less potent to A. mellifera than coumaphos, they were also much less potent to A. tumida. The results of this study suggest that a beneficial future direction would be to clone the A. tumida ace-1 gene, as this is the gene shown to produce the majority of A. tumida AChE (Kim et al.,

2018). Although the crystal structure of AChE is known in humans and Drosophila melanogaster (Pang, 2014), cloning this gene could then aid in elucidation of the crystal structure of A. tumida AChE. This knowledge will allow for optimization of insecticides that could provide good future controls, with increased potency and selectivity toward A. tumida. Low mammalian toxicity should also be of paramount concern in such studies, as this was one of the attractive features of the three experimental compounds in this study (Swale et al., 2014). Despite the lack of potency observed in the three experimental AChE inhibitors, this research does provide a useful comparison of coumaphos to other carbamate insecticides with varying moieties that, unlike many organophosphates, have not yet been tested against A. tumida (Kanga and Somorin, 2012). Knowing the toxicity of these compounds against A. tumida will be a valuable asset for further expansion of the A. tumida pharmacology knowledge base, which can help lead to the discovery of an effective future control for these destructive invasive beetles. 70 3.6 TABLES AND FIGURES
II III I IV V VI VII VIII Figure 1.
Chemical structures (and International Union of Pure and Applied Chemistry (IUPAC) names)
of known AChE inhibitors used in this study: (I)
aldicarb (2-Methyl-2-(methylthio)propanal O-(N-methylcarbamoyl)oxime), (II) bendiocarb ((2,2-Dimethyl- 1
,3-
benzodioxol-4-yl) N-methylcarbamate), (III) carbaryl (1-naphthyl methylcarbamate), (IV)
carbofuran (2,2-Dimethyl-2,3- dihydro-1-benzofuran-7-yl methylcarbamate),
(V) coumaphos (O,O-Diethyl
O-3- 71 chloro-4-methyl-2-oxo-2H- chromen -7-yl phosphorothioate), (VI) coumaphos-oxon ((3-chloro-4-
methyl-2- oxochromen -7-yl) diethyl
phosphate), (VII) propoxur (2- Isopropoxyphenyl N-methylcarbamate), and (VIII) thiodicarb
(methyl (1E)-N- [methyl-[methyl-[(E)-1-methylsulfanylethylideneamino]Oxycarbonylamino]
sulfanylcarbamoyl]oxyethanimidothioate).
I II 72 III Figure 2. Chemical Structures of novel AChE inhibitors: (I) PRC 331, (II) PRC 408, and (III) PRC
421. A B 73 C Figure 3. A. tumida colony rearing, with container for adults and egg laying (A), from which second and third instar larvae are removed and placed in a container with sand/soil (B), after which some larvae and placed in 50 mL conical tubes as pupation containers (C). A B C D D E E 74 Figure 4. Steps of the Ellman Assay: A. mellifera heads, or A. tumida heads and thoraces are removed on ice (A), homogenized in 0.1 M sodium phosphate buffer (B),
centrifuged at 10 ,000 x g and 4 °C for 10 minutes (C), supernatant is pipetted into a
clear flat-bottomed microplate, with inhibitor, acetylthiocholine iodide, and DTNB (D), plate is then read in a SpectraMax M2 microplate reader on the kinetic setting, measuring absorbance over 30 minutes at 405 nm (E). Figure 5. Reaction that takes place in the Ellman Assay: acetylthiocholine iodide is hydrolyzed by AChE, forming thiocholine and acetic acid. Thiocholine reacts with DTNB to form yellow 5-thio-2-nitrobenzoic acid and mixed disulfide products. A strong yellow color in microplate wells after this assay indicate low inhibition of AChE. 75 AChE Inhibitors Aldicarb Bendiocarb Carbaryl Carbofuran Propoxur Thiodicarb Coumaphos Coumaphos-oxon A. mellifera Ki A. tumida Ki Selectivity Ratio (μM) (μM) (SR) 18.0 47.0 0.38 0.53 1.13 0.47 0.21 0.42 0.50 0.82 1.88 0.44 0.79 4.23 0.19 1.11 0.51 2.18 537 317 1.69 0.46 0.29 1.61 Table 1. Inhibitory constant (Ki) values obtained through the Ellman Assay for both A. mellifera and A. tumida. The darker gray color indicates the carbamates used, while the lighter gray color denotes the organophosphates coumaphos and coumaphos-oxon. The selectivity ratios (SR) on the far right were obtained by dividing the Ki of A. mellifera by the Ki of A. tumida for each treatment. A SR greater than 1 indicates selectivity against A. tumida. 76 Compound A. mellifera A. tumida Selectivity Ratio IC50 (μM) IC50 (μM) Coumaphos-oxon 0.16 0.13 1.14 Propoxur 0.92 1.67 0.55 Thiodicarb 3.27 3.12 1.05 PRC 331 0.04 0.11 0.41 PRC 408 8.94 16.9 0.53 PRC 421 4.25 3.82 1.11 Table 2. This graph depicts the inhibitory concentrations of 50% of the enzyme activity, or IC50 values, discovered through use of the Ellman Assay. The selectivity ratios (SR) can be seen in the far-right column of the graph, which were found by dividing the IC50 of A. mellifera by the IC50 value of A. tumida for each treatment. An IC50 value greater than 1 indicates greater selectivity toward A. tumida. 77 A B C Figure 6. Topical application of treatment to the thoracic area of A.

tumida on filter paper with a 25 μL repeating dispenser syringe (delivers 0.5 μL) can be seen in (A). Injection of the A. mellifera abdomen with a 10 μL repeating dispenser syringe is shown in (B). The 2 oz containers used to contain A. tumida are depicted in (C), along with the setup used to treat these insects, which only differed in A. mellifera treatments through the use of paper 32 oz containers. 78 Compound Aldicarb Bendiocarb Carbaryl Carbofuran Propoxur PRC 331 Coumaphos Coumaphos- oxon Thiodicarb PRC 408 PRC 421 A. mellifera Topicals LD50 (ng/mg) 0.05 0.12 4.39 0.40 1.02 1.34 >43 >43 >43 >43 >43 A. mellifera Injections LD50 (ng/mg) - - - - - - 6.3 3.3 0.7 18.5 32.6 A. tumida Topicals LD50 (ng/mg) 46 42 >276 40 >276 >276 17 >276 >276 >276 >276 Table 3. This table shows the LD50 values for each compound tested via topicals and injections with A. mellifera, and all compounds that were topically applied to A. tumida. The compounds that had LD50 values greater than 43 ng/mg in A. mellifera and greater than 276 ng/mg in A. tumida are depicted because after treatment with this concentration of insecticide (10,000 ppm) injection bioassays became the preferred choice of treatment. 79 * Figure 7. This figure shows injections of 2 μg of each compound vs. topical application of 2 μg of each compound (±SEM). An Ellman assay was performed 2 hours post treatment (with topical insects washed in ethanol). All results were normalized to non-treated control insects. The asterisk denotes a significant difference between all treatments and the topical and injection controls ($P<0.05$), while there is not a significant difference between the compounds administered topically or via injection. Chapter 4. 80 GENERAL CONCLUSIONS AND FUTURE DIRECTIONS 4.1 GENERAL CONCLUSIONS 4.1.1 Varroa mite (Varroa destructor, Anderson and Trueman) Population Dynamics and Acaricide Resistance One of the key contributors to Colony Collapse Disorder (CCD) in European honey bee (Apis mellifera L.) colonies is the ectoparasitic mite V. destructor (Rosenkranz et al., 2010). Colony damage is caused by transmission of numerous viruses (Hung, 1996) as V. destructor feeds upon the fat body of pupating and adult bees (Ramsey et al., 2018). Historically, V. destructor was controlled primarily by synthetic acaricides, with three of the most popular being amitraz, coumaphos, and tau-fluvalinate (De Mattos et al., 2017). However, heavy and repeated treatment application led to observations of V. destructor resistance to these three compounds (Kamler et al., 2016; Pettis, 2004; Hillesheim et al., 1996). V. destructor resistance in three geographic regions of Virginia were investigate by first examining mite infestations levels at three to four-week intervals. The three geographic regions included several apiaries in Blacksburg, and at collaborating apiaries in Richmond and Virginia Beach. Results showed mite population increases throughout the sampling season at all three sites, as would be expected, based on previous reports (Beaurepaire et al., 2017) and the reproductive cycle of V. destructor (Ruijter, 1987). A glass vial contact bioassay was used to investigate resistance at these three sites against the acaricides amitraz, coumaphos, and tau-fluvalinate. Mortality of mites in the resistance 81 bioassays at 24 hours was significantly different from control mites in acetone-treated vials, indicating that these mite populations are not resistant to the three acaricides. In accordance with the toxicity bioassays, there was not a difference in biochemical assays investigating metabolism. Specifically, there was not a significant difference between the three geographic regions for general esterase activity or cytochrome P450 monooxygenase activity. Since mites collected at Price Fork (Blacksburg apiary) was not previously exposed to these acaricides, or really any acaricide treatment on a regular basis, we can assume this is a naïve population of mites. These biochemical levels provide a baseline for continued investigation of acaricides resistance throughout the Commonwealth. 4.1.2 Small Hive Beetle (Aethina tumida, Murray) Susceptibility to Known and Novel AChE Inhibitors A. tumida has risen in importance over the past 20 years, as these beetles have spread throughout the United States, leaving a trail of fermenting waste-filled hives (Hayes et al., 2015) and significant economic loss (Hood, 2004) in their wake. Of the control methods attempted to date, chemical controls are still underdeveloped (James & Ellis, 2016). This study tested various carbamates, along with the organophosphate coumaphos, which is currently used for A. tumida control (Cuthbertson et al., 2013), through a series of in vitro and in vivo bioassays.

Three experimental compounds PRC 331, PRC 408, and PRC 421 where investigated against A. tumida and compared to A. mellifera. These experimental carbamates were previously designed for and displayed selective 82 toxicity and cholinesterase inhibition against mosquitos and mammals (Swale et al., 2014). To

date, A. tumida AChE has not been characterized, and this study resulted in an increased knowledge of A. tumida acetylcholinesterase pharmacology. Furthermore, one of the experimental carbamates (PRC 421) displayed selective inhibition of A. tumida acetylcholinesterase, albeit weak selectivity. However, PRC 421 was still 29-fold less potent than coumaphos- oxon. The in vivo bioassay results further supported conclusion, in that coumaphos was more selective, topically, against A. tumida. These results indicate that, of the insecticides tested in this study, coumaphos was the most selective acetylcholinesterase (AChE) inhibitor towards A. tumida. However, because coumaphos can detrimentally affect A. mellifera, (Gregore et al., 2018) the quest for effective A. tumida controls will need to be continued in the future. 4.2 FUTURE DIRECTIONS 4.2.1 Continued V. destructor Acaricide Resistance Studies To glean a more thorough picture of acaricide resistance throughout Virginia, continued surveillance is needed to monitor both mite infestation levels but also acaricide resistance. The use of pre-treated glass vials that were used in this study could be deployed to apiaries throughout the Commonwealth. It would also be useful to study mites from hives that are frequently treated with these acaricides, which could be compared with mites from hives that receive no, or less frequent, acaricide treatment administration. Also, in addition to testing 83 esterase activity and cytochrome P450 monooxygenase activity, Glutathione S- transferase (GST) activity biochemical assays should also be performed, as increased GST activity can be correlated with organophosphate resistance, which would correspond to coumaphos resistance in this study (Yao et al., 2018). It would be especially useful to use these three bioassays to compare enzyme activity between populations that display resistance and those that do not. In a previous study, performed using mites from one of the Blacksburg apiaries (Prices Fork Apiary, from which mites were collected for this study) some resistant populations were observed (Vu, 2016). Although there was no reported use of any of the synthetic acaricides at the Prices Fork Apiary prior to this study, the difference in resistance at this site between this study and the 2016 mentioned above indicate that perhaps resistance development is costly to the mites and, when acaricides are no longer present, resistance is quickly lost. Therefore, continuing to study these mite populations over time can help in establishing the rate and incidence of acaricide resistance development, including an investigation of novel point mutations that have been reported in the V. destructor sodium channel gene (Wang et al., 2002), both locally and throughout Virginia. 4.2.2 Optimization of A. tumida Chemical Treatments In order to obtain better insights into the nature of A. tumida organophosphate and carbamate sensitivity, cloning the A. tumida ace-1 gene should take place, as well as discovery of the A. tumida AChE crystal structure, allowing for better design of A. tumida selective insecticides that could be performed in silico. In a 84 previous study, no point mutations that are commonly seen in other insects that demonstrate organophosphate resistance were observed in the ace-1 gene (Kim et al., 2018), however, this should also be tested in A. tumida populations found in the Commonwealth. One voltage-gated sodium channel gene has also been identified in A. tumida, and, although no resistance-associated mutations were observed (Kim et al., 2018), this would also be an important gene to examine in A. tumida populations of Virginia. The laboratory colony established for this project will continue to be maintained and will be available for future experimentation. 4.2.3 Integrated Pest Management (IPM) Impacts on V. destructor and A. tumida The acaricide resistance that has caused such distress to apiculturists when dealing with V. destructor infestations can be contributed, in large part, to the repeated use of these acaricide treatments that was historically observed (De Mattos, et al., 2017). To reduce risk of resistance development, IPM methods are now being advocated, which attempt to keep pest populations below seasonal economic thresholds (Honey Bee Health Coalition, 2015) through cultural and mechanical controls, treatment rotation using organic acids and, when all else fails, rotated synthetic acaricide treatments (Vandervalk & Nasr, 2017). A. tumida management is complicated by lack of a clear economic threshold (Neumann et al., 2016) and well-established population estimate, therefore, establishing and studying an IPM model for A. tumida control could be highly beneficial and reduce risk of resistance development. Future studies should, therefore, include 85 the testing of IPM methods in both V. destructor and A. tumida control, and examining colony strength, disease prevalence, and pest populations. Combining this knowledge of V. destructor acaricide resistance, A. tumida pharmacology, and successful IPM methods can lead to a future where these pest populations are kept in check by informed apiculturists. 86