Targeting Proctolin Signaling Pathways to Control the Small Hive Beetle

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Abstract

The small hive beetle, *Aethina tumida*, is an invasive pest responsible for extensive damage to the European honey bee, *Apis mellifera*. This pest damages hives by feeding on honey and defecating its excrement contains yeast microbes that ferment the honey, causing it to leak from the hive. Fermented honey is irreparable, and if enough honey is lost, colonies will abscond in search of a new hive. The loss of honey and colonies can have a heavy impact on beekeepers and farmers, since *A. mellifera* -pollinated crops contributed to between $11.68 and $19.22 billion in yearly crop yield in the U.S. alone as of 2009. Current measures to combat *A. tumida* are often ineffective, can harm *A. mellifera* populations, negatively impact native species, or leave residues in honey. This underlines the need for a safe, effective, and targeted control method against the pest. RNA interference (RNAi) is a promising molecular tool for targeted pest management. By introducing double-stranded RNAs, essential physiological processes can be disrupted in a target organism without harming other species, such as *A. mellifera*. Prior research indicates that *A. tumida* has a robust RNAi response, suggesting that targeting a gene that *A. mellifera* lacks could prove fruitful for targeted pest control. Another burgeoning means of pest control is peptidomimetics. Peptidomimetics are synthetic peptides that can artificially disrupt the normal cascades of various systems by mimicking neuropeptides. These neuropeptide mimics can potentially nullify or extend processes, leading to a dysregulation of vital systems. Previous research suggests that Proctolin peptidomimetics are an effective control method for another hive pest, *Varroa destructor*, as application of the peptidomimetic achieved 100% mortality within 48 hours of exposure. If both RNAi and peptidomimetics are effective against *A. tumida*, this could provide two new control options against the pest. Initial trials demonstrated the effectiveness of *proctolin receptor* (*Proc-R*) dsRNA injection against *A. tumida*, with

mortality rates comparable to those observed in groups injected with *vacuolar-type ATPase subunit A* dsRNA. Once *Proc-R* was established as a viable target, proctolin peptidomimetic injections and topical assays were conducted to determine the effectiveness of these peptidomimetics against *A. tumida* larvae. While the topical assays were inconclusive, the injection assays resulted in significant effects, including twitchy behavior and increased speed, which eventually would lead to complete mortality in one of the three experimental groups. These conclusions help support the idea that both RNAi and peptidomimetics are feasible means of control in *A. tumida* populations, suggesting further research is needed to develop these as commercial control methods.

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Chapter 1: Literature Review

1.1. European Honey Bee

The European honey bee, *Apis mellifera* Linnaeus, makes significant contributions to the national and global economy and health through its roles in the production of valuable resources and pollination efforts (Khalifa et al., 2021). Regulating services, in the form of pollination, are greatly impacted by *A. mellifera*. *A. mellifera*-mediated pollination contributed between $11.68 and $19.22 billion in crop yield in the U.S. alone as of 2009 (Khalifa et al., 2021). Roughly 75 crop species rely solely on *A. mellifera* for their pollination, including almonds, soybeans, cotton, apples, and many citrus fruits (Khalifa et al., 2021). With such heavy reliance on *A. mellifera*, there could be great losses in agriculture if they were to decrease drastically due to invasive hive pests.

Alongside pollination efforts, *A. mellifera* also benefits the pharmaceutical field through the production of a variety of useful items. Beeswax is a compound directly secreted by the bees used for the structural integrity of the hive and comb. Beeswax and bee venom both have notable antimicrobial and antifungal effects when added to foods and act as an organic pesticide (Fratini et al., 2016). Propolis, a substance bees produce by mixing their saliva with tree and plant saps, has been shown to have anti-inflammatory and antioxidant effects and to show promise in Alzheimer's and cancer studies (Duffy 2020). Overall, bee products have a variety of agricultural and pharmaceutical benefits.

1.2 Honey Bee Losses

A. mellifera losses were previously fairly consistent, ranging from 15-30% in 2006-2007, yet by the current year, annual *A. mellifera* colony losses have increased to 55% (Genersch et al., 2010; Nearman et al., 2025). This decline in population is attributed to a variety of different causes depending on the source. The most consistent cause of decline is the increased uptake of pesticides into honey and hives, and increased pests and pathogens of bee (Hristov et al., 2020). These various factors have different effects on bees, both immediate, lethal effects along with systemic effects such as continuous hindrance of generations or overall nuisance.

In approximately 161 commonly used apiary pesticides, 124 remained in the pollen, 95 in wax, and 77 in honey and/or nectar. Of these groups, only five pesticides were found to be of significant risk to *A. mellifera* populations (Mullin et al., 2010). There was significant risk, with half or more of populations dying off after only two days of exposure to thiamethoxam or lindane, neonicotinoid and organochlorine (Mullin et al., 2010). These pesticides have long-term effects on *A. mellifera*, including neurological and muscular deterioration. About 12.4% of American beekeepers say their hives are stressed by pesticides, though this does not always result in hive loss. Currently available data does not explore the overall hive losses across the country due to pesticides, pests, etc., as these studies are fairly small-scale (USDA 2024)

Among the invasive pests that threaten *A. mellifera*, three species are particularly destructive: *Galleria mellonella* (greater wax moth), *Varroa destructor* (*Varroa* mite), and *Aethina tumida* (small hive beetle). The greater wax moth adults will infiltrate a hive, lay eggs, and those eggs will hatch into destructive larvae (Kwadha et al., 2017). These larvae will feed on the honey, tunnel through the wax, and produce silk that can entangle bees and damage the hive. The *Varroa* mite acts similar to a tick, yet they will attach to bees and exclusively feed on their

fat reserves, draining the bee of nutrients and energy (Noël et al., 2020). These mites reproduce in bee hive cells, usually attaching to fresh larvae and sapping them of their nutrients until they emerge deformed. Finally, the small hive beetle is a nuisance that causes damage to the honey through feeding and defecating in it, leading to fermentation by yeast microbes in their feces (Roth et al., 2022).

Many viral, bacterial, and fungal pathogens are significant threats to *A. mellifera*. Deformed wing virus (DWV) is a destructive pathogen that causes significant developmental defects in infected bees. Any bee larvae infected with DWV will grow and, upon pupation, emerge with small, deformed wings and abdomens, typically lethal within 76 hours post-emergence (Lanzi et al., 2006). Acute bee paralysis virus (ABPV) can paralyze and kill pupae and adults 3-5 days post-infection. Aside from these pathogens, *Nosema ceranae* is a common fungal pathogen that can be carried by a variety of pests, including *Aethina tumida*. This pathogen will infect healthy adults and replicate in gut cells before bursting and being expelled in feces, which will then be exposed to other bees, continuing the replication process and leading to increased *A. mellifera* mortality during fungal replication (Dussaubat et al., 2012).

1.3 Small Hive Beetle

The small hive beetle, *Aethina tumida* Murray, is a significant hive pest in the U.S., first found in Charleston, South Carolina, in 1996, followed by a second influx across all of Florida in 1998. These pests are hypothesized to have come from infested beehives originating in central Africa (Sheridan 2020). By 2008, the pest could be found ubiquitously in all U.S states except Alaska (Cuthbertson et al., 2013). This spread has largely been attributed to the mass movement of managed hives (Sheridan 2020). For example, a colony of small hive beetles may be present

in Florida, and then the hive is then shipped to California for the almond pollination season. During this mass congregation of millions of hives, the beetles will hop between hives, develop, grow, and produce offspring that will fly to other hives, all of which are heading back home across the U.S. (Cuthbertson et al., 2013).

1.4 Life Cycle and Damages

Upon first infection, adult hive beetles will remain in proximity to this original hive or find a new hive to propagate. Small hive beetles can fly two miles a day, or 8-9 miles per week on average (Cornelissen et al., 2024). During flight, beetles are searching for volatiles and pheromones primarily produced by honey bees and bumble bees, such as ethyl linolenate and ethyl palmitate from pollen dough or oleamide and tetracosane found in fermenting honey (Hayes et al., 2015). Once adults have located a hive, they will mate within the hive, and gravid females will lay clutches of 10-30 eggs in cracks and crevices throughout the hive, or they will pierce the brood comb and deposit eggs inside (Roth et al., 2022). Each female is capable of producing up to of 300 eggs across their lifetime, meaning small hive beetles can rapidly infest a hive. Eggs will take 2-3 days to hatch, after which the larvae will feed and develop for 10-14 days, molting through three instars. Once larvae have reached the third instar, they enter a "wandering" larval stage during which they will leave the hive and burrow into the soil near the hive. Once the larvae are settled, they will undergo pupation, which can take 15-33 days, or overwinter for months if proper conditions are not met. After pupation, newly emerged adults will return to a hive and feed. After five days of post-eclosion, adults are sexually mature and can reproduce (Cuthbertson et al., 2013; Roth et al., 2022). Depending on temperature, these beetles can have upward of 4-6 generations per year (Sheridan et al., 2012). Within this life

cycle, the larval stage is responsible for the majority of damage to hives. When they feed and excrete in honey, their excrement contains *Kodaea ohmeri* yeast microbes, which will ferment the honey. Fermented honey becomes a slurry that seeps from the hive, and bees do not attempt to fix afflicted honey or comb, as they will abscond from the hive if the infestation becomes too severe (Roth et al., 2022).

1.5 Control Methods

There is a wide array of control methods for the small hive beetle, ranging from mechanical to chemical and biological approaches, each with its own advantages and limitations in the apiary setting. As a mechanical control, hood traps involve placing a small pitfall-trap-like container between frames of the hive with a grated top for beetles to fall into. These containers will be filled with an attractant, such as apple cider vinegar, which attracts and drowns beetles (Ellis, 2002; Ellis, 2004). Similar to hood traps, bottom board traps are removable bottom boards with a grated top and an attractant, designed to capture and drown hive pests, including small hive beetles, that fall from the hive as part of a defense response. Both traps use grates that allow hive pests, such as the small hive beetle, to enter but not exit. Entanglement traps are simple, usually consisting of a piece of cloth laid along the uppermost frames of the hives that entangles any beetles that attempt to climb to the hive lid (Ellis, 2002; Ellis, 2004). While all three types of traps are effective, they only target adults, allowing larvae to continue destroying comb and honey. These traps can occasionally attract, drown, or entangle bees (Roth et al., 2022).

Since 1999, coumaphos and fluvalinate, two common insecticides, were the primary tools for small hive beetle management until 2007. These pesticides were sprayed in hives to eliminate both adults and larvae, while permethrin was applied to the surrounding soil to kill pupae (Hood

2000). Other pesticides were notably stronger than coumaphos, yet they all had severe off-target effects on other insects (Kanga and Somorin, 2012). Coumaphos was added to CheckMite+ strips, a type of strip that could be hung between the frames of a hive, fumigating the hive with the pesticide and killing any hive beetles or *Varroa* mites. In 2007, Levot tested a plastic, tamperproof harborage containing corrugated cardboard soaked in fipronil, an organic phenylpyrazole, and its efficacy against the small hive beetle (Levot 2007). Marketed under the trade name Apithor™, this product is considered one of the most effective chemical defenses against the small hive beetle. It demonstrates high efficacy against beetle populations, while maintaining metabolite residual levels below the legal limit for fipronil, which is 1 µg/kg (Levot and Haque, 2006).

Since around 2003, two primary forms of biological control have gained traction: soil minerals and entomopathogenic fungi. Minerals such as slaked lime and diatomaceous earth are known to drastically alter the pH of soil, which can disrupt anything attempting to pupate in the soil. It must be noted that these minerals only work for a very brief period in the life cycle, as the larvae must ingest either mineral for their pH to be altered, yet larvae only leave the soil when they are no longer feeding. This implies that, unless soil is introduced inside the hives, it is unlikely to lead to high mortality rates in small hive beetles. As for entomopathogenic fungi (EPF), few species of EPF were known to be broadly effective against many coleopteran pests, including the small hive beetle. The introduction of common fungi such as *Aspergillus niger* and *Aspergillus flavus* to pupating larvae led to a 32% mortality rate in replicates (Ellis et al., 2004). With deficiencies in these preexisting methods, a new form of control is necessary to combat *A. tumida*. Remaining of this chapter will describe relatively new technology being developed for pest control in bee hives, which includes RNA interference and peptidomimetics.

1.6 Overview of RNAi

RNAi (RNA interference) is a conserved gene-silencing mechanism that operates through post-transcriptional mRNA degradation (Hannon 2002). This process begins when double-stranded RNA (dsRNA) enters a cell and is cleaved by RNase III Dicer-2 (Dcr2) into small interfering RNAs (siRNAs) (Hannon 2002). These siRNAs are incorporated into Argonaute2 (Ago2) with the aid of the cofactor Dicer-2. This assembly, known as RNA-induced silencing complex (RISC), unwinds siRNAs, degrades the sense strand, and retains the antisense strand to guide the degradation of complementary mRNAs (Hannon 2002), thereby preventing mRNA translation and protein synthesis.

1.7 Developments in RNAi

First described by Andrew Fire in 1998, injections of dsRNA targeting the *unc22* (uncoordinated-22) gene were conducted in the nematode *Caenorhabditis elegans*, leading to the expected twitching phenotype due to a lack of myofilament protein production (Fire et al., 1998). Later the same year, Kennerdell and Carthew extended RNAi research into insects by injecting *Drosophila melanogaster* embryos with dsRNA that ultimately led to deformed body types in flies, showing that RNAi is functional in insects (Kennerdell and Carthew, 1998). In 1999, the first RNAi experiments in *T. castaneum* demonstrated that RNAi is a reliable tool that can be used in coleopterans (Brown et al., 1999). In 2007, a major advancement occurred when it was proven that dsRNAs can be delivered to some coleopterans in multiple oral routes (Baum et al., 2007). Multiple targets were tested, including *Diabrotica vigifera*, the western corn rootworm, *Diabrotica undecimpunctata howardii*, the southern corn rootworm, and *Leptinotarsa*

decemlineata, the Colorado potato beetle. This was a major development demonstrating that dsRNAs can be administered to variety of pest insects orally and non-orally, providing a potential means of control in a field setting. Further insect RNAi research can be seen in below (Table 1).

Table 1. Notable RNAi applications in beetle species: Target genes, life stages, and experimental outcomes

This table depicts the most notable examples of RNAi across different beetle species over the past decades, including which genes were targeted, which insect stages were targeted, and how successful each experiment was.

Family	Species	Target genes	Delivery Method	Target Stage	Results	Source
Tenebrionidae	*Tribolium castaneum*	*TcDfd, Mxp, Hth, Exd*	Injection	Larvae	Successful	Brown et al., 1999
Tenebrionidae	*Tribolium confusum*	*CPR, CYP4G7, CYP4G14*	Injection	Larvae	Successful in CYP4G7 and CYP4G14	Huang et al., 2021
Tenebrionidae	*Tenebrio molitor*	*Vermillion, TmCad1*	Injection	Larvae	Successful	Oppert et al., 2019
Tenebrionidae	*Zophobas atratus*	*Met, Kr-h1, malE*	Injection	Adults	Successful in Met and malE	Toga et al., 2021
Brentidae	*Cylas puncticollis*	*ATPsynβ, Vha68-2, Syb, Pfk*	Injection, Feeding, Soaking	Larvae	Injection successful; Feeding successful	Prentice et al., 2016

					in prosα2, RpS13, snf7	
Brentidae	*Cylas brunneus*	*ATPsynβ, Vha68-2, Syb, Pfk*	Injection, Feeding	Larvae	Injection successful; Feeding successful in prosα2, RpS13, snf7	Christiaens et al., 2016
Buprestidae	*Agrilus planipennis*	*AplaScrB-1, AplaScrB-2*	Injection	Adults	Successful in AplaScrB-2	Zhao et al., 2015
Curculionidae	*Anthonomus grandis*	*AntgCHS1*	Injection	Adults	Successful	Firmino et al., 2013
Curculionidae	*Dendroctonus ponderosae*	*MPB*	Feeding and Absorption	Adults	Both Successful	Kyre et al., 2020
Chrysomelidae	*Diabrotica virgifera*	*V-ATPase-A*	Feeding	Larvae	Successful	Baum et al., 2007
Chrysomelidae	*Phyllotreta striolata*	*AK*	Feeding	Larvae	Successful	Zhao et al., 2008
Chrysomelidae	*Leptinotarsa decemlineata*	*β-Actin, sec23, V-ATPase-E, V-ATPase-B, COPβ*	Feeding	Larvae	Successful	Zhu et al., 2010
Chrysomelidae	*Phaedon cochleariae*	*Cact, srp54K, rop,*	Injection and Feeding	Larvae	Injection and feeding successful	Mehlhorn et al., 2021

Chrysomelidae	*Callosobruchus maculatus*	*Laccase1*	Injection	Adult	Successful	Segers et al., 2023
Dermestidae	*Dermestes maculatus*	*prd*	Injection	Larvae and Adult	Successful	Xiang et al., 2016
Scarabaeidae	*Popillia japonica*	*BAPC*	Feeding	Adult	Successful	Carroll et al., 2023
Nitidulidae	*Carpophilus hemipterus*	*Vermillion*	Injection	Larvae	Successful	Bansal et al., 2023
Nitidulidae	*Carpophilus hemipterus*	*Vermillion*	Injection	Larvae	Successful	Bansal et al., 2022
Nitidulidae	*Aethina tumida*	*V-ATPase-A, Laccase2*	Injection and Feeding	Larvae, Eggs	Injection successful; feeding unsuccessful	Powell et al., 2016
Nitidulidae	*Aethina tumida*	*JHAMT*	Injection and Feeding	Larvae	Injection successful; Feeding successful	Gu et al., 2025

1.8 Recent Studies in Small Hive Beetle

In 2016, initial RNAi trials provided dsRNAs to *A. tumida* wandering larvae via injection and oral application of *Laccase2* and *V-ATPase-A* dsRNAs, acting as the non-lethal positive control and lethal control gene, respectively. Expected mortality was observed in the injected groups; however, no mortality was observed in the dsRNA-fed groups, indicating that the feeding assay was unsuccessful (Powell et al., 2016). The largest gap in knowledge was how to deliver dsRNAs to *A. tumida* larvae orally, which was achieved in 2025 by encapsulating the dsRNAs in a nanoparticle that was then fed to wandering larvae (Gu et al., 2025). Current goals

for the continuation of RNAi in *A. tumida* should be to determine if there is a way to allow the oral uptake of these dsRNAs without the need of nanoparticles, or if other compounds may be better suited for the control of this pest, such as peptidomimetics.

1.9 Overview of Peptidomimetics

Peptidomimetics are synthetic peptides designed to mimic certain neuropeptides commonly found in the target organism while overcoming many limitations of natural peptides (Gante 1994). Natural peptides are commonly degraded by proteases, have limited activity due to specificity, and are constrained by poor stability. Compared with standard peptides, peptidomimetics exhibit improved stability, greater resistance to native proteases, and fine-tuned hydrophobicity/hydrophilicity, binding, and specificity through the additions of non-natural amino acids being added to their structures (Gante 1994). This means that peptidomimetics can bind to a receptor and continuously elicit a response, leading to severe dysregulation and mortality in a target organism. Peptidomimetics are a promising tool in hive pest control, as they can be designed to target specific receptors vital to important functions *in A. tumida* or the *Varroa* mite without harming *A. mellifera*, since the bees lack the corresponding peptide receptors.

1.10 Developments of Peptidomimetics

Early work established a framework for peptidomimetic design by converting agonists of the pheromone biosynthesis-activating neuropeptide (PBAN) into antagonists through backbone and terminal modifications that enable binding without receptor activation. These stable

antagonists, which bind efficiently without eliciting any effect, were among the earliest peptidomimetics (Gilon et al., 1997).

Alongside PBANs, other diverse types of peptidomimetics have been developed, such as kinin-based peptides (Holman et al., 1986). Recent work focused on the *Varroa* mite, with different peptidomimetics being tested on both the *Varroa* mite through injection and topical application on the palps, and injection in adult bees. The best peptidomimetics were those altered from the proctolin peptide. While this topical/oral application to *Varroa* mites elicited a near-100% mortality, the bees experienced no adverse effects or increased mortality (Jindal et al., 2022). This indicates that these peptidomimetics can be designed with such specificity that they can target a given hive pest without harming nearby bees. While the success of this peptidomimetic is evident against the *Varroa* mite, it remains unknown how it affects other hive pests, such as *A. tumid*a.

The next step in the development of this project was to determine the efficiency of this peptidomimetic when exposed to *A. tumida*, to see if it affected this pest as severely as it had affected the *Varroa* mite. Once specific peptidomimetics from that paper were selected based on their distinct modifications to the peptide, they would be reused and tested against *A. tumida* larvae. This new project involved determining that *A. tumida* was susceptible to proctolin dsRNA and proctolin-based peptidomimetics. We hypothesized that knockdown of the *proctolin receptor* (*Proc-R*) via RNA interference would result in significant larval mortality comparable to *V-ATPase-A* suppression, and that proctolin-based peptidomimetics would induce similar neuromuscular dysregulation in A. *tumida* larvae.

Chapter 2: dsRNA and Peptidomimetic Application in *A. tumida*

2.1 Introduction

The decline in *A. mellifera* Linnaeus populations is a multifaceted issue driven by various factors, including pests such as *A. tumida*. *A. tumida* Murray has been shown to have significant effects on A. mellifera populations, as seen in Chapter 1, mainly through honey sliming and pathogen transmission. All control methods for *A. tumida* have significant drawbacks, including limited range or toxicity. Given how inefficient traps and chemicals are against *A. tumida*, new methods need to be developed to combat this pest. Evidence of RNAi's effectiveness against common coleopteran pests, including *A. tumida*, and of the effects of peptidomimetics against similar hive pests laid the groundwork for this research. The *proctolin receptor* gene mediates the effects of proctolin through muscular contractions and neural signaling. Initial tests examined how RNAi-mediated knockdown of this gene would affect the behavior, development, and mortality of the beetle. Subsequently, the peptidomimetics effective against the *Varroa* mite were tested on *A. tumida* to determine whether they yield similar results. As seen in chapter 1, the use of RNAi would help determine how susceptible *A. tumida* should be against the selected peptidomimetics. We hypothesized that knockdown of the *proctolin receptor* (*Proc-R*) via RNA interference would result in significant larval mortality comparable to *V-ATPase-A* suppression, and that proctolin-based peptidomimetics would induce similar neuromuscular dysregulation in *A. tumida* larvae.

2.2 Materials and Methods

2.2.1 *A. tumida* Acquisition and Rearing

Throughout this project, Valor Honey collaborated with K-State, providing access to the hives and permitting the collection of any *A. tumida* found. From the span of Fall 2025 to Spring 2026, 20-30 field collections were conducted to collect hive beetle adults. A variety of sites were used, ranging across Riley County, KS. Each week, hives at multiple sites in Manhattan, Kansas, were inspected for beetles. Live beetles were collected and brought back to the lab, where they were placed in the appropriate enclosures. Initial attempts included keeping the adults in large 17” x 12” containers with a sieve lid for airflow. These containers were kept in complete darkness and were provided nondescript amounts of water via soaked paper towels which would be placed along the bottom of the plastic container, while the beetles would also be provided whole frames of bee comb to feed on and lay eggs within. Beetles would be provided pieces of pollen patty *ad vitam*, depending on how many beetles were present and how fast they were feeding day-to-day. Other alterations made between test runs were spraying bee comb in Nasonov, releasing live bees into the terrarium, spraying the walls of the enclosure instead of adding soaked cloth, altered the size of terrarium, and changing how many beetles would be added per terrarium. All of these were proven to not aid in encouraging *A. tumida* breeding. This is the following enclosure that worked: 1) Provide *A. tumida* adults with a standard 32 oz mason jar, then add a 1” x 3” piece of comb and 200 g of pollen patty. 2) Place this jar in a 35℃ incubator with no access to light, artificial or natural. 3) Place an open mason jar filled with purified water next to the jar to increase humidity in the incubator. 4) As the adults produce larvae, immediately remove these larvae from the terrarium and place them in a separate 32 oz mason jar with 300 g of pollen patty. All of these alterations allow for adults to produce a high

quantity of larvae without producing too many, which would lead to overcrowding and unsanitary conditions. Providing these small amounts of feed and comb keep the populations low and manageable. High humidity and temperature also expedite the breeding process. Providing larvae with a higher quantity of food will encourage faster growth for use in experiments, but the highest priority is keeping the original breeding adults clean and breeding continuously. As the adults produced many larvae, and these larvae fed and grow, they would become what is called wandering larvae. This was characterized by their large size, sizable dorsal spines, and lack of feeding. These wandering larvae were moved to pupation jars, characterized as 32 oz major jars filled to the top with sandy soil in a 20:80 mix of autoclaved play sand and potting soil. For maximum efficiency, the number of larvae allowed per pupation jar did not exceed 40 larvae; any higher would lead to decreased pupation/development speed. Larvae that pupated and successfully reached adulthood would be added to new breeding chambers with comb and pollen patty. In this cycle, and excess larvae that were not needed to continue producing adults would be used for injected experiments.

2.2.2 Primer Designs

Primers were designed using UGENE and Primer3 software for Nested PCR and qPCR Analysis of *Glyceraldehyde-3-phosphate dehydrogenase* (*GADPH)* , *vermilion*, *V-ATPase-A*, and *Proc-R* in *A. tumida*. GAPDH primers were used as a reference gene in qPCR protocols, as reported in Gu et al. (2025), who compared it with other RNAi targets against *A. tumida*. *Vermilion, V-ATPase-A*, and *Proc-R* primers were designed based on conserved regions found in *A. tumida, A. mellifera, D. virgifera, D. melanogaster*, *T. molitor, and T. castaneum.*

Table 2 Sequences of primers used for *Vermilion*, *V-ATPase-A*, and *Proc-R* subcloning, dsRNA synthesis, and qPCR

Primer	Primer Sequence 5'-3'
Nested PCR	
Vermilion F1	CTTGGAGCCCAGAGAATG
Vermilion R1	TAACCTGATGAACCGCCAG
V-ATPase-A F1	GGTAAACCCTTGTCCGTCG
V-ATPase-A R1	CCACGTGATCTTGTTCTC
Proc-R F1	CACTGGCGATAGCGGATATTC
Proc-R R1	TTCAGTTGCCTCCGAGTTTTC
Vermilion F2	TAATACGACTCACTATAGGGTGTACGGCAAGAAAATCGTG
Vermilion R2	TAATACGACTCACTATAGGGTGAACCCTTCCAACTCAAGC
V-ATPase-A F2	TAATACGACTCACTATAGGGAAACACAAAATCGTCCTGCC
V-ATPase-A R2	TAATACGACTCACTATAGGGAGACACTTAACACGACCGGC
Proc-R F2	TAATACGACTCACTATAGGGCGCATTGGATCTGTGATGCAG
Proc-R R2	TAATACGACTCACTATAGGGAATATGGTGGTTGTGGCG
qPCR	
GAPDH F	GAAAACTGTCGACGGTCCCT
GAPDH R	TGGGACACGGAAAGCCATAC
Vermilion F	GAAAGTGAACCCTCATTGGC
Vermilion R	ACAGCATCCTGATATTTG
V-ATPase-A F	AACGGGAGGTGACATCTACG
V-ATPase-A R	CAGGCCACACTTGCAACATAG
Proc-R F	GAAGAATGAGAAGCTCCA
Proc-R R	TGCATCACAGATCCAATGCG

2.2.3 dsRNA Synthesis

Total RNA was initially extracted from wandering larvae using Direct-zol RNA Miniprep (Zymo Research, Irvine, CA). First-strand cDNA was synthesized using the First Strand

Synthesis (Thermo-Fisher, Waltham, MA). Nested PCR reactions were performed using the inner primer set per each gene set, each with a T7 sequence attached. PCR reactions were carried out separately for *Vermilion*, *V-ATPase-A*, and *Proc-R* groups, due to their different annealing temperatures of 53℃, 55℃, and 57℃, respectively. The PCR product from the first round, for each cDNA set, was diluted 1:20 and 1 µL would be used in each following nested reaction. The second round of PCR followed the same reaction setup as before, including the different annealing temperatures. The PCR product was then purified according to Clean and Concentrator-20 (Zymo Research). Final product concentration was 1 to 2 µg/µL in a 20 µL solution, which was determined based on the use of a NanoDrop (Thermo Scientific). The purified PCR product would then be used for dsRNA synthesis according to MEGAscript RNAi (Thermo Fisher).

2.2.4 dsRNA Injection

Wandering larvae were anesthetized by cooling on ice for 10-15 minutes. Afterwards, the larvae would be individually injected using a microsyringe fitted with a needle-tipped capillary tube. These needles were standard capillary tubes, mounted with 34-gauge needles, and melted together using a Bunsen burner. Larvae were injected with 100 nl of 20 ng of *Vermillion*, *V-ATPase-A*, or *Proc-R* dsRNA or PBS solution. Larvae were injected between the fifth and sixth abdominal segments, angled between the dorsal and lateral sides of the body, to avoid damaging the nerve chord or trachea during injection. Once injected, the needle would remain in the larvae for 30-60 seconds to reduce fluid expulsion, as reported by Powell et al. (2016). Larvae were placed into bare plastic cups and remained in a 35℃ incubator without access to light for 24 hours to ensure that there were no post-injection complications. All larvae were then moved to

glass “ant farm” enclosures that ensured the larvae had access to soil while also being able to view every step of their metamorphosis.

2.2.5 RNA Isolation

After 24 hours post-injection, three larvae from each group were collected, frozen, and used for Trizol RNA Extraction according to the Direct-zol RNA Miniprep kit (Zymo Research) previously used. This allowed for three biological replicates per injection replicate. Quantification of the RNA was determined using a NanoDrop. Each sample was around 1 to 2 µg/µL, which would later be diluted to 50 ng/µl for use in qPCR. Relative expression was subsequently analyzed using qPCR.

2.2.6 qPCR

Quantitative real-time PCR was performed on previously isolated RNA samples from each trial replicated using the Verso SYBR One-Step qRT-PCR ROX Mix Kit. Subsequent cycle threshold (Ct) values would be collected, and the relative expression of *Vermilion*, *V-ATPase-A*, and *Proc-R* was determined based on ΔΔCt value calculations. Reaction mixtures (20 µL) contained Luna® Universal Probe qPCR Master Mix, 10 µM of qPCR primers, and 100 ng of RNA or water as a negative control. Final results were normalized to *GAPDH*, as described by Gu et al. (2025).

2.2.7 Experimental Enclosure Conditions

As previously mentioned, injected larvae would be added to “ant farm” enclosures for high visibility. These enclosures were each built by taking two 6.5” x 7.25” glass panes and

inserting 6.25” x 7.25” x 0.5” cotton foam pads between three of the four sides, leaving one side without pads. Once all pads were aligned with all corners of the glass panes, they were secured using construction tape and 2.4-inch binder clips. Each empty chamber was completely filled with autoclaved sandy soil (20% play sand; 80% potting soil), ensuring each enclosure was free of contaminants and natural soil bacteria that could have affected metamorphosis results. After all larvae were added to their chambers, the exposed tops of each enclosure were covered with construction tape and punctured 10 times with a needle to ensure the larvae had minimal air and humidity circulation, without allowing them to escape. All “ant farms” were maintained in 35℃ incubators alongside all rearing and pupation jars.

2.2.8 Peptidomimetic Synthesis (2022)

In 2022, Jindal et al. developed a new acaricide designed to target *Varroa* mites without harming *A. mellifera*. The team developed 21 different peptidomimetics from the same RYLPT peptide, of which three were chosen. Each compound was named based on the changes made to it, with the pieces in parentheses being the alterations to the standard RYLPT compound. The three chemicals chosen were R[β3F]L{HyP]T, R[Phe(4-F)]L[HyP]T, and Ac-R[Phe(4-NO2)]L[HyP]T, which will be referred to as “2438,” “2441,” and “2442” from here on for ease. These three were selected due to their high mortality rates when topically applied to the gnathostome of *Varroa* mites.

2.2.9 Peptidomimetic Injection

Identical to the dsRNA injection protocol, wandering larvae were anesthetized by cooling on ice for 10-15 minutes. Afterward, the larvae would be individually injected using a micro

syringe fitted with a melted capillary needle. Larvae were injected with 50 nl of the 40 μM of 2438, 2441, or 2442 peptidomimetic solution or PBS. Peptidomimetics 2438, 2441, and 2442 were previously stored at -20℃ and required redissolution in deionized water. Once injected, the needle would remain in the larvae for 30-60 seconds to reduce fluid expulsion. Larvae were placed in bare plastic cups and incubated at 35℃ in the dark for 24 hours. All larvae were then moved to glass "ant farm" enclosures identical to the enclosures used in the dsRNA injections.

Figure 1**. Melted capillary gauge-needle.** This figure depicts the needle used in the injection experiments, which is a 34-gauge needle melted to the front of a capillary needle cut to six inches in length.

2.2.10 Peptidomimetic Behavior Observation

Groups of five larvae were injected with peptidomimetics according to Section 3.8; however, these larvae were added to clear plastic cups rather than to ant farms. From time point zero upon initial injection, and every 12 hours following onward, larvae would be lined up along

a ruler and the time it took the larvae to crawl two inches of space would be measured. Between data collection, the larvae were stored in the plastic cups in the 30℃ incubators alongside all other concurrently running trials.

2.2.11 Peptidomimetic Topical Application

Groups of five wandering larvae were treated with 1 μl of either the peptidomimetic or deionized water on their dorsal side, depending on the group. Larvae were then moved to the 30℃ incubator and remained for 24 hours to allow the solution to soak into and penetrate their integument. After 24 hours, the larvae would be added to a sterile ant farm and sealed within it to avoid escape from the chamber. Development would be noted daily for 10-15 days until all larvae were either dead or developed into adults.

2.3 Results

Through detailed management of larval development and qPCR analysis, the efficiency of RNAi was determined based on gene knockdown data (Table 2). Gene knockdown was assessed using qPCR by comparing experimental Ct values with control Ct values to calculate ΔΔCt, which was converted into expression percentage. All qPCR results were verified using standard curves to confirm primer efficiency (Figure 2). Based on the slopes, three of the four falls within the acceptable range of the slopes, in between -3.1 to -3.3, whereas the control is over 100% efficient due to its lower slope. Phenotype expression was evaluated based on adult outcomes at the end of 30 days for each group, and on the presence of specific symptoms: *vermilion* caused a change in eye color, *V-ATPase-A* resulted in impaired development, and *Proc-R* produced twitchy movement. Two of the three vermilion groups showed a significant

change in *vermilion* mRNA expression, as indicated by qPCR, and exhibited white-eye phenotype, with few adults expressing red-eye phenotypes. The white eye phenotype indicates that there was strong knockdown of *vermilion* mRNA expression, leading to no tryptophan production, ultimately leading to no expression of tryptophan 2,3-dioxygenase. In the red eye phenotype, however, there is still some brown pigmentation occurring, creating a red eye color. The final group did not appear to have any notable genotypic changes when compared with the control. In the V-ATPase-A group, all larvae across all three replicates died before reaching adulthood, indicating that this treatment was effective, as supported by the qPCR results. In the Proc-R group, two of the three groups showed a noticeable difference in expression across both categories, whereas replicate one did not. Meanwhile, replicate two could not be categorized because all larvae died before reaching adulthood.

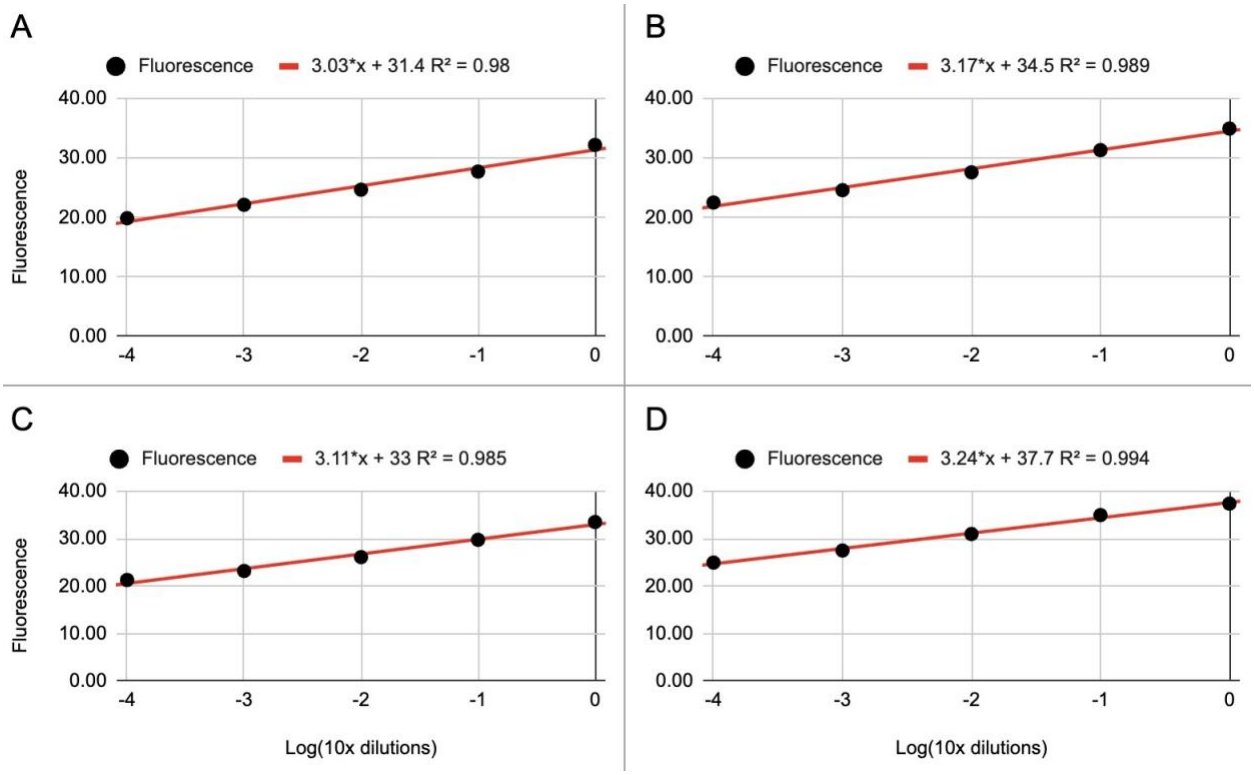

Figure 2**. qPCR standard curves for all experimental groups.** This figure presents the standard curves and their corresponding slopes for (A) Control groups (primer efficiency: 114%), (B) *Vermilion*-dsRNA-injected groups (106%), (C) *V-ATPase-A*-dsRNA-injected groups (110%), and (D) *Proc-R*-dsRNA-injected groups (104%), illustrating the amplification efficiency and reliability of the qPCR assays for each group.

Table 3. Gene knockdown success rate among different groups. Each group is divided according to the injection received, as specified by the sublabel for Trial #. The control was injected with PBS, whereas *vermilion*, *V-ATPase-A*, and *Proc-R* were injected with their respective dsRNAs in PBS. Gene knockdown was calculated from ΔΔCt values and expressed as percentages. Phenotype Expression was calculated as the number of surviving adults exhibiting the phenotype associated with RNAi knockdown of each sequence. All dashes in the three control rows are due to no gene or phenotype data being collected, as there were no alterations to the control being observed. The dashes in Vermilion Trial #3 and Proc-R Trial #1 are due to unsuccessful knockdown according to the original Ct values. The dash in Proc-R Trial #3 is due to all larvae dying before reaching adulthood, meaning that no data could be inferred of their phenotype.

	Gene Suppression (%)
Vermilion Trial #	
1	99.6
2	88.5
3	-
V-ATPase-A Trial #	
1	92.2
2	100
3	82.1
Proc-R Trial #	
1	-
2	87.9
3	84.7

It was important to include development rates alongside the mortality rates in these trials, since the rate of development could indicate factors beyond mortality. Every day, the numbers of larvae, pupae, adults, and intermediate stages were catalogued, and the process continued until all larvae had either developed into adults or died. Both the control and *vermilion* groups had a low 15-20% mortality rate, meanwhile all larvae in the *V-ATPase-A* groups and the majority in

the Proc-R groups died (Fig. 3). All four groups share similar development times between life stages as well.

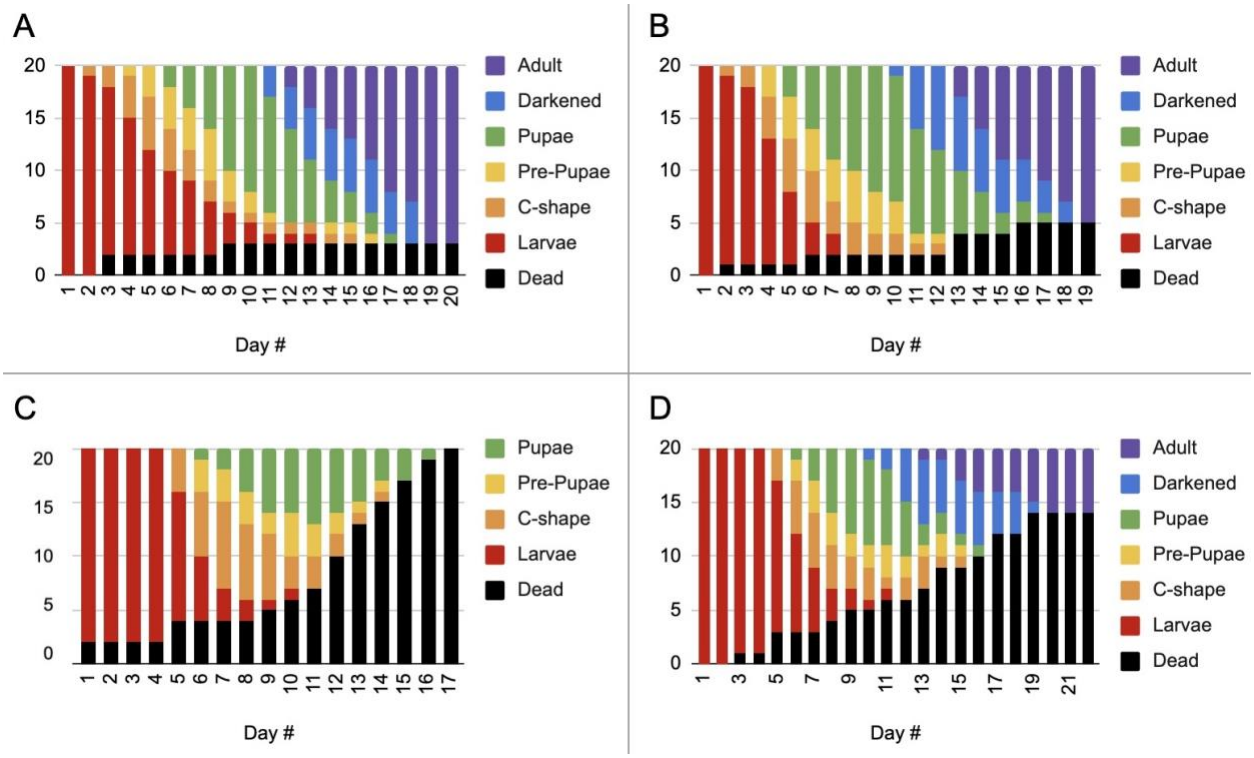

Figure 3**. Development and mortality rates of dsRNA-injected *A. tumida* larvae.** The graph depicts the development rates of (A) uninjected, (B) *Vermilion* dsRNA-injected, (C) *V-ATPase-A* dsRNA-injected, and (D) *Proc-R* dsRNA-injected larvae expressed as different colors for each developmental stage alongside the mortality rates expressed in a different color alongside the others.

Moving on to peptidomimetic assays conducted after the RNAi studies, these experiments included five groups: the control injected with PBS, and groups 2438, 2441, and 2442 injected with their respective peptidomimetics as previously mentioned in section 3.8. Initial studies focused on determining how the peptidomimetic affected the larvae, as it was unknown at the time whether these compounds would affect *A. tumida* larvae. The initial behavioral assay concluded that there was a significant hyperactive effect, as larvae would vigorously twitch and move significantly faster than non-injected individuals (Fig. 3). After

around 36-48 hours, most larvae would resume normal action, yet larvae injected with peptidomimetic 2442 did not slow down (Fig. 4). Further replicates focusing on larvae injected with just the peptidomimetic 2442 showed similar results, showing that larvae injected with 2442 had long-lasting twitchy movements (Fig. 4). These larvae twitched continuously until they either died or successfully pupated, although some were able to successful pupate, and those who did successfully pupate showed zero negative effects in the adult stage.

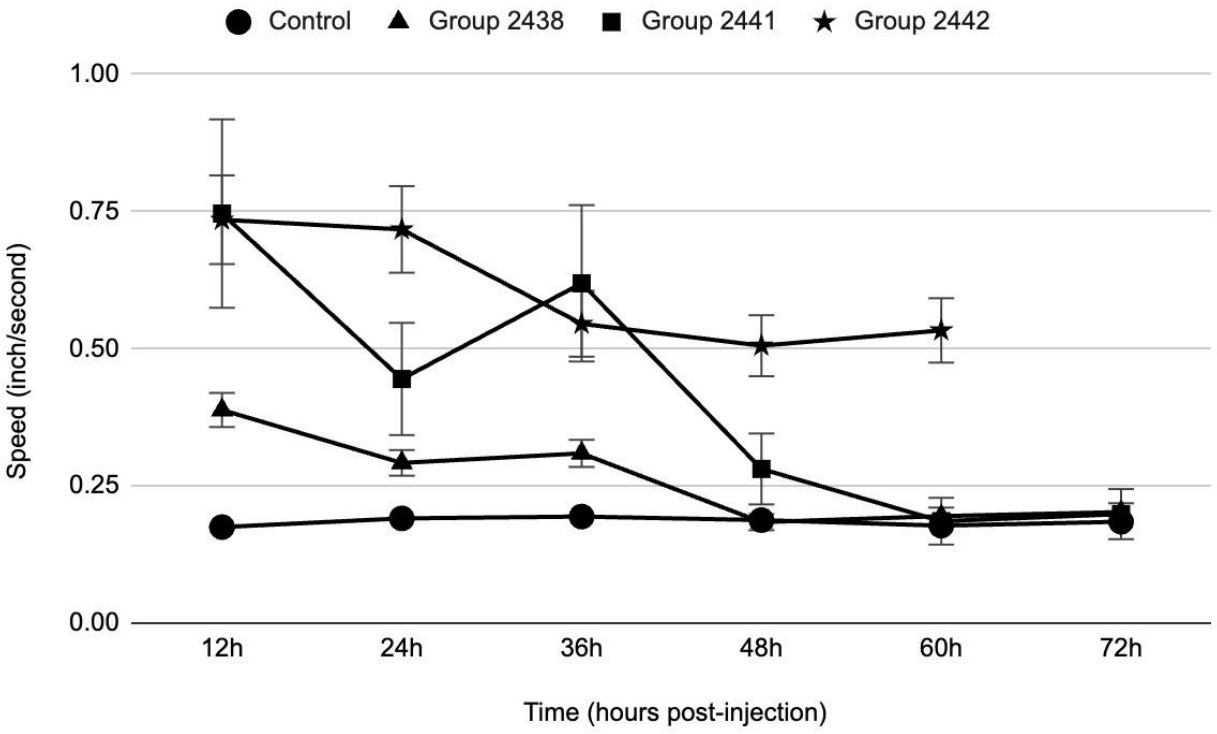

Figure 4**. Peptidomimetic-injected larvae speed Over 72 hours.** This figure depicts the movement speed of larvae injected with either PBS or peptidomimetic solution 2438, 2441, or 2442. Speed was determined by the number of seconds it took each larva to crawl 2 inches. The group number (n) for each group was ten larvae. While group 2438 and 2441 eventually returned to a similar rate as the control, group 2442 did not, as all died by 60 hours post-injection.

2.4 Discussion

Previous successful RNAi experiments in *A. tumida* support that dsRNA compounds could be used as a biopesticide for managing *A. tumida* in hives (Powell et al., 2017). While *V-ATPase-A* and *Juvenile hormone acid methyltransferase* (*JHAMT*) are effective, both have drawbacks, with *V-ATPase-A* being too broad in its effect against pests and surrounding insects such as *A. mellifera*, and *JHAMT* not having any mortality effects on *A. tumida* adults (Powell et al., 2016; Gu et al., 2025). Proc-R has been shown to be specific to *A. tumida*, along with causing significant mortality rates in *A. tumida* larvae, with this experiment showcasing mortality rates of around 60% (Fig. 5). The majority of these deaths occurred in the larval stage, yet few occurred in the pupal and adult stages, indicating that effects can affect multiple life stages.

Previous research suggests that proctolin-based peptidomimetics are effective pesticidal tools against *V. destructor*, a notable hive pest (Jindal et al., 2022). While mortality rates up to 70% after 72 hours. *A. tumida* larvae showed severe “twitchy” behavior as soon as 12 hours after injection, with larvae injected with peptidomimetic 2442 exhibiting the twitchy phenotype up to 72 hours until 70% died on average (Fig. 5). Compared to the injected groups, there was far less success, with only 20% of larvae topically applied with the peptidomimetic dying. This indicates that, while the peptidomimetic is efficient at eliciting a quick, lethal response, it shares a similar issue to dsRNA in that it needs to enter the organism successfully to be effective.

Chapter 3: Conclusions

3.1 Overall Conclusions

Over the past two decades, multiple countries have made attempts to contain and control *A. tumida*, with varying results. Whether through traps, pesticides, or biological means, all control methods fall short of being effective due to small range throughout the life cycle of the pest, harm to bees or humans, and/or limited research on the effectiveness of the control method (Roth et al., 2022). With global warming predicted to only increase the spread of hive pests in the coming decades, it is of great importance to determine an effective means of control against different hive pests, including *A. tumida* (Cornelissen et al., 2019). With the advent of RNAi and peptidomimetics, both of these have opened up new avenues for means of control against not just *A. tumida*, but all hive pests. This is due to the specific nature of both of these tools, given that neither bees nor humans have the neuropeptide proctolin, making either dsRNA or peptidomimetic exposure harmless to either. Based on the results of this study dsRNA and peptidomimetic injections were effective in eliciting a high mortality rate of 60% and 70%, respectively. However, when the peptidomimetic was topically applied, the larvae experienced zero mortality and no significant effects in bodily function as adults, unlike the injected adults which remained twitchy as adults. Given these results, this experiment supports the initial hypothesis that the *Proc-R* injections would perform similar to that of the *V-ATPase-A* injections. Meanwhile, the topical application of the peptidomimetic did not work. For alterations to this experiment, it would have helped to have gotten a successful colony of insects sooner, as that would have saved a year of time and allowed for more experiments. Alongside with more time, it would have been interesting to see how both the dsRNA and peptidomimetic behave when topically applied effectively. While topical peptidomimetic applications were conducted, further

added depth was necessary. It would have helped to determine how topical application is affected based on where it is applied, whether it be ingesting droplets placed on its mouthparts or soaked into their integument, for example. dsRNA injection would have been interesting to conduct if the dsRNA could be kept stable in a nanoparticle, since naked dsRNA topical application was shown to have zero effect on any larvae (data not shown).

I think that to control *A. tumida* and other hive pests, multiple control methods must be combined. Control methods like dsRNA or peptidomimetics, traps, and pesticides need to be integrated together in future experiments to see how these hinder or improve their effects. Given that 31.7% of beekeepers use pesticides in or around their hives and only 21.9% of beekeepers practice integrated pest management (IPM), the practice of using multiple control methods for pests at once, further development of alternative means of control than traps or pesticides are necessary (Taha et al., 2025). This is because, if many beekeepers only use a single control method such as pesticides, pests like *A. tumida* can become immune, ultimately making the control methods pointless and creating resistant strains of the pests. Further studies involving RNAi and peptidomimetics across *A. tumida* or *V. destructor* within this lab/department should consider the value in IPM and how these compounds can be enhanced or degraded when in contact with common pesticides and other tools already on the market, as it seems few beekeepers are willing to change from their standard course of action.

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