Sprayable formulations of dsRNA as a tool for management of *Popillia japonica* (Coleoptera: Scarabaeidae)

by

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Abstract

RNA interference (RNAi) mediated by environmental dsRNA presents a promising, target-specific approach to providing plant-protection against damage from invasive and outbreak insect pests. However, this approach has not been optimized for most species and multiple limitations still need to be addressed. These limitations include preliminary degradation by abiotic and biotic degradative factors and poor cellular uptake and transport to target cells.

The primary objective of this study was to develop a platform for sprayable formulations of dsRNA for the management of Japanese beetle (*Popillia japonica*) by evaluating target genes and technologies that could aid in overcoming limitations with stability and transport. First, in collaboration with Dr. Avila-Flores lab, I evaluated whether complexing dsRNA with peptide nanoparticles named Branched Amphiphilic Peptide Capsules (BAPCs) would increase the efficacy of gene silencing of a peritrophin gene majorly expressed in midgut tissue in adults. Then, I determined the primary transport mechanism of cellular uptake of BAPC-dsRNA complexes into the larval midgut tissue. Lastly, in collaboration with Dr. Reddy Palli's lab, I conducted field experiments to determine whether dsRNA targeting an actin gene specific to *P. japonica* would provide plant protection to roses when applied as a foliar spray. Overall, this study provides insights that may aid in decision making when considering target life stage, gene, and necessary inert ingredients for producing gene silencing and efficient mortality.

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BAPC's	Branched Amphiphillic Peptide Capsules
CPZ	Chlorpromazine
d	Days
DAT	Days after treatment
DPI	Days post ingestion
dsRNA	Double stranded RNA
h	Hours
RNAi	RNA interference
ZP	Zeta potential

Chapter 1: Introduction and Literature Review

1.1 Invasive insects: A global economic and environmental challenge

Globalization, both commercial and social, has opened doors for invasive alien insect species to spread, posing challenges to global well-being in terms of economic loss, disease dissemination, and environmental harm [1]. Alien species are those that move naturally or have been moved across biogeographic barriers and into a novel region by deliberate or inadvertent human-mediated dispersal [2]. Alien species are considered invasive when they cause, or have the potential to cause negative impacts on the environment, economy, or human welfare [3,4]. The successful establishment of alien invasive insect species has been generally considered a low probability outcome (1 in 1000 chance of success) [5], While the successful establishment of invasive insect species was initially considered unlikely, recent trends indicate an increasing rate of establishment, particularly in the U.S. forest systems [6].

The rise in global trade since the 1950s has correlated with a surge in first records of non-native insect establishment, particularly in terrestrial ecosystems where invasive insects dominate as the most common and damaging invaders [7,8]. Invasive insects can negatively affect the economy [7,9], the environment [10,11], and human health and welfare [12,13].

The economic toll of invasive insects globally is substantial, with estimated losses of US$70 billion annually, affecting goods, services, and health. Notably, North America bears the highest annual estimated cost (US$27.3 billion), surpassing Europe by 7.5 times [7]. Other studies have estimated global cost of alien invasive species across all taxa to have been between US$47 billion to US$163 billion in 2017, with insects accounting for between 13%- 45% of the total economic losses [9]. The global economic losses attributable to insects are likely to be miscalculated as estimates have not been produced in many regions of the world outside of North

America and Europe [7,9]. National and international policies have mitigated frequencies of invasions [6], however, implementation of some policies may indirectly impact economic growth in developing countries by precluding international trade via sanitary restrictions [14].

The ecological consequences and economic impacts of insect invasions vary across species, regions, and ecosystems [15]. This variability in impact can be ascribed to the scale of the impact, the abundance and trophic level of the invasive species relative to native species, and aspects of their biology such as voltinism, reproductive capacity, dispersal capabilities, host range, and facilitation interactions with other invasive species [16–18].

Direct effects to the environment typically occur when invasive insects kill or outcompete native fauna and flora within the environment, leading to local extirpation or extinction of native species [2]. Direct negative effects can occur quickly after an invasive insect becomes established, resulting in cascading losses of biodiversity and subsequent degradation of ecosystem function [19]. This was observed in the Upper Huron River watershed, where 99% of Ash trees (*Fraxinus spp.*) died within the five years post-invasion of the emerald ash borer (*Agrilus plannipensis*, EAB) [19]. EAB-induced ash mortality has resulted in increased abundance of invasive plants in the understory layer and rapid accumulation of woody debris [20,21]. Indirect impacts resulting from invasive insect species include facilitation of plant damage by a secondary species resulting from weakening of the plant and the spread of pathogens to naïve, susceptible species [11].

Management responses to biological invasions are prediction, detection, containment/eradication, and control [22]. Prediction aims to identify pathways and climatic regions vulnerable to invasion based on dispersal and life history of a pest species in an attempt to prevent establishment/spread [23,24]. Prediction and prevention of invasive species remain the priority of biological invasion plans and rely on preventative methods and early detection of the

species. However, many factors reduce the likelihood that an invasive species will be detected including low population levels, non-native species resembling native species, and the cost of large-scale monitoring programs [22,25]. In practice, prevention safeguards are often breached, and many non-native insects are already established by the time they are reported [2]. For many invasions, once a population has become established, eradication efforts are extremely expensive and are unlikely to be successful [26]. Furthermore, little is initially known about the biology and management of many non-native insect species when they are first discovered [6,25]. This results in indiscriminate, non-specific management practices, such as applications of systemic insecticides, that can result in non-target effects on beneficial organisms and human health [27–29]. Our reliance on systemic insecticides for invasive species management has resulted in an accumulation of literature of the non-target effects of these products, including effects on beneficial species, accumulation of product in groundwater, and development of resistant pest populations [30–32].

Sustainability is within the overarching definition of Integrated Pest Management (IPM), as it considers the short-term and long-term impacts on the three components: society, economy and the environment [33]. Although the importance of these pillars is generally acknowledged, the implementation of tactics that reduce negative impacts resulting from invasion responses is largely ignored, resulting in increased pesticide concentrations in water bodies, soils, air, and inside residences [31,34–36]. For invasive insect management plans to follow the principles of IPM, introduction of novel, target specific approaches that efficiently provide control to a similar degree as conventional (chemical) insecticides must be incorporated.

1.2 *Popillia japonica*: The physiology and socio-economic impacts of an old and new invasive species

1.2.1 History and status as a pest species

Popillia japonica is an invasive scarab beetle first discovered in the U.S. during routine nursery inspection in South New Jersey in 1916 [37]. Initial eradication efforts did not occur until two years after the beetle was discovered, and consisted of widespread use of sodium cyanide and arsenate in soil and on crops, which contaminated the soil and poisoned humans [38].

Despite multiple efforts to eradicate, and subsequent efforts to restrict its invasive range, *P. japonica* has succeeded in spreading throughout much of the U.S. [37]. Reproducing populations are found in 28 states, including all states east of the Mississippi River, apart from Florida, and as far west as Oregon [39]. Also in North America, six Canadian provinces have resident populations [39,40]. Interestingly, a small reproducing population near Axis, (Mobile Co.) AL may be the most southern population of this species in North America [39]. The global distribution of *P. japonica* outside of North America includes Japan, Switzerland, northern Italy, the Kuril Islands of Russia, and the Azores Islands of Portugal [41,42]. Data suggests that the recent expansion of *P. japonica* into Azores and the European mainland resulted from transportation from Southeastern and Northeastern U.S. populations, respectively [41]. The populations in northern Italy appear to be the first detection of this species in mainland Europe [43]. *Popillia japonica* has potential to establish in many territories that have suitable climates such as Europe, Asia, the Pacific Islands, South America, New Zealand, Australia, and Africa [37,44].

Before being introduced into the U.S., *P. japonica* was considered only a minor, regional pest in its native region of the Japan archipelago [45]. This was likely a result of limited habitat suitable for larval development and adequate natural enemy pressure to provide population checks [37]. Introductions into the northeastern U.S. provided the beetle with a generally suitable climate with expansive grassland for larval development, and over 300 species of adult food

plants [45]. Economic damage has been documented on >100 plant species [46]. It is the most destructive pest to turfgrass and woody ornamental plants in the eastern U.S. and supposedly a cause of US$450 million in economic loss through plant injury, control and plant replacement costs annually, according to a 2003 estimate [37]. Fruit and flower feeding habits in particular cause considerable damage to many fruit, garden and field crops [37].

Although previous models suggested that the area between the Rocky Mountains and the Sierra Nevada are unsuitable environments for establishment [44], detections have occurred in multiple states across this region [39,47]. Mass trapping efforts and insecticide applications have worked to eradicate localized populations previously in California and Oregon [47–49]. Establishment of *P. japonica* in California could have significant effects on U.S. agriculture as the state produces over a third of the country's vegetables and two-thirds of its fruits and nuts [50]. *Popillia japonica* has not yet established in all suitable territories within the U.S., and human-mediated transport and land management practices (such as overwatering turfgrass) are likely facilitators of invasions into suitable and unsuitable ranges [44,51].

1.2.2 Lifecycle and phenology

P. japonica is univoltine, having only one generation per year throughout most of its invasive range within North America [45]. Adult emergence varies by region in North America. In the southeastern U.S., adult emergence typically occurs in May coincident with the first bloom of Natchez crape myrtle [52]. Females can oviposit up to 60 eggs into the soil during the 4-6 week adult lifespan which will hatch into first instar grubs within 12-14 days [45].

P. japonica spend most of their lives, between 18-30 weeks, in the soil as root feeding larvae [45]. They undergo two molts before pupation. Adult beetles have metallic green bodies with brown

elytra, and are typically 8-11 mm long [45]. Grubs can be identified by spines arranged in a characteristic V-shaped pattern on the ventroposterior tip of their terminal abdominal segment [53,54].

1.2.3 Gut morphology and physiology

Beard (1945) reported the gut morphology of *P. japonica* larvae. The esophagus is housed in the head and expands posteriorly to form a simple crop. The ventriculus is the straight, tube-like midgut region of the larvae. From it arise three concentric rings of folds, or "diverticula", which are referred to as gastric caeca. The third gastric caecum delineates the midgut from the hindgut. The hindgut is composed of three regions. Malpighian tubules arise from the anterior region of the hindgut, which compresses posteriorly at its connection to the enlarged rectal sac. The rectal sac ultimately leads to the rectum, which houses the anus at its posterior.

The only study describing the gut morphology of adult *P. japonica* in detail is Swingle (1930). The generalized anatomy of the digestive tract is described as the foregut, midgut, and hindgut, in the anterior and posterior, respectively. The digestive tract of adults is about twice the length of the body, with a diameter ranging between 0.5 – 1 mm. The foregut is approximately 2 mm in length and is housed almost entirely within the head. The midgut, between the foregut and the hindgut, ranges in length from 20-25 mm and is the longest section of the digestive tract. This region begins at the head, and continues into the abdomen, where it is coiled about itself beneath the hindgut mass. It has a width of 0.5 to 1 mm, and the width gradually becomes narrower toward the posterior end. The midgut is composed of an outer row of columnar epithelial cells, and a region composed of circular muscle fibers and bulbous organs called nidi. Nidi function to form new midgut epithelium cells. The discontinuation of the midgut into the

hindgut can be distinguished by the presence of four Malpighian tubules, string-like organs that function in excretion, osmoregulation, transport of organic molecules, and detoxification of chemicals [56]. The pyloric valve is a region of muscular tissue, posterior to the Malpighian tubules, functioning to regulate passage of particles into the hindgut compartment. Posterior to the valve, the hindgut gut widens. This section of the hindgut is yellow and wrinkled in texture. The hindgut terminates at the rectum, which is muscular tissue that attaches to the anus.

The insect gut is composed of a single layer of epithelial cells (typically columnar cells) and a basal lamina, which is supported by surrounding muscle tissue [57]. The foregut and hindgut are derived from ectodermal tissue, and thus, serve mainly for storage of food and waste, respectively [58]. The midgut region is where the majority of digestion and nutrient absorption occurs in insects, and a it is also a site of exposure to toxins, bacteria, and viruses [59,60]. Thus, the midgut functions for food digestion, nutrient absorption, and immunity [57]. This species possesses multiple adaptations functioning for digestion and immunity, including an alkaline gut pH in both life stages, secretion of a peritrophic matrix (PM), and digestive and detoxification enzymes [61–64]. The gut environment of *P. japonica* is alkaline; the pH is 9.5 and ~ 7.4 in the larvae and adults, respectively [65]. Alkaline midgut pH is suggested to be an adaptation in herbivorous insects that functions to break down hemicellulose, however, could also aid in the breakdown of pathogens [60,65].

Detoxification enzymes have been identified in adults and larvae, with the greatest activity observed in the midgut [62]. These enzymes include cytochrome p450 (P450), glutathione S transferases (GST), and carboxylesterases (CoE) [61,62,66]. These are large enzyme superfamilies that use reactions to increase polarity and water solubility of toxins, making them more readily excreted. P450 activity has been shown to be induced by feeding, reaching maximum levels

within 24 h after feeding on host plants [62]. Furthermore, P450 and GST activity is greater when feeding on non-preferred host plants and when feeding on preferred hosts [61].

1.3 RNA interference as a pest management tool: History, advancements, and current challenges

1.3.1 Cellular function and discovery of RNA interference

In eukaryotic organisms, RNA interference (RNAi) is a cellular mechanism that results in post-transcriptional gene silencing and is important for cellular function and communication [67]. It involves silencing of specific genes or gene regions with the use of small RNA (sRNA) molecules complimentary to endogenous mRNA [67]. In insects, the evolutionary arms race between nucleic acid parasites and host has resulted in the diversification of small RNA (sRNA)-directed RNA silencing pathways in insects into three related pathways [68,69]. These pathways can be differentiated based on the origin and characteristics of the initiator sRNA molecule, enzymatic requirements, and functions (**Table 1**) [68].

Table 1. A summary of the RNAi pathways known in insects. Note that primary functions are dependent on RNA mapping, although functions between each pathway may overlap depending on species and developmental stage.

RNAi pathway	Initiator molecule	Length (bp) of initiator molecule	Dicer enzyme	Argonaute enzyme	Primary functions
Small interfering RNA	dsRNA	21-23	Dicer-2	Argonaute2	Antiviral defense, transposon silencing, gene regulation
MicroRNA	Hairpin dsRNA	21-23	Dicer-1	Argonaute1	Gene regulation, pathogen defense
Piwi interacting	ssRNA	27-32	Dicer independent	Piwi	Germline protection against transposons, antiviral defense, oogenesis, spermatogenesis, embryogenesis

The microRNA (miRNA) pathway and the P-element induced wimpy testis (Piwi)-interacting RNA (piRNA) pathway typically respond to small RNAs encoded within the genome, either parasitic or regulatory [70–72]. The former pathway functions in regulation of gene expression for cell physiology, communication, and pathogen suppression, and can be found in most cell types [73–75]. The latter pathway, PIWI, is typically restricted to the germ-line cells and is reported to function in protection of the genome against transposable elements, although recent findings have suggested this pathway to have regulatory functions within somatic cells in some insects [76]. The third pathway, termed the small interfering (siRNA) pathway, is unique in that it is initiated by exogenous (viral) or endogenous (transposable) strands of dsRNA molecules. This is the primary defense pathway against DNA and RNA viruses and is the typical pathway manipulated for RNAi [77].

RNA interference was first observed by Napoli et al. [78] after injecting single-stranded RNA (ssRNA) homologous to the chalcone synthase gene into petunias. Their objective was to overexpress chalcone synthase to determine whether it was a rate limiting enzyme in the production of pigment molecules (anthocyanins). However, they observed the opposite effect; the introduction resulted in a 50-fold reduction in chalcone biosynthesis and petunias with phenotypes exhibiting a mosaic of white and purple petals [78]. Guo and Kemphues [79] were the first to observe RNAi in animals while evaluating the role of the Par-1 gene in asymmetric cell division in early-stage embryos of *Caenorhabditis elegans*. Injection of sense or antisense ssRNA homologous to the endogenous Par-1 gene into the gonads of adults resulted in arrested development and morphological deformities in ~50% of the progeny [79].

A few years later, Fire et al., [80] used well-known phenotypic markers and reporter proteins to show that double-stranded RNA (dsRNA) produced much stronger phenotypic effects

at halved molar concentrations relative to ssRNA molecules in all genes examined. However, these effects were observed only when both strands of the dsRNA sequence were homologous to the gene of interest, suggesting that the dsRNA sequence must match perfectly to the target region. For their discovery of dsRNA mediated RNAi, Craig C. Mello and Andrew Fire won the Nobel prize in physiology and medicine in 2006. Since then, RNAi has become a fundamental tool for studying functional genetics and has led to applications for pest management.

1.3.2 Mechanism of RNA interference

Uptake of environmental dsRNA from the gut lumen or extracellular space and into the cell is required to initiate a cell-autonomous, or localized, RNAi response [81,82]. Studies have shown that the suppression of cellular dsRNA uptake confers resistance and refractoriness of RNAi in insect species, demonstrating the importance of cellular uptake in RNAi [82,83]. Two pathways mediating dsRNA cellular uptake have been described in insects. The first pathway is a transmembrane-protein mediated pathway. In *C. elegans,* a luminal transmembrane channel protein termed systemic RNA interference defective 1 (Sid-1) functions to endocytose double stranded nucleic acids via passive transport [84]. The presence and relative importance of the sid-1 gene in dsRNA uptake seems to be evolutionarily conserved in some insect species and lost in others. For example, sid-1 homologs are required to achieve RNAi response in Colorado potato beetle *(Leptinotarsa decemlineata,* CPB), but not in closely related species western corn rootworm (*Diabrotica virginifera,* WCR) and red flour beetle (*Tribolium castaneum*) [85–88]. Furthermore, a comparative genome survey revealed that Sid-1 homologs are not present in 12 species of *Drosophila* and two species of mosquitoes, which suggests sid-1 channels may have been evolutionarily lost in Diptera [85].

Clathrin receptor-mediated endocytosis (CDE) is a well-known pathway utilized for cellular uptake of dsRNA in insects. CDE is the formation of an intracellular vesicle dependent on invagination and engulfment of cargo by the plasma membrane [89]. Formation of the vesicle is structurally aided by the assembly of proteins such as actin and clathrin that coat the outer surface [90]. CDE has been reported to be a key endocytic pathway for long dsRNA in species within orders Diptera [81,83], Coleoptera [86,88,91], Hemiptera [92], and Lepidoptera [93]. Although clathrin receptor-mediated endocytosis is the most well-known endocytic pathway used for dsRNA uptake in insects, its role in dsRNA uptake may vary depending on the insect species and the length and structure of the dsRNA [94].

Once inside the cell, the long dsRNA strands are cleaved into short silencing intermediates termed short- interfering RNA (siRNA) by the first component of the RNAi machinery, Dicer-2 (Dcr-2) enzyme [95]. The RNase III domain of Dcr-2 recognizes and cleaves dsRNA at specific points, producing short heteroduplex structures consisting of 19 base paired nts with 2-nt overhangs on the 3' ends [96,97]. The efficiency of siRNA processing is dependent on the length of the long dsRNA strand, with long strands up to 500 bp being more efficient than that of shorter strands (<100 bp), and no processing occurs after the introduction of exogenous dsRNA below 39 bp [97]. This length dependence may reflect a means to prevent an undesired activation of RNAi by endogenous cellular RNAs [97].

The siRNA duplexes are then carried by the RISC loading complex (RLC), consisting of Dcr-2 along with the R2D2 RNA-binding protein, which facilitates transfer of the siRNA to the RNA-induced silencing complex (RISC) [98]. The argonaute protein, commonly referred to as "slicer", is the catalytic enzyme of RISC and is characterized by two conserved domains; a N-terminal PAZ domain and a C-terminal PIWI domain [99,100]. In *Drosophila*, the argonaute protein

forms a clamp-like structure in the PAZ domain with two amino acid residues (Phe 50 and Phe 72) being critical for RNA binding [99]. Once loaded into the RISC, siRNA duplex is cleaved endonucleolytically by an argonaute protein into ssRNA [101]. Separation of the two strands is initiated by a thermodynamic difference between strands; the 5' antisense region is relatively unstable, resulting in flexibility for the RISC to initiate unwinding at the 5' antisense region [102]. Once separated, the passenger strand is degraded, and the guide strand is incorporated into RISC [103]. Using the retained guide strand, the activated RISC complex scans cellular mRNAs, and an argonaute protein (Ago2) within RISC cleaves transcripts with complementarity to the siRNA, ultimately suppressing gene expression [104].

Cell autonomous response , or the localized response of cells that uptake environmental dsRNA, is conserved in eukaryotes [106]. Systemic RNAi is the process by which the RNAi signal is spread from one cell to another or one tissue to another [107]. Systemic RNAi is not conserved in eukaryotes and studies suggest that systemic responses vary between insect species. In CPB (Lepd-SL1) cells, exogenous long dsRNA strands are encapsulated in exosomes [108]. Silencing genes involved in exosome production (Rab 4 and Rab 35) results in a subsequent decrease in phenotypic change in the cells when targeting a marker gene (IAP) using dsRNA [108]. In red flour beetle (TcA) cells, extracellular vesicles (EV's) consisting of exosomes and microvesicles have been shown to encapsulate siRNA's (21-23 nt) derived from luciferase reporter gene [109]. Introduction of these isolated EV's to new TcA cells resulted in a silencing effect like that of cells exposed to long dsRNA [109].

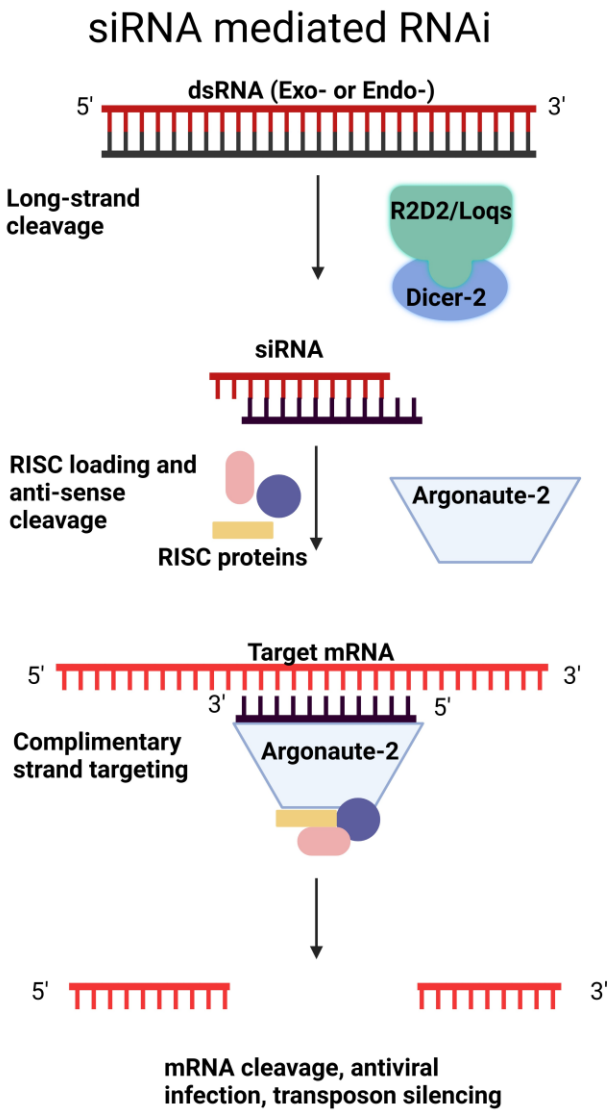

Figure 1. Cellular mechanism of siRNA-mediated RNAi. Figure modified from Zhu and Palli [105].

There are likely many variations in the cellular mechanism of RNAi in insects brought about by the adaptive radiation of insect species and subsequent co-evolution with viral pathogens [101]. Understanding the variation in the siRNA pathway among insects will likely aid in the advancement of RNAi for managing pest insects.

1.3.3 RNA interference for pest management of herbivorous insect species

RNAi has emerged as a powerful and promising tool for managing herbivorous insect populations [110]. Although injection, soaking, and topical applications are commonly used in laboratory assays, ingestion is the route of exposure with the greatest potential for pest management [111]. Delivery methods that could be deployed to manage herbivore insect pests in the field include transgenic methods such as host-induced gene silencing (HIGS) and viral-induced gene silencing (VIGS), and non-transgenic methods such as spray-induced gene silencing (SIGS) [112]. Although HIGS provides a potential for prolonged efficacy resulting from constitutive expression of pest dsRNAs *in-planta*, the regulatory timeline and economic input required make it unfeasible for most economically important plant species [113]. Furthermore, the plant cellular physiology may compromise the efficiency of dsRNA-mediated RNAi via low levels of expression or pre-processing of the long strand into siRNA by the plant [114]. Lastly, constitutive expression may contribute to resistance breakdown over time [114].

The use of RNAi for insect pest management provides a critical tool to be incorporated into integrated pest management, offering multiple advantages which are related to its unique mode of action. First, RNAi currently represents the most species-specific approach to targeting herbivorous pest insects resulting from the ability of dsRNA to silence essential regulatory genes in a target-specific fashion. The propensity for non-target effects depends on the

complementarity between the siRNA derived from the dsRNA and mRNA. Off-target effects occur when the siRNA hybridizes with unintended mRNA within the target species, while non-target effects occur when unintended off-target effects occur across multiple species[115]. Chen et al. [115] conducted a study using a combination of chimeric dsRNA and dsRNA mutation experiments to investigate the complementarity rules governing off-target and non-target effects. Their results showed that a 16-base pair (bp) contiguous match between a dsRNA derived siRNA and mRNA is the minimal length needed to produce silencing effects in off-target genes and non-target species. Furthermore, almost perfectly matched segments of at least 27 nt with ≥5 bp repeated segments of contiguous matches linked by single mismatches, or ≥8 bp linked by mismatch couplets, were sufficient for inducing RNAi response in off-target genes and species [115]. This information can be used to specifically design dsRNA's to avoid effects on non-target species. It also opens the possibility to design a dsRNA that targets multiple homologous genes within a single species [115].

Second, RNAi is a technology that can be combined with other IPM approaches, which will aid in reducing the overall reliance on chemical insecticides and prevent resistance. For example, the adoption of transgenic Bt-cotton since 1995 has resulted in global reduction of over 330 million kg of insecticide active ingredient for cotton management [116]. However, it has also resulted in the evolution of Bt-resistant cotton bollworm (*Helicoverpa armigera*) populations [117,118]. The development of transgenic plants that endogenously express cry proteins and dsRNA has shown to have a synergistic effect against Bt-resistant populations of cotton bollworm [119]. Because dsRNA and cry proteins have independent modes of action, cross-resistance is highly unlikely [120].

RNAi technology has been used to successfully suppress gene expression in Blattodea [121] Coleoptera [122], Hemiptera, [123,124], Diptera [125,126], Blattodea [121], Lepidoptera [127,128], Orthoptera [129]. Nevertheless, a large degree of variability in RNAi efficiency is observed between different insect orders. Lepidoptera and Diptera are orders particularly recalcitrant to RNAi, resulting from rapid nuclease and lysosomal degradation, poor cellular uptake, and deficient or functionally divergent core machinery enzymes [83,130–135]. RNAi technology has made the greatest strides when used for the management of coleopteran pests.

1.3.4 Success of RNAi in controlling coleopteran pests

Environmental dsRNA is highly efficient in producing a systemic RNAi response in multiple species of Coleoptera. The red flour beetle has been the model organism for insect functional genetics studies because of its high sensitivity to systemic RNAi post injection or feeding of dsRNA [136,137]. This species has also been used to screen lethal RNAi targets that may be useful for managing other pest coleopteran species [138,139].

Baum et al. (2007) were the first to demonstrate the sensitivity of leaf beetles (Coleoptera: Chrysomelidae) to RNAi mediated by ingestion of dsRNA by conducting LC_{50} assays on WCR and CPB. The most active dsRNA products yielded LC_{50} values of 0.57 ng/cm^2 and 5.2 ng/cm^2 for WCR and CPB, respectively [140]. Following this seminal paper, dsRNA-based insecticides have been, or are currently undergoing the process of being, commercialized using both HIGS and SIGS application methods.

Multiple collaborative studies between Monsanto and Dow AgroSciences led to the EPA acceptance and commercialization of SmartStax Pro®, a dsRNA producing transgenic-corn strain, in 2017 [120,141–145]. This strain includes two Bt proteins targeting Coleoptera (cry34Ab1,

cry34Bb1) plus DvSnf7, a dsRNA targeting WCR sucrose non-fermenting protein-7 mRNA. DvSnf7 is a key protein in the ESCRT (Endosomal Sorting Complex Required for Transport)–III complex, which is essential for internalizing, sorting, and transporting ubiquitinated transmembrane proteins for lysosomal degradation [143,146]. SmartStax Pro® has shown to provide protection to corn roots against root damage in Bt-susceptible and Bt-resistant WCR populations [145,147,148].

Building off previous RNAi studies demonstrating the susceptibility of CPB to RNAi, greenlight biosciences have developed the first sprayable dsRNA-based biopesticide named Calantha™. This product contains a dsRNA, named Ledprona, that targets the CPB proteasome subunit beta 5 (dsPSMB5), a subunit of the proteosome 20S catalytic core machinery that is responsible for substrate recognition and hydrolysis of damaged proteins [149,150]. In greenhouse assays, applications of Ledprona to potato (*Solanum tuberosum*) foliage resulted in 100% mortality of CBP first-instar larvae by 14 days after treatment (DAT) [151]. Furthermore, foliar treatment of Ledprona conferred whole-plant protection similar to that of Spinosad and Chlorantraniliprole, despite the lag effect in mortality observed in larvae exposed to Ledprona [152]. One year later, a combination of lab and field experiments conducted by Pallis et al. (2022) determined that neonate and second instar larvae, the life stages that consume the least plant tissue, are most susceptible to Ledprona. Furthermore, the rate of feeding was slowed by 4-5 days after exposure for all developmental stages at higher concentrations [151]. Thus, plant protection likely results from the feeding cessation and mortality of early stage larvae which consume relatively less foliage than later developmental stages [151]. The Ledprona formulation invented by Greenlight Biosciences Inc. (Calantha™) was classified as a new mode of action

group of insecticide (RNAi-mediated targeted suppressors) and was approved for the use on potatoes in 2023 [152,153].

Other studies have validated the sensitivity of multiple other economically important species within Chrysomelidae to RNAi via environmental dsRNA [154,155]. Systemic RNAi post ingestion has also been documented in families of beetles outside of Tenebrionidae and Chrysomelidae including Coccinellidae [156,157], Curculionidae [158,159], Nitidulidae [139,160], Buprestidae [161,162], and Cerambycidae [163]. However, Willow and Veromann (2021) point out that few families have been tested for oral sensitivity to RNAi, and only few species within those families have been evaluated. Furthermore, species within the same family and genus have shown differences in susceptibility to oral RNAi [156,158,165]. This necessitates evaluation of oral RNAi sensitivity on a greater diversity of species to understand the full potential of RNAi-based management of coleopteran pest.

1.3.5 Limiting factors in the use of RNAi for pest management

The main barrier to the use of RNAi for insect pest management is the variability in silencing efficiency between insect species, life stages, tissues, target genes, and routes of exposure [166]. These limitations get more complex when considering how to apply nucleotide-based insecticide products to the field and avoid rapid, preliminary environmental degradation before it is consumed by the pest population. Environmental dsRNA may undergo degradation before it is ingested by the insect, via microbe-derived nucleases and UV exposure [167]. In soils with varying characteristics, dsRNA has a half-life of less than 30 h, and the rate of degradation is independent of the concentration applied [168]. Similarly, dsRNA applied to soybean foliage in

field conditions rapidly dissipates with a half-life of less than 1 day, and a 95% reduction by 3 days [167].

Multiple species that are refractory, or insensitive, to oral RNAi have internal environments that rapidly degrade dsRNA before it is internalized by cells or do not have the ability to undergo one or more of the steps necessary to achieve systemic silencing. These barriers to RNAi may include preliminary nucleolytic degradation inside the insect, lack of cellular uptake, endosome entrapment, insufficient core machinery, or lack of systemic spread of silencing siRNAs [82,93,130,169–171].

Before interacting with the midgut cells, dsRNA must pass through the mouth, foregut, and in some insects, the PM. Multiple studies have documented premature degradation of dsRNA by dsRNases in the salivary canal, midgut, and hemolymph of multiple species, overall contributing to an inefficient RNAi response [172–174]. For example, the saliva and midgut luminal liquids of southern green stinkbug (*Nezara viridula*) require only 10 minutes (min) and between 30-60 min to completely degrade dsRNA, respectively [174]. Silencing the only identified dsRNase in southern green stinkbug via dsRNA injection increased the efficacy of gene suppression and mortality effects, while also showing to have decreased degradation efficacy relative to control groups [175].

The pH of the insect midgut has been pointed out previously as a potential factor contributing to dsRNA degradation [176]. This has assumed that the range of pH environments by which dsRNA is stable is consistent with ssRNA. Single-stranded RNA is most stable in pH environments between 4-5 and prone to hydrolysis of the phosphodiester bonds in alkaline environments [177]. However, recent evidence supports added stability provided by the duplex structure of dsRNA in preventing rapid degradation in alkaline environments up to a pH of 12.4

and against metal-catalyzed hydrolysis [178]. The pH environment that the dsRNA will interact with is likely more important in the contexts of the enzymatic optimums of residing nucleases, and the release of the dsRNA from inert ingredients used for dsRNA protection and transport [176,179,180].

As described previously, cellular internalization of long dsRNA strands is an essential step in the process of systemic RNAi (see section 1.3.2.). Variations in the endocytic capacity of dsRNA among insects may be one driver of variability in the sensitivity to RNAi. In *Bactrocera dorsalis*, primary exposure to dsRNAs targeting endogenous genes induces a temporal, systemic refractory period, blocking silencing induced by secondary exposure to dsRNA with the same or different sequences [83]. Fluorescent microscopy confirmed that the refractoriness to dsRNA in *B. dorsalis* resulting from the inability of secondary dsRNAs to accumulate inside midgut cells [83]. Further evidence comes from the first documented dsRNA resistant WCR lab population [82]. Inheritance of an autosomal recessive gene on a single resistance locus conferred a ~130-fold increase in resistance against dvSnf7 dsRNA in WCR and cross-resistance to dsRNA targeting other genes [82]. Resistance coincided with a lack of dvSnf7 derived siRNAs in WCR tissue, suggesting a general dsRNA resistance mechanism resulting from impaired endocytosis [82]. Overall, these studies suggest that lack of cellular uptake of dsRNA could be a major barrier for targeted RNAi activity in many non-model species.

Once inside the cell, dsRNA must interact with core machinery of the RNAi pathway (see section 1.3.2). If dsRNA enters the cell by means of endocytosis, it must first escape from endosomes to avoid lysosomal degradation and to interact with the core machinery enzymes. In two species of noctuid moths, long dsRNA strands accumulate inside endosomes and are not

converted into siRNAs, indicating endosomal entrapment and subsequent digestion by lysosomes [93,132].

Alternatively, core machinery genes may be expressed at deficient levels or functionally divergent. Inefficient RNAi could be a result of deficient expression or upregulation of enzymatic core components of the siRNA pathway. For example, in the silkworm moth (*Bomyx mori*), expression of two RNA binding proteins, *translin* and *R2D2*, are deficiently expressed in silk moth larval and pupal tissues, likely arising from deletion mutations [181]. Interestingly, overexpression of Dcr2 and Ago2 increases silencing efficacy in silkworms, while overexpression of R2D2 in Bm5 cells does not [131,171,181]. The core machinery proteins of the siRNA pathway are more prone to mutations relative to the core machinery proteins in the miRNA pathway, with much of the variability in these enzymes occurring between insect orders [135]. Multiple structural mutations on functional motifs of core machinery proteins in Lepidoptera may provide insight into the inefficient RNAi response observed in this order [133,135]. Significant divergence in the structure of functional protein motifs may also occur below order level, such as was found between the dipteran suborders Brachycera and Nematocera [135]. This suggests that the understanding of RNAi in model organisms may not translate to the understanding of RNAi in non-model species. Assuming mechanisms of RNAi in model organisms are conserved among taxonomic groups could hinder the broad use of RNAi for insect management.

Lastly, failure to achieve a systemic response may result in reduced efficacy of gene silencing. Failure to achieve a systemic response may be an indirect result of a failure in an upstream event necessary to achieve systemic silencing, as those described in the previous paragraphs of this section [182]. Outside of model species (see section 1.3.2), little has been investigated regarding transcellular transport of dsRNA. The use of "RNAi-of-RNAi"

experiments to identify genes that may be used in intracellular trafficking of dsRNA have been conflicted by the lethal phenotypes produced by silencing these genes [183,184]. Additionally, the lack of RNA dependent RNA polymerases (RdRPs) in insects results in the inability to amplify secondary siRNA signals that is observed in other model invertebrates such as *C. elegans* [85]. Thus, a small dose may not be adequate to affect all cells of a tissue necessary to produce a robust and systemic response. This may be a reason why longer strand sizes and higher concentrations of dsRNA have shown to increase knockdown efficiency and duration in amendable species [185,186]. Overall, barriers lie in the way of RNAi-based insect management for many species evaluated. Better understanding the species-specific cellular mechanisms behind RNAi will aid in developing technologies to overcome these barriers.

1.4 Nanoparticles and their uses in pest management

1.4.1 Defining and characterizing nanoparticles

Nanoparticles (NPs) are defined as particles that are between 1-100 nm in diameter, however, it's worth noting that terminology and definitions can vary between different scientific disciplines and contexts [187]. What is of greater importance is the unique properties that materials at a nanoscale exhibit due to small size, high surface-area-to-volume ratio, and various shapes of the materials, which make them versatile tools applicable across multiple disciplines. Furthermore, many types of NPs self-assemble spontaneously in water and thus can be easy to manufacture at a large scale.

The types of NPs used for pest management include organic (peptide, chitosan, liposomal), inorganic (Bioclay, metal), synthetic linear and branched co-polymers and dendrimers [188,189]. Nanoparticles are customizable in size, shape, electrostatic charge, and

material, providing specific benefits for pest management. For example, many cationic nanoparticles can adhere to plant tissue, resulting from electrostatic and structural forces, preventing run-off of insecticides via rainwater [190]. Furthermore, NP's with porous structures such as hollow porous silica nanoparticles (HPSN's) can increase insecticide loading capacity by more than 200%, provide extended slow release of the insecticide, and protect the active ingredient from UV degradation [191–193]. Additionally, nanoparticles can be designed using biodegradable, non-toxic materials such as chitin, peptides, and lipids that can aid in reducing environmental pollution from insecticide inputs [194].

1.4.2 Uses of nanoparticles for pest management

Apart from NP uses in ultrasensitive detection of insecticides [195–197], NPs can also be used directly and indirectly to manage insects [198,199]. Nanoparticles have been studied for their direct insecticidal effects on pest insects and as inert ingredients for the protection and transportation of insecticidal molecules [199,200].

Many inorganic NPs have been shown to exhibit direct lethal effects on insects. For example, silver nanoparticles have been reported to have larvicidal effects on multiple species of disease vectoring mosquitoes, with LC-50 values between 12 and 15 µg/mL [201]. Zinc, zinc oxide, silica, silica dioxide, titanium dioxide, aluminum oxide, and other nanomaterials have been tested for direct lethal and sublethal effects on insect pests [198,200,202]. The main limitations of using these products to kill insects in agricultural settings is the high LC-50 values observed in bioassays and the accumulation of these products in plant tissues leading to phytotoxic effects [200,203,204].

The potentially more promising application of nanoparticles for insect pest management is their indirect use as a nano-transporter and nano-protectant of synthetic and biorational insecticidal compounds. Nanoparticles can encapsulate conventional insecticides and Bt proteins, conferring protection against environmental degradation and prolonged bioavailability via slow release of the product [190,205]. Furthermore, the development of nano-based smart delivery systems has great potential to increase efficacy and decrease the input of insecticides for pest management. These nanoparticles react to environmental stimuli such as temperatures, light intensities, and pH, resulting in conformational changes in the NPs and subsequent release of insecticide [206–208]. This has led to the development of "smart-insecticides" that provide on-demand release in response to environmental cues elicited during insect herbivory. For example, engineering a smart nano-carrier that has a high release rate in alkaline pH environments can result in prolonged effectiveness in managing Lepidopteran pests, which typically have highly alkaline midgut pH environments [206]. This approach could also reduce off-target human toxicity by suppressing release in acidic or slightly basic environments, such as human gastrointestinal environments and blood, respectively [209].

In the last decade, multiple reports have found that nanoparticles can also increase the efficacy of orally delivered RNAi by overcoming various barriers associated with recalcitrant species [210–212]. Much like with other inert ingredients added to conventional insecticides, the increased success of RNAi when complexing dsRNA with nanoparticles is largely resulting from the enhanced stability, prolonged bioavailability, and transport of the active ingredient to the target tissues [213].

Nanoparticles that have been explored typically encapsulate dsRNA strands via ionotropic gelation or attach at the surface via electrostatic interactions between the anionic

phosphodiester backbone of the dsRNA and the cationic surface of the nanoparticle [212,214]. This process can isolate dsRNA from the environment and protect it from abiotic and nucleolytic degradation [176,214]. Encapsulation of dsRNA by chitosan nanoparticles have shown to protect dsRNA from UV and heat degradation for at least 29 h, while degradation of naked dsRNA occurred under the same conditions by 4 h [214]. Further assays have suggested that chitosan-dsRNA complexes can adhere to leaf surfaces and remain stable for ≥ 5 d, with ~85% of the dsRNA still bound to the complexes, suggesting prevention of rapid environmental degradation [176] Most reports investigating the protective role of NPs against environmental degradation factors have been conducted with chitosan nanoparticles. Chitosan nanoparticles, polymer-based nanoparticles, protamine-lipid nanoparticles, carbon quantom dots (CQDs), and liposomes have been reported to protect dsRNA from nucleolytic degradation using *ex vivo* experiments [127,210,215,216]. These studies provide evidence that nanoparticles can stabilize dsRNA in the environment and provide residual efficacy that is needed to produce an economically feasible pesticide formulation.

Nanoparticles can also overcome the limitations regarding cellular uptake and transport [127,217,218]. Multiple cationic nanoparticles enhance uptake into midgut cells, however the mechanism behind increased uptake is currently unknown. One potential mechanism could be via the electrochemical attraction with the negatively charged surface of the cell membrane and the cationic surface of the nanoparticle-dsRNA complexes [111]. Once Inside the cell nanoparticle-dsRNA complexes can be released from early endosomes before they are trafficked to the lysosome for degradation, a cellular mechanism known to be a limiting factor of RNAi for multiple Lepidopteran species [187,216,219]. The mechanisms resulting in endosomal escape are

unknown, however, multiple hypotheses have been made. These include the proton sponge effect, and destabilization of the endocytic membrane followed by polymer swelling [220,221].

Nanoparticles have displayed potential as an inert ingredient that can prolong environmental persistence of dsRNA, overcome physiological barriers to RNAi, and increase efficacy of RNAi for insect pest management. However, further evaluation of nanoparticle-dsRNA complexes with unique properties on both model and non-model species is needed to understand the broader application of nanoparticles for the future of dsRNA-based pest control.

Branched amphiphilic peptide capsules (BAPCs) are nanoparticles formed by the combination of two branched amphiphilic peptides, bis(Ac-FLIVI)$_2$–K–K$_4$–CONH$_2$ and bis(Ac-FLIVIGSII)$_2$–K–K$_4$–CONH$_2$, which spontaneously assemble in water [222]. BAPCs display similar size and charge characteristics to other nanoparticles that have been used in previous experiments to increase RNAi-mediated knockdown in insects. BAPCs contain amino groups with pKa values between 9-13, conferring stability in neutral and alkaline environments [111]. BAPC-dsRNA complexes have been reported to distribute into distal tissues such as the fat bodies and malpighian tubules in *T. castaneum* adults post-ingestion [212]. Furthermore, BAPCs have been used to enhance gene suppression in recalcitrant species. Oral delivery of dsBiP-BAPC complexes in the pea aphid (*Acyrthosiphon pisum*) lead to significant gene suppression by 5 days post ingestion (DPI), following premature death relative to dsRNA alone [212]. Further research is needed to determine the extent of applicability for BAPCs as a nano-delivery system of dsRNA for insect pest management.

1.5 Rationale and objectives

It is well-known that dsRNA-mediated RNAi can be a tool for insect pest management, with multiple products now registered against significant pest species. However, most knowledge about the mechanisms, efficiency, limitations, and subsequent technologies to overcome limitations of RNAi have relied on model organisms. To gain a better understanding of RNAi and how it may be developed for the control of non-model species, we set up objectives with a broader goal of developing a platform for sprayable formulations of dsRNA for the management of *P. japonica*. Our objectives were to 1) Determine whether complexing dsRNA with Branched Amphiphillic Peptide Capsules (BAPCs) could enhance the silencing effect of a peritrophin gene expressed in the midgut tissue of *P. japonica*, 2) Evaluate whether BAPCs can enhance cellular uptake and transport of dsRNA across the larval midgut of *P. japonica* using an *ex vivo* experimental set-up, and 3) evaluate whether foliar sprays of dsRNA targeting an actin gene in *P. japonica* to rose plants (*Rosa spp.*) would provide plant protection against adult *P. japonica* feeding damage.

Chapter 2: Gene silencing in adult *Popillia japonica* through feeding of double-stranded RNA (dsRNA) complexed with Branched Amphiphilic Peptide Capsules (BAPCs)

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Abstract

Gene silencing by feeding double-stranded (dsRNA) holds promise as a novel pest management strategy. Nonetheless, degradation of dsRNA in the environment and within the insect gut, as well as inefficient systemic delivery are major limitations to applying this strategy. Branched amphiphilic peptide capsules (BAPCs) complexed with dsRNA have been used to successfully target genes outside and inside the gut epithelium upon ingestion. This suggests that BAPCs can protect dsRNA from degradation in the gut environment and successfully shuttle it across gut epithelium. In this study, our objectives were to 1) Determine whether feeding on BAPC-dsRNA complexes targeting a putative peritrophin gene of *P. japonica* would result in the suppression of gut peritrophin synthesis, and 2) gain insight into the cellular uptake mechanisms and transport of BAPC-dsRNA complexes across the larval midgut of *P. japonica.* Our results suggest that BAPC-dsRNA complexes are readily taken up by the midgut epithelium, and treatment of the tissue with endocytosis inhibitors effectively suppresses intracellular transport. Further, assessment of gene expression in BAPC- peritrophin dsRNA fed beetles demonstrated significant downregulation in mRNA levels relative to control and/or dsRNA alone. Our results demonstrated that BAPCs increase the efficacy of gene knockdown relative to dsRNA alone in *P. japonica* adults. To our knowledge, this is the first report on nanoparticle-mediated dsRNA delivery through feeding in *P. japonica*.

2.1 Introduction

Invasive species account for significant ecological and economic impacts [9,14]. In 1916, a small, metallic-colored beetle from Japan, *Popillia japonica* Newman, was first detected near Riverton, NJ, USA. *P. japonica* is currently established in 28 states in the US, and continues to expand its range west and north in North America into previously non-infested states, territories, and provinces, likely through human-mediated transport [39,44]. The polyphagous nature, feeding on >300 plant species, and capable of forming large feeding aggregations on host plants are key factors in the success of *P. japonica* adults as pests in the extended geographic range [37,45]. This species is a target for substantial insecticide usage in both larval and adult life stages, especially in areas with large monocultures of turfgrass such as roadsides, golf courses, and urban landscapes [37]. Insecticide use targeting *P. japonica* adults and larvae are associated with secondary pest outbreaks and interference with host finding by introduced natural enemies [30,223].

Targeting insect pests, especially beetles, with double-stranded ribonucleic acid (dsRNA) molecules has shown great promise as an alternative to chemical insecticides [145,224,225]. Exogenous dsRNA activates the RNA interference (RNAi) pathway, which is a conserved and innate biological defense mechanism in eukaryotic organisms against viruses and transposons via post-transcriptional gene silencing [101]. Unlike common chemical insecticides, dsRNAs are designed to target mRNA sequences unique to the target pest because of the necessity to have high sequence fidelity for gene silencing to occur [224]. Furthermore, dsRNA has a low potential for persistence in the environment, including soil, sediment, and surface water compartments, because of its instability in environmental conditions and rapid microbial degradation [141,167,224].

The most field-applicable route of dsRNA delivery is via ingestion by the target insect [111]. Currently, the only commercially available dsRNA product for insect control is facilitated by

genetically modified plants [145]. However, these products involve plant transformation, which is not feasible for all plants/crops due to the cost and time of production, and extensive regulatory processes to evaluate environmental risk [114]. Thus, exogenously applied products in the form of bio-pesticides may be a more feasible and cost-effective method for pest attacking multiple plant/cropping systems. The general use of dsRNA as an insecticide has been forestalled by the variability in efficacy of RNAi among species, life stage, dosage, delivery method, and target gene [111,166,226,227]. The observed variability in efficacy may be contributed to degradation of dsRNA in the environment and in the insect gut, inefficient uptake by the gut epithelium, defective RISC complex, and impaired systemic delivery [111,171,173,211]. The enzymes present in body fluids of *P. japonica* are highly efficient at degradation of dsRNA relative to other beetles that have been successfully targeted [170]. Hence, it is imperative to provide a protectant to dsRNA for silencing effects to occur.

Nanoparticles can help to overcome the technical challenges associated with the oral delivery and efficiency of dsRNA. Nanoparticles are typically defined as particles ranging between 1 and 100 nm in size made of a variety of materials (i.e., lipids, peptides, polymers and metals) [111,228]. In most cases, nanoparticle-dsRNA complexes are formed by electrostatic interactions between the cationic groups of the nanoparticles and the negatively charged phosphate groups of dsRNA [212]. Nanoparticles can prevent degradation of the dsRNA nucleotides by nucleases in the salivary glands and in the gut by blocking target sites for RNases [127,229]. Furthermore, the overall net charge of the complexes are typically positive, which is suitable for uptake by cell membranes [212,230]. Our research team developed branched amphiphilic peptide capsules (BAPCs) [212]. BAPCs are formed through the spontaneous assembly of two branched amphiphilic peptides, bis(Ac-FLIVI)–K–K_4–$CONH_2$ and bis(Ac-FLIVIGSII)–K–K_4–

$CONH_2$ in water [217]. Ingestion of BAPC-dsRNA complexes targeting a major genes associated with the unfolded protein response resulted in significant knockdown of gene expression levels and enhanced mortality rates in the red flour beetle (*Tribolium castaneum*) and in the pea aphid (*Acyrthosiphon pisum*) [212]. The properties of BAPCs also make the synthesis scalable to large scale production as they can be stored for extended periods, self-assemble in pure water, and are effective at low μM concentrations.

In this study, BAPC-dsRNA complexes were evaluated for their efficiency in 1) knockdown of the peritrophin expression and subsequent mortality post-ingestion in adult *P. japonica*, and 2) uptake and transport across the larval midgut epithelial cells. Silencing of peritrophin can result in increased susceptibility of the insect gut tissue to insecticides, phytochemicals and pathogens affecting their metabolism, growth, development, and survival [231]. We also analyzed BAPCs and BAPC-dsRNA size in a buffered solution with pH ~7.4 (similar to the adult *P. japonica* midgut). Finally, we explored the cellular mechanism for uptake of BAPC-dsRNA complexes and transport across larval midgut [231]. According with the literature review, this is the first report on gene knockdown in adult *P. japonica* using nanoparticle-mediated dsRNA delivery.

2.2 Materials and methods

2.2.1 Specimens

Larvae of *P. japonica* were sourced from a commercial sod farm near Murfreesboro, TN and collected in April 2022. Larvae used for midgut assays were maintained by placing them in individual cells of ice cube trays and fed with carrot strips. In July 2022, field collected, adult female *P. japonica* used in the dsRNA feeding trials were shipped overnight from Michigan

State University. The beetles were maintained in a container with sifted soil and fed on a diet of crape myrtle (*Lagerstroemia indica*) and rose (*Rosa* spp.) flowers. Female and male beetles were separated based on the morphology of the tibial spur and only insects that were free of obvious morphological defects or injuries were used in experiments [45].

2.2.2 Preparation of BAPCs and BAPC-dsRNA complexes

To form the BAPCs, two monomeric peptides, bis(Ac-FLIVI)-K-K_4-$CONH_2$ and bis(Ac-FLIVIGSII)-K-K_4-$CONH_2$, were synthesized using solid phase peptide synthesis as previously described by Avila et al., (2018) [212]. These peptides are referred to as H5 and H9, respectively, after the number of residues in the hydrophobic branches. After synthesis, dried peptide was dissolved in trifluoroethanol (TFE). Concentration of each peptide was determined by measuring the absorbance of phenylalanine and subsequently dividing that value by two to account for the two phenylalanine residues per peptide. The two peptides were then mixed in equimolar ratios to create a 1mM final stock. TFE was then evaporated off using a FreeZone2.5 and refrigerated Centrivap Concentrator vacuum system (LabConco). BAPCs were assembled by adding 1 mL nuclease-free water and allowing the solution to sit at room temperature for 5 min, followed by an incubation at 4°C for at least 1 hr. At room temperature, the peptides spontaneously assemble into a bilayer and fuse, and the shift to 4°C slows the fusing of complexes and locks the BAPCs in a size range of 50-250 nm. BAPC-dsRNA complexes were formed by mixing the appropriate concentration of BAPCs with 1µg dsRNA and allowing the mixture to sit for 15 min at room temperature.

To form rhodamine-labelled BAPCs (Rh-BAPCs), half of the bis (Ac-FLIVI)-K-K_4-$CONH_2$ component was substituted with the same peptide labeled with the N-hydroxysuccinimide ester of rhodamine B covalently attached to the ε-amino group of the C-

terminus lysine (bis (Ac-FLIVI)-K-K_3-K(Rh)-$CONH_2$). This resulted in a final molar ratio of 1 H9: 0.5 H5: 0.5 Rh-H5. Rh-BAPC-dsRNA complex were formed as described for unlabeled BAPC-dsRNA complexes.

2.2.3 Dynamic Light Scattering (DLS) and electrophoretic retardation assay of BAPCs and BAPC-dsRNA complexes

BAPCs and BAPC–dsRNA complexes were suspended in a buffer simulating midgut pH, then size was measured via DLS using the Zetasizer Nano ZS (Malvern Instruments Ltd., Westborough, MA). A 500μM stock of BAPCs was prepared following the protocol previously described. BAPCs were then complexed with 1μg of dsRNA if needed, and the BAPCs or BAPC-dsRNA complexes were transferred to phosphate buffered saline solution (pH = 7.4). Samples were incubated at room temperature for 5-10 minutes prior to analysis, and all DLS measurements were performed in triplicates. For the gel retardation assay, BAPC-dsRNA complexes of 20μM and 60μM BAPCs complexed with 1μg dsRNA were assembled and incubated in pH 7.4 buffer as described above. Following, complexes were mixed with RNA gel loading dye (Invitrogen, Waltham, MA) at a 1 : 1 ratio. Samples were then resolved onto a 2% agarose gel composed of 1× MOPS buffer and SYBR green stain, then electrophoresed at 100 V for 30 min. Control wells containing 1 μg of dsRNA only and BAPC concentrations of 20μM and 60 μM without dsRNA were included. The gel was imaged using ImageQuant LAS 4000 (GE Healthcare, Pittsburgh, PA, USA).

2.2.4 Selection of target gene and dsRNA synthesis

Due to availability of only five known mRNA sequences of *P. japonica* in NCBI database, we chose peritrophin, one of the available sequences, as a target for RNAi. Peritrophins play key protective roles during food processing in feeding life stages, growth, and development

of larvae. To synthesize dsRNA, first total RNA was extracted from the gut tissue of *P. japonica* using a commercially available TRIzol reagent. After purification of total RNA, the RNA was reverse transcribed to cDNA using SuperScript II First-Strand Synthesis System, and the genomic DNA was removed by DNase I treatment (Invitrogen). The synthesized cDNA was then used as a template for the amplification of the peritrophin gene segment using following primers; forward primer: GCTGGTACCTACTTCAATCC, reverse primer: CATACAACCTGCATCTT-CGG. Both primers were designed manually to amplify the peritrophin gene segment of ~300 bps with T7 promoter sequence flanking at 5' end of both primer sequences. Upon amplification and purification of T7 flanked peritrophin DNA, sense and antisense RNA strands were synthesized separately as per manufacturer protocol using TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific^{TM}, Carlsbad, CA, USA). After transcription, the sense and antisense single-stranded RNA (ssRNA) were purified using LiCl precipitation, quantified using nanodrop and resuspended in duplex buffer (Integrated DNA Technologies Inc., USA). For annealing, both RNA strands were mixed in 1:1 molar ratio and annealed as recommended by the supplier. The quality of dsRNA was verified by 1% agarose gel electrophoresis and using a Nanodrop technique. In addition, we synthesized a GFP dsRNA sequence non-specific to *P. japonica* and it was used as a non-specific control.

2.2.5 Adult feeding and survivorship assay

One day prior to the experiment, 120 adult female beetles were randomly selected from a container and transferred to a plastic cup (Dart, Mason, MI) and deprived of food for 24 hr prior to feeding. Whatman GF/A filter papers (25 mm diam., Cytivia life sciences, Marlborough, MA) were cut into quarters and pinned between two 5 x 5 mm pieces of transparency film (Tri-state Visual Products, Highland Heights, KY) using stainless steel insect pins. Each filter paper

quarter was treated with 40 µL of a 1 M sucrose solution and allowed to dry for 12 hr prior to applying the treatment to promote adult beetles feeding. Adult *P. japonica* will feed on filter papers amended with 1 M sucrose [232]. Upon drying, filter papers for seven different treatment groups and 15 biological replicates were prepared by applying 40 µL of BAPC-dsRNA complexes or dsRNA alone. In the treatment group containing non-specific dsRNA, only 10 biological replicates were prepared.

On day 0, 85 food-deprived beetles were selected for the survivorship assay. Individuals were tested for vigor by flipping them on their dorsum and used only if they could right themselves within 5 min. Selected beetles were then transferred into an individual wax-bottomed plastic cup and randomly assigned to one of the seven treatments. Three extra beetles were randomly selected, deprived of food for 24 hr and their midgut tissues were isolated and preserved in TRIzol to assess effect of starvation on peritrophin gene expression. Once the beetles were transferred to wax-lined cups, filter paper quarters with BAPC complexes were placed inside and all beetles were transferred into a growth chamber at 25±0.5° C. Beetles were allowed to feed until a treatment group had consumed either an average of 2/3 (66%) of the filter paper or 24 hr, whichever occurred first. Post feeding, all filter papers were replaced with leaf disks (20 mm diam.) taken from freshly collected Virginia creeper (*Parthenocissus quinquefolia*) foliage, followed by replacement of these leaf disks daily until day 6. Until the end of experimental protocol or observed mortality, beetles were tested daily for vigor as previously described. Beetles that failed to provide a vigor response were considered dead and eliminated from the study. Three beetles from each group were selected at random and dissected to isolate gut tissue for RNA extraction and analysis.

On day 7, a second dose of respective treatments was administered to surviving beetles through filter papers using the same methodology as described above. Data on survivorship was collected every 24 hr until day 14. Filter paper consumption was calculated using Image-J [233] by collecting the filter papers quarters after both doses. The area of filter paper after the assays was measured, then a percentage of area consumed was calculated based on the initial area. The initial area was the average of six filter paper quarters not provided to beetles.

2.2.6 Quantitative polymerase chain reaction

Total RNA was isolated from adult *P. japonica* gut tissue with TRIzol Reagent (TRIzol (Ambion, Inc., Carlsbad, CA, USA). RNA concentration was measured using nanodrop and quality was evaluated using 260/280 and 260/230 ratio. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer instructions and used as a template for the RT-qPCR. Each RT-qPCR sample contained 10 µL of synthesized cDNA, 0.8 µL of each primer (10 µM forward and reverse), 0.9 µL of nuclease free ddH2O, and 12.5 µL of Perfecta Sybr Green Supermix (Quanta Biosciences, Gaithersburg, MD, USA); totaling 25 µL. All reactions were performed using SYBR Green Master Mix and amplified under the following cycling conditions: beginning cycle at 95 °C, 40 cycles at 95 °C for denaturation, followed with 30 s at 65 °C for annealing and extension, and ending with generation of a melting curve consisting of a single peak to rule out non-specific product and primer dimer formations. Each treatment group contained three biological replicates and two technical replicates. The expression levels of peritrophin and the number of transcripts per sample was estimated based on the Ct value. Due to unavailability of housekeeping gene sequences in *P. japonica*, we used a β-actin gene sequence from the closely related species *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) to design primers and used as an internal loading

control. Generated cDNA was then used to quantify changes in gene expression levels among different treatment groups by RT-qPCR. The expression levels of the genes were determined by $2^{-\Delta\Delta Ct}$* proportional calculation method. (The fold changes in peritrophin transcript levels relative to the β-actin).

2.2.7 Larval midgut transcytosis assay

To elucidate if transcytosis is involved in the translocation of BAPCs through midgut epithelium cells, live third instar *P. japonica* larvae were dissected to isolate the midgut tissues. The preparation for assays in the Ussing chamber have been described in our previous work [217]. Larvae used in this assay were stored in a fridge at 4°C for 48 hr prior to dissections to stabilize the gut tissues. Incisions were made along the lateral-medial line of the larvae from the anterior to the posterior using corneal scissors. Insect pins were then used to secure the integument onto the dissection tray and expose the digestive tract. Incisions were performed on the midgut by making lateral-medial incisions anteriorly from the third gastric caecum. The tissues were then isolated from the body, rinsed with insect physiological solution described previously [217], and immediately mounted onto a modified 0.01 cm^2 slide. Dissected midgut tissue from larvae was inserted into a tissue holder slide which was placed inside of a Ussing chamber. This chamber creates an *ex vivo* gut environment through which transport of molecules across tissue may be studied. Buffer containing rhodamine dye labelled BAPCs was added to the luminal side of the tissue, and transcellular transport was determined by measuring rhodamine dye fluorescence on luminal as well as hemolymph side at discrete time points. Dissections were conducted with meticulous effort to avoid tissue punctures and to conserve orientation of the tissue relative to the lumen and the hemolymph.

After mounting, slides were slotted into the two-sided chamber, where tissues were then perfused with 3 mL of lumen or hemolymph buffer according to tissue orientation [217]. To study the effect of inhibiting endocytosis on the transport of BAPC-dsRNA complexes, three replicate tissues were pre-incubated with 10µM chlorpromazine (CPZ) for 30 min before the addition of Rh-BAPC-dsRNA complexes. A final concentration of 50 µM Rh–BAPCs with or without 1 µg dsRNA was then added to the lumen side. Tissues were exposed for a period of 120 min, after which fluorescence was read using a BioTek Cytation 3 plate reader (λex = 544 nm; λem = 576 nm). Change in relative fluorescence over time was plotted to visualize the transport of Rh-BAPCs due to transcytosis. Fluorescence was measured in arbitrary fluorescence units (AFU's). To account for the variability of relative fluorescence between replicates, data were normalized using proportions.

2.2.8 Statistical analysis

Data analysis and plots were done using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, San Diego, California USA). To estimate the sample size in the survivorship assay, we employed the "resource equation method" [234]. For survival curves we used the Log Rank Test. For the gene expression analysis, consumption of filter papers, and DLS experiments, we used one-way ANOVA using Tukey as post-test. Transcytosis experiments were analyzed using two-way ANOVA and Tukey as post-test. An alpha level of $P<0.05$ was used for all analyses.

2.3 Results

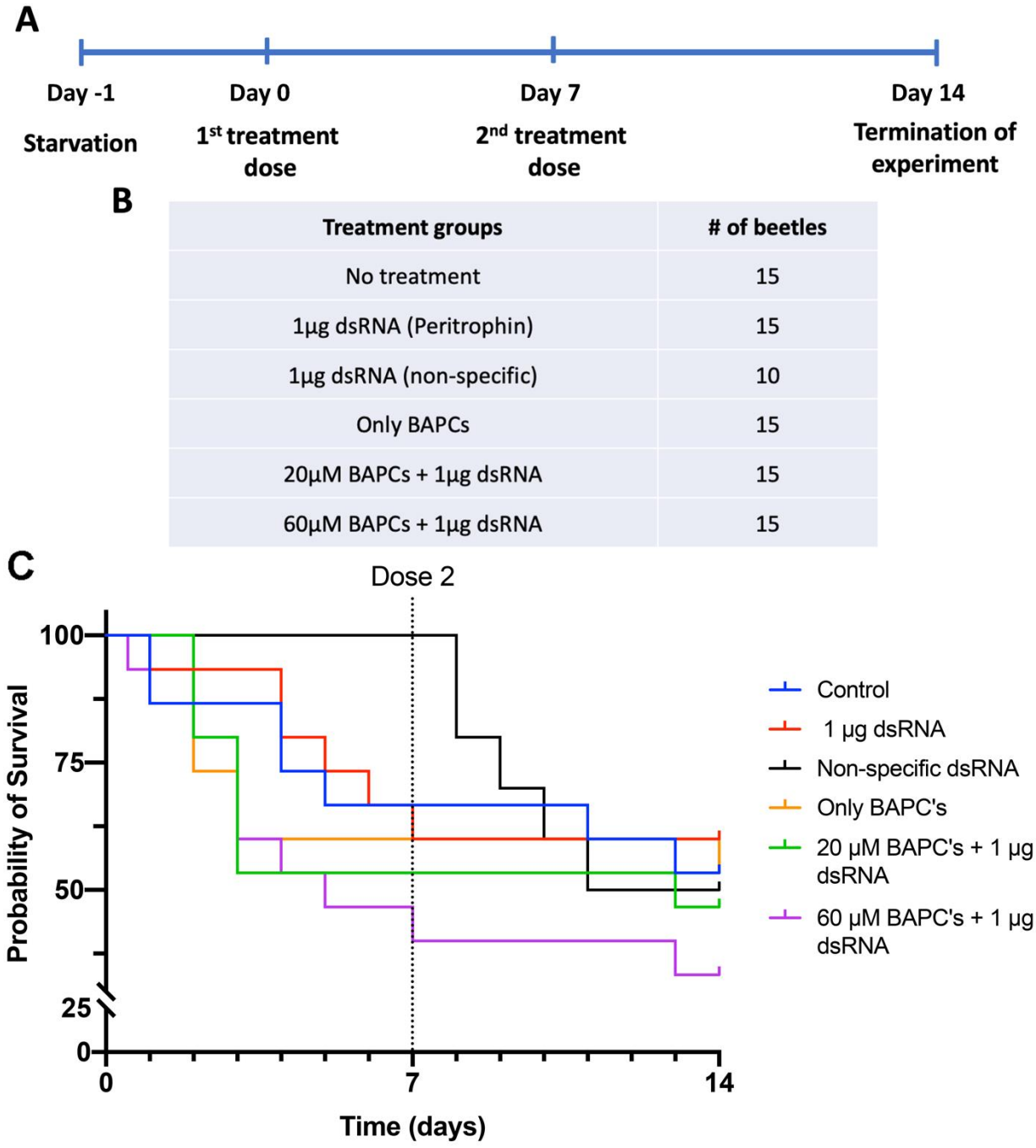
2.3.1 *P. japonica* artificial diets supplemented with BAPC-dsRNA complexes.

We delivered two doses of BAPC-dsRNA complexes through feeding on day 0 and day 7, allowing them to feed on the treatment up to 24 hr **(Figure 1A and B)**. Subsequently, we monitored all beetles daily for mortality up to 14 days (normal life span of adult *P. japonica* is 30-45 days). Survivorship by 14 days was only 33% in insects fed on diets containing 1 μg dsRNA+60 μM BAPCs ($t_{1/2}$= 7 d). On the other hand, 60% and 53% survivorship were observed at 14 days in beetles fed only dsRNA and untreated control groups, respectively. This difference in survivorship was not significant as determined by a log-rank test (Day 7: *P= 0.12* & Day 14: *P = 0.6, df= 1*). Feeding of beetles on a diet of non-specific (non-peritrophin) dsRNA or with lower BAPCs concentration (1 μg dsRNA+ 20 μM BAPCs) also had no effect on survival ($t_{1/2}$= 14 d) **(Figure 1C)**.

Consumption of filter papers vary greatly among individuals within same treatment group, therefore the average dosage delivered would be lower than provided. We calculated surface area of filter paper consumed as a proxy for dose ingested within 24 hr before being replaced with a diet of Virginia creeper leaf discs. On average, 38% of the filters were consumed across treatments, with an average of 22% of the filter paper consumed in the control dsRNA treatment (**Figure 2**) (*P<0.01, Df= 6, 139, F = 3.087)*. Thus, the amount of dsRNA ingested was lower than the amount applied, and dose likely varied among each female adult beetle.

2.3.2 Assessment of peritrophin-mRNA levels isolated from *P. japonica* midgut.

We quantified the peritrophin transcript levels by RT-qPCR analysis to confirm that dsRNA induced gene silencing in the targeted gene. Ingestion of 20 μM BAPCs+ 1 μg dsRNA resulted in a 30-fold decrease in peritrophin gene expression, which was significantly different *($P<0.05$, $Df= 4, 10$, $F= 6.840$)* from the dsRNA alone group. Similarly, 60 μM BAPCs+ 1 μg dsRNA had the greatest gene silencing rate, with knockdown of expression by approximately 34-fold relative to non-treated control group (**Figure 3**). Although quantification of the mRNA transcripts is not statistically congruent with the trends observed in the survivorship study, our results support the concept of BAPC nanoparticles acting as dsRNA stabilizer, and cellular uptake enhancer. Furthermore, we also analyzed the integrity of the BAPCs formulations by measuring the size in a buffered solution with a pH similar to the *P. japonica* midgut (pH=7.4) [45].



Treatment groups	# of beetles
No treatment	15
1µg dsRNA (Peritrophin)	15
1µg dsRNA (non-specific)	10
Only BAPCs	15
20µM BAPCs + 1µg dsRNA	15
60µM BAPCs + 1µg dsRNA	15

Figure 2. Survival curves of *P. Japonica* post-ingestion of BAPCs formulations: Experimental outline of dsRNA feeding assay in *P. japonica* adult females (A). Treatment groups included in the feeding assay (B). The survival curve of *P. japonica* females upon feeding on BAPCs complexed with peritrophin-dsRNA (n = 15). The data were analyzed using a log rank test. There were no significant differences between treatment groups ($P > 0.05$).

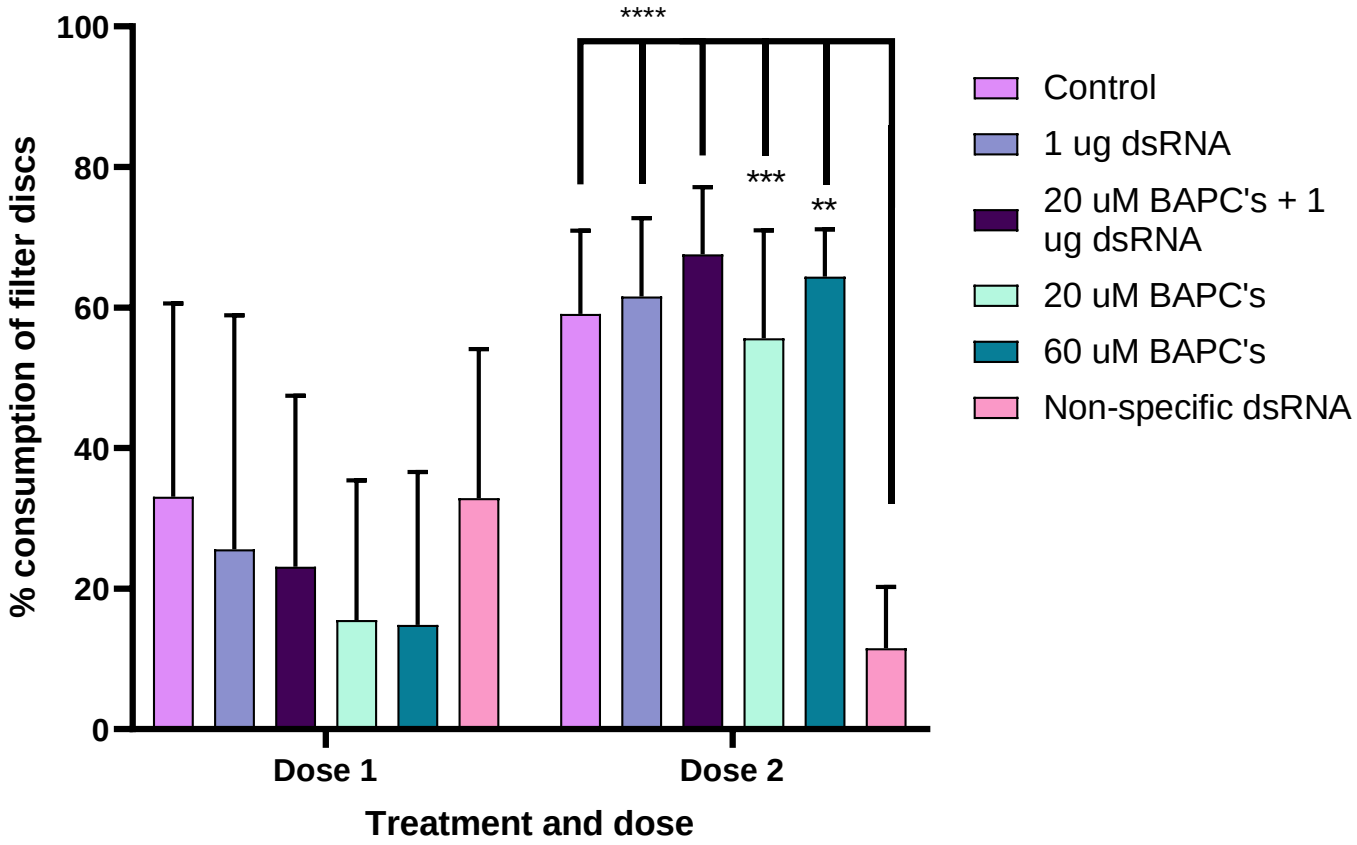


Figure 3. Average feeding of the filter paper by adult *P. japonica* within a 24-hour period. This data was obtained using image-J software. The data was analyzed using a one-way ANOVA. Significant difference in feeding occurred between replicates for both doses, and significant differences occurred between treatments for the second dose ($P > 0.05$).

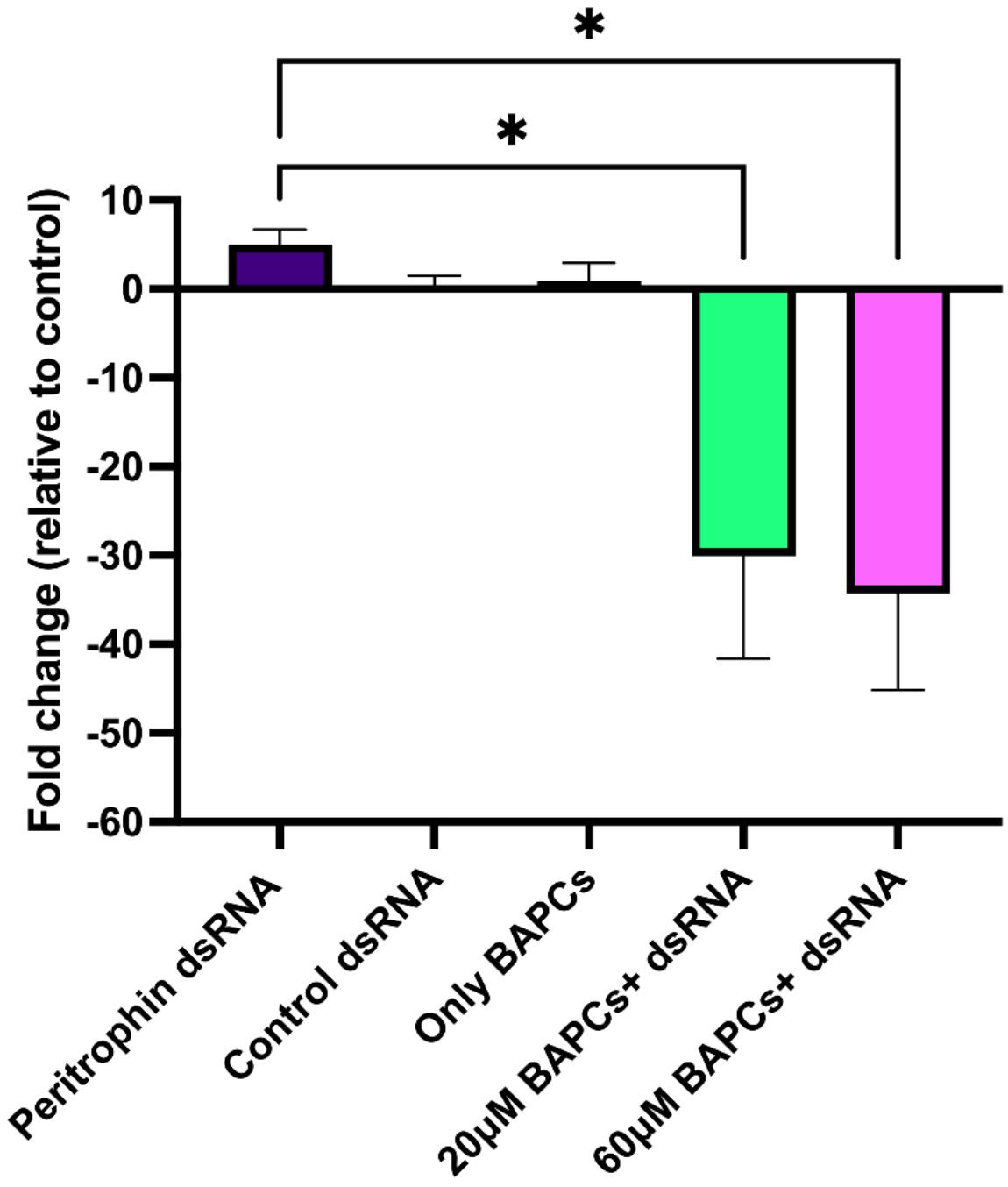


Figure 4. Analysis of gene expression upon dsRNA mediated gene silencing: peritrophin-mRNA transcript levels in the midgut of *P. japonica* upon feeding on BAPCs complexed with and without dsRNA analyzed using RT-qPCR. Fold change in peritrophin expression is normalized using β-actin as an internal control. Differences between values were compared by one way ANOVA using Tukey as post-test. Statistical significance: (*) $P < 0.05$; (**) $P < 0.01$. Non-statistical significance (*ns*) was considered when $P > 0.05$.

2.3.3 Biophysical characterization of BAPCs and BAPC-dsRNA complexes

From a biophysical perspective, the stability or integrity of nanoparticles is used to describe the preservation of a particular nanostructure property (i.e., size). We assessed BAPCs stability by incubating in buffer of pH 7.4 using dynamic light scattering (DLS), According with **Figure 4A**, the BAPCs–dsRNA complexes displayed a size ranging between 250 to 350 nm, a size that is consistent with our previously reported DLS measurements performed in distilled water [217]. BAPCs (60 μM) not associated with dsRNA exhibited a significant *(P= 0.0002, Df= 3, 20, F = 10.45)* smaller hydrodynamic diameters than the BAPC-dsRNA complexes, confirming that the association of dsRNA increases the size of the BAPCs or causes BAPCs to cluster together. Furthermore, these results indicate that the complexes do not dissociate or aggregate in the buffered solution, proving structure stability in a pH environment consistent with the gut of *P. japonica* adults. Although multiple variables can play a role in nanoparticle stability inside the midgut, the pH is critical since it can lead to variation in nanoparticle charge and oxidation state. Regarding nuclease degradation of dsRNA, studies performed in mammalian cells support the notion that BAPCs protect dsRNA against nuclease degradation [217,235]. Target sites of RNAs might no longer be accessible to the catalytic core of RNases after the association with the BAPCs surface [236]. It is important to mention that nucleases exclusively affect the dsRNA structure and not BAPCs. The downregulation of the peritrophin transcript levels also support the notion of nuclease protection conferred by BAPCs.

To elucidate the dsRNA binding capacity of BAPCs at concentrations used for the survivorship assay, we evaluated their electrophoretic mobility in a 2% RNA agarose gel. Our

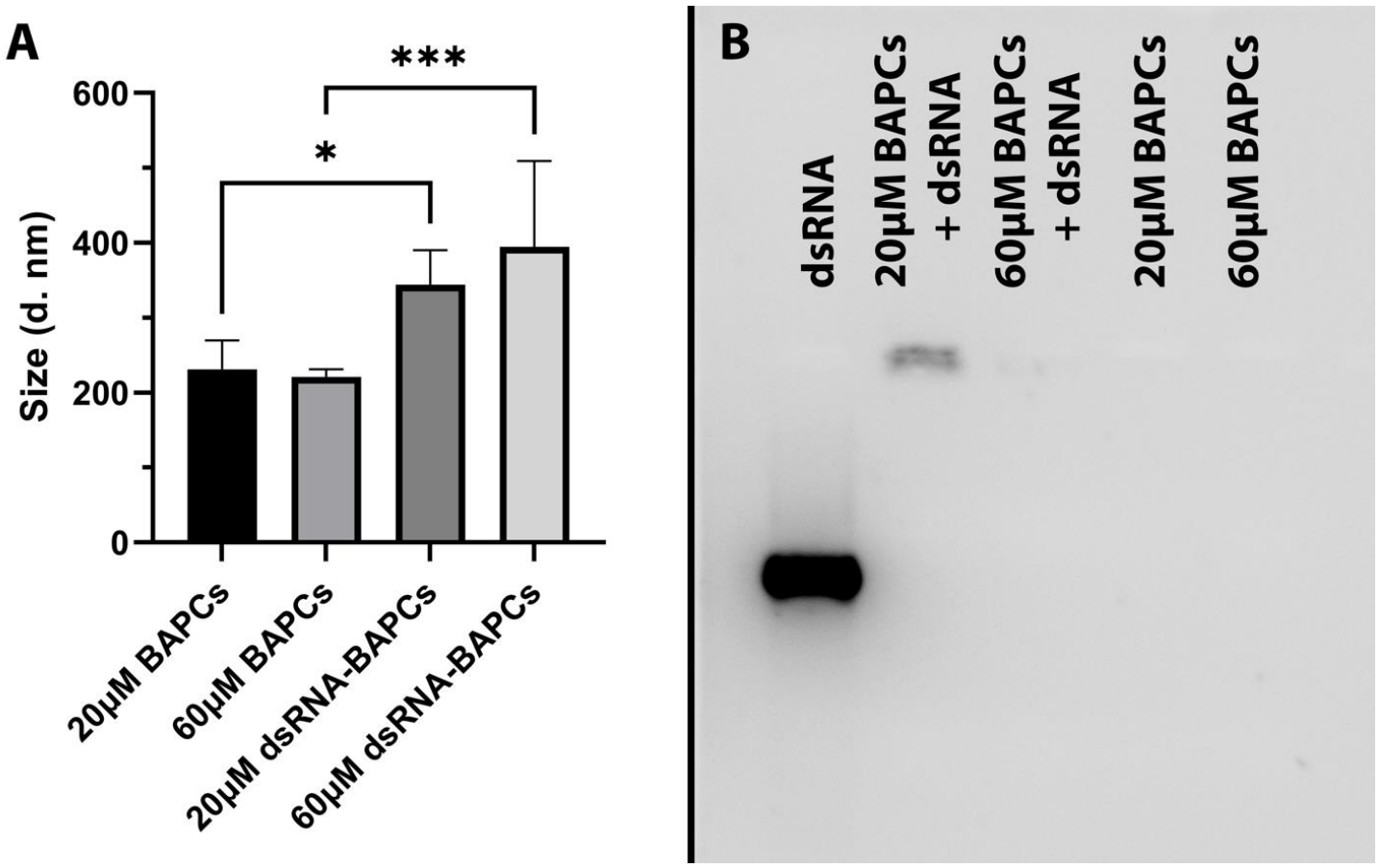


Figure 5. (A) Stability assessment of BAPCs and BAPC-dsRNA complexes in a buffer mimicking *P. japonica* gut (pH 7.4). (B) BAPC loading capacity assessed by the electrophoretic mobility shift assay. BAPC-dsRNA complexes were formed by mixing 20µM and 60µM BAPCs with 1µg dsRNA. Controls containing only 20µM or 60µM BAPCs without dsRNA were also run to show they did not produce background signal. Differences between values were compared by one way ANOVA using Tukey as post-test. Statistical significance: (*) $P < 0.05$; (***) $P < 0.001$. Non-statistical significance (ns) was considered when $P > 0.05$

results indicated that association of dsRNA with the BAPCs surface led to a decreased migration of dsRNA that was dependent on BAPC concentration (**Figure 3B**). The formulation with the highest BAPCs concentration (60 μM) displayed a barely visible band, suggesting that all added dsRNA has firmly adhered to the BAPCs surface, which resulted in a poor interaction with the SYBR green dye. However, lower concentrations of BAPCs yielded a more visible dsRNA band, indicating more availability for SYBR green binding due to a weaker interaction of BAPCs and dsRNA. As expected, BAPCs not complexed with dsRNA showed no signal in the well.

2.3.4 Midgut cellular uptake mechanisms of BAPC−dsRNA complexes

Through the many dissections for the reported experiments, we noted less tissue strength of the adult gut leading to those tissues tearing and shearing more easily than the larval midgut tissues (**Figure 5A and B**). The width and sensitivity of the adult midgut tissue was an obstacle to study BAPC-dsRNA complexes cellular uptake and transport across gut tissue. Consequently, only the larval midgut tissues were used to study these mechanisms in *P. japonica*. For larval dissections, we used the third gastric caecum to delineate between midgut and hindgut tissues (**Figure 5A**).

The transport of BAPCs and BAPC-dsRNA complexes into and across the gut tissue was assessed with the help of Ussing chamber (**Figure 6A&B**). Both formulations are actively transported across the gut tissue, with around 50% reduction in rhodamine fluorescence in the luminal compartment **(Figure 6C)**. However, BAPCs complexed with dsRNA slows significantly *($P<0.05$, $Df= 2, 6$, $F= 5.841$)* the rate of transcellular transport compared to only BAPCs, and transcellular transport of the complexes was similar in the presence or absence of chlorpromazine **(Figure 6D)**.

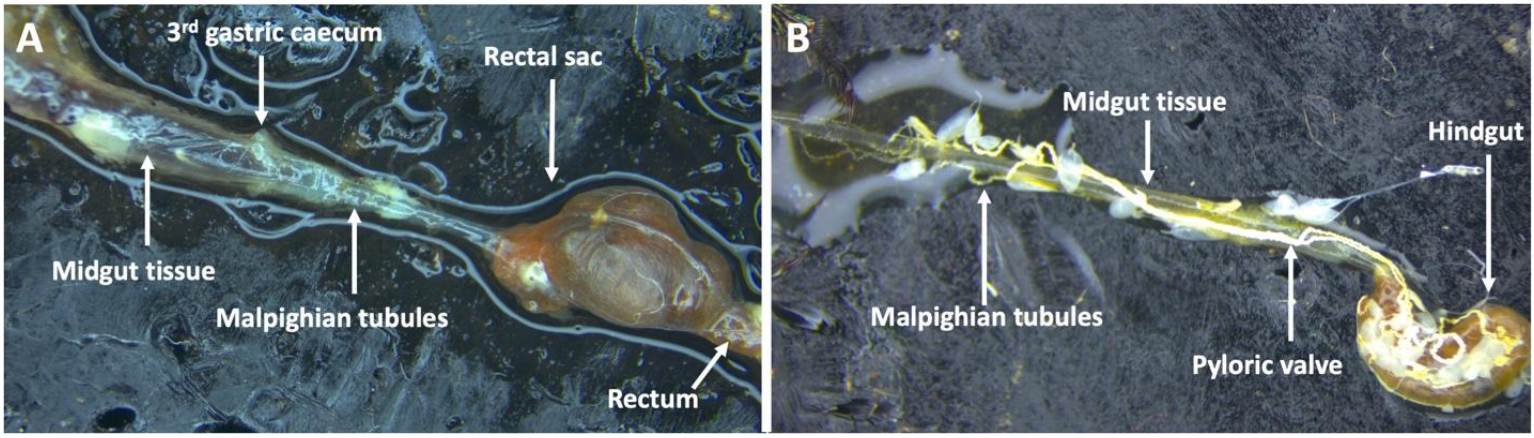


Figure 6. Digestive tract of *P. japonica*. A) Larval digestive tract including midgut tissue, 3rd gastric caecum, Malpighian tubules, rectal sac, and the rectum. B) Adult digestive tract including midgut tissue, hindgut, the pyloric valve, and Malpighian tubules (White, string-like organ).

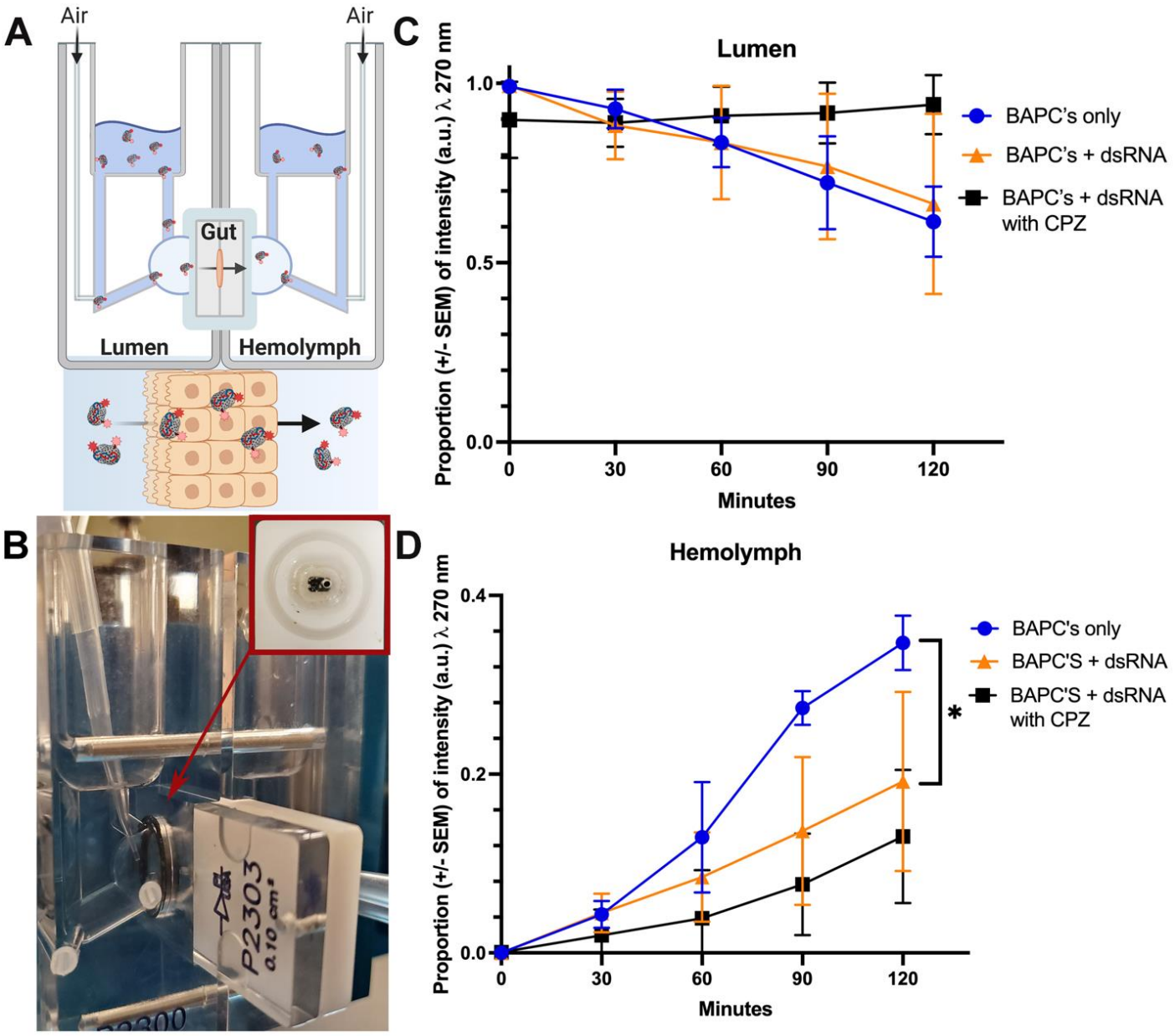


Figure 7. Cellular uptake assay. Mechanism of Rh–BAPCs and Rh–BAPC-dsRNA cellular uptake by *P. japonica* midgut cells. (A) & (B) graphical representation and actual set up of Ussing chamber used for *ex vivo* analysis of BAPC-dsRNA complexes uptake and transport across *P. japonica* midgut tissue. (C) Mean relative fluorescence of Rh-BAPCs complexes on luminal side buffer and (D) Mean relative fluorescence of Rh-BAPCs complexes on hemolymph side buffer over 2 hr. Differences between values were compared by two-way ANOVA using Tukey as post-test. Statistical significance: (*) $P < 0.05$.

To assess the role of clathrin-mediated endocytosis on the uptake of BAPC-dsRNA complexes, gut tissue was pre-incubated for 30 min with chlorpromazine, an inhibitor of clathrin-mediated endocytosis before adding Rh-BAPC-dsRNA complexes to the luminal chamber. Treatment of gut tissue with endocytic inhibitor abrogated BAPC-dsRNA uptake from luminal side with no change in rhodamine fluorescence up to 2h **(Figure 6C)**. Similarly, there was no significant increase in rhodamine fluorescence observed on hemolymph side **(Figure 6D).** Overall, these results indicate that cellular uptake of BAPCs and BAPC-dsRNA complexes is mediated, in part, by clathrin coated endocytic vesicles. Nonetheless, cellular uptake is a complex process and potentially other mechanisms can also be involved in the uptake of BAPC-dsRNA complexes.

2.4 Discussion

In summary, we reported the first gene knockdown study in adult *P. japonica* by feeding of BAPC-dsRNA complexes. Although there was a numerical (20%) difference in survivorship between the 60 μM BAPCs +1 μg of peritrophin-dsRNA treatment and the non-treated control group by 14d, no statistical differences were observed between treatment groups. Here, we discuss the potential reasons for the lack of observed mortality in groups that ingested dsRNA. Peritrophic matrix proteins (PMP's) and analogs are diverse in Coleoptera, and few have been experimentally demonstrated to have significant effects on the structure and function of the peritrophic matrix. A total of 11 genes encoding PMP's have been identified and screened for phenotypic and mortality effects in *Tribolium castaneum,* of which only two resulted in lethal phenotypes during early and late pupal stages post-injection [237,238]. Thus, targeting of peritrophin genes alone may not be sufficient to achieve high mortality rates, but could be involved in important roles including protection, detoxification, absorption of nutrients, and increasing RNAi

efficiency when targeting secondary genes [124,237,239]. Despite peritrophin silencing making the insect gut more susceptible to chemicals and pathogens affecting their metabolism, growth, and development, these effects might not be as lethal as other genes such as vATPase, tubulin, or inhibitor of apoptosis.

The concentration of body fluid required to degrade 50% of dsRNA (CB50) within one hour is between 45-94 fold lower relative to the CB50's of *Tribolium castaneum* and *Leptinotarsa decemlineata* [170]. These are two model coleopteran species that account for most of the knowledge on how dsRNA impacts beetles. *P. japonica* has a broad ecological host range, and utilizes a suite of detoxification enzymes induced by feeding to detoxify phytochemicals [61] . For these reasons, ingested dsRNA faces a complex biochemical environment in the gut of *P. japonica.* When verifying gene expression, naked dsRNA resulted in no significant difference in expression relative to the non-treated control group. Although ingestion of naked dsRNA did not lead to gene silencing, we observed that the fold change in peritrophin expression in both BAPC-dsRNA treatment groups was significantly lower ($P < 0.05$) compared to the control groups (non-treated control and dsRNA only). These results suggest that increasing molar concentrations of BAPCs (>20 μM) improves efficiency of dsRNA delivery, resulting in the desired biological response. Nonetheless, our previous work with BAPCs in different organisms indicate that BAPCs concentrations >60 μM may trigger cytotoxicity [217,235]. Thus, higher BAPCs concentrations were not tested. While the presence of cationic moieties facilitate binding with the cell membrane, excessive cationic charge can also disrupt cell membrane's potential and lead to cell death [240–242]. Therefore, the optimal dose of BAPCs and any other cationic nanoparticle must be carefully optimized to avoid undesirable outcomes. Our results support that BAPC

nanoparticles are effective protectants of dsRNA in insect midgut environments and suggest protectants may be required for efficient RNAi in *P. japonica*.

After ingestion, dsRNA passes into the *P. japonica* midgut [111]. The midgut is composed of three types of epithelial cells: columnar cells, endocrine cells, and stem cells. Presumably, it is in these cells where dsRNA uptake and processing take place. The alimentary tract of adult and 3rd instar larval stages of *P. japonica* have only been described in separate publications with supporting hand illustrations [55,243] respectively. More recent photo images of the digestive tract of neonate and 3rd instar grubs have been published [244]. Here, for the first time we present comparative images of the 3rd instar larval and adult alimentary tracts juxtaposed to highlight the morphological differences (Gryphax® Series Avior microscope camera, Jenoptic, Jena, Germany). These images confirm previous descriptions stating the adult midgut is narrower in width relative to the larval midgut [243,245]. The morphological differences conferred structural differences in these tissues between life stages. The morphological differences between life stage may also confer physiological differences, such as relative thickness of the peritrophic membrane, which may lead to differences in rates, or the overall ability, of dsRNA uptake and transfer across these tissues.

In the experiment using 3rd instar larval *P. japonica* midguts in an Ussing chamber, it was observed that fluorescence of BAPCs and BAPC's-dsRNA complexes decreased in the lumen compartment in a time-dependent manner consistent with previous reports in different insect species [217]. However, the transcellular movement of the complex was significantly slowed. The increase of fluorescence of the complexes into the hemolymph chamber was similar with or without the addition of chlorpromazine, which was significantly lower relative to the BAPC nanoparticles alone. A plausible reason for the diminished transport can be related to the binding

of the negatively charged dsRNA to the surface of BAPCs. The dsRNA association blocks a portion of the positively charged lysine residues exposed on the BAPCs surface thus becoming less cationic, and reducing cellular uptake by epithelial cells [246].

This decrease in transcellular transport when dsRNA is complexed to BAPC nanoparticles may be a hindrance to effectively targeting genes that are highly expressed outside of midgut tissues. This is further suggested by results obtained from larval feeding studies. In this, 3rd instar *P. japonica* were force-fed 5 μL of a 0.5 mg/μL solution of dsRNA targeting an inhibitor of apoptosis gene or Ras opposite, two genes which have previously resulted in high mortality rates post-ingestion in other pest species [247–250]. These dsRNAs were force fed, in complex with BAPCs or alone, and two doses were provided one week apart. The experiment resulted in no mortality observed in any treatment group by the termination of the experiment at 18 DPI (Carroll et al., unpublished). Previous studies have shown that ingestion of dsRNAs targeting these genes can result in between 70-100% mortality by between 10-18 DPI [247,249,250]. Although we cannot rule out the potential of silencing a non-essential region of these genes, it may also be possible that the BAPC-dsRNA complexes remained trapped in the midgut tissue or were preliminarily degraded once in contact with the hemolymph. Future studies comparing multiple nanoparticle types and dsRNA housed in heat-killed bacteria will aid in elucidating the best inert ingredient for enhancing RNAi and subsequent mortality [230,250].

It has been reported in insects that two main mechanisms are involved in the cellular internalization of dsRNA: receptor mediated uptake or endocytosis. Clathrin-mediated endocytosis is one of the major pathways by which cells transport extracellular cargo from outside the cell membrane to the interior via the formation of clathrin-coated endocytic vesicles and has been the reported mechanism of dsRNA uptake in other insect species. In the presence of

CPZ, a clathrin-mediated endocytosis inhibitor, there was a noticeable lack of change in the relative fluorescence in the lumen and smaller increase in hemolymph fluorescence relative to BAPC's alone. Formation of a clathrin-coated pit is initiated by the rearrangement of various accessory and cytoskeletal proteins followed by subsequent creation of a clathrin-coated pit at the inner surface of the cell membrane. CPZ inhibits the anchoring of clathrin and adaptor protein 2 (AP2) complex to endosomes, thereby preventing the assembly of these coated pits [251]. This suggests that clathrin-mediated endocytosis may play a significant role in the uptake of NP in the gut, but other pathways are also likely present as some Rh-BAPCs movement was still observed. Despite the increased number of articles demonstrating nanoparticles-dsRNA mediated gene silencing, fundamental mechanisms such as uptake by midgut cells or transport to the hemolymph are not widely reported. Thus, our findings are particularly relevant as they suggest mechanisms that could potentially enable systemic delivery or can lead to a more tailored nanoparticle design for gene silencing.

Overall, BAPCs provide a means of reliably protecting dsRNA through oral delivery to *P. japonica* midgut tissues. BAPCs are a new class of biomaterial developed by our research group that stands out in the crowded field of nanoparticle delivery systems due to two crucial features: 1) they are assembled exclusively in water, and 2) they contain four free lysine Ɛ-amino groups with pKa values between 9 and 10.5, which makes them stable in neutral and alkaline insect guts. According with DLS, the BAPCs-dsRNA complexes form compact clusters with size ranging from 250 – 350 nm in a pH environment consistent with the gut of *P. japonica* adults. The use of dsRNA and nanoparticles currently appears expensive when compared with relatively low cost of common insecticides. It is unlikely that dsRNA technology will replace the use of conventional insecticides for the management of *P. japonica.* However, providing targeted

control will reduce the negative impacts on non-target arthropods associated with the use of insecticides to control both economically important life stages of *P. japonica*.

Chapter 3: Evaluation of sprayable dsRNA for in-field plant protection against adult *P. japonica* feeding damage

Abstract

The purpose of this study was to evaluate whether sprayable formulations of dsRNA targeting an essential actin gene in *Popillia japonica* could provide plant protection against adult feeding damage on knockout roses. We set up two experiments to evaluate: 1) one-month post-treatment residual efficacy of dsRNA in providing plant protection and 2) whether weekly applications of dsRNA to rose foliage could provide plant protection. The results of this study showed that actin dsRNA did not provide significant plant protection to roses in either experiment relative to a non-treated control group. The potential reasons for this could relate to the feeding ecology of adult *P. japonica* and is discussed further in the conclusions section.

3.1 Introduction

RNA interference (RNAi) is a promising, target specific approach for managing multiple coleopteran pest species including WCR and CPB [145,151]. In-lab mortality resulting from gene silencing in these species has conferred protection to crops against feeding damage caused by these species when incorporating these products in the field [145,147,152]. These dsRNA products are now commercialized, one as a transgenic corn strain and another as a sprayable dsRNA formulation.

Transformative methods have worked in multiple systems, conferring plant protection against insects, plant pathogens , and nematodes [113,145,252]. For example, SmartStax Pro® is a transgenic corn variety that expresses dsRNA targeting western corn rootworm Snf7 gene coding for an essential subunit of the endosomal sorting complex (ESCRTT-III). This effectively provides plant protection against root damage caused by WCR and has aided in overcoming Bt-resistant breakdown of SmartStax®, the parent product of SmartStax Pro® [145]. However, this approach is not feasible for most plant systems resulting from regulatory rules, variability in plant transformability and transformation protocols, genetic stability of the transformed plant, and public acceptance of genetically modified crops [114]. Furthermore, host-induced gene silencing poses a high risk of producing resistant insect populations because of the constitutive expression of the dsRNA by the plant cells throughout the growing season [82,114].

Spray induced gene silencing (SIGS) is an alternative approach to using dsRNA to control insect pest populations without incorporating heritable changes into the host-plant genome. Sprayable formulations of dsRNA can fast-track the development of new insecticides because it does not involve several years of developing and gaining regulatory approval of a

genetically modified crop [114]. However, limitations to the SIGS approach include a required repetitive administration of the product as the plant grows and rapid inactivation of the product via environmental degradation, which increases input costs to stakeholders. It also raises the issue of whether the dsRNA will remain stable for a period sufficient to control the target pest [114].

Ledprona, the active ingredient of a sprayable dsRNA-based insecticide (Calantha™) targeting Proteasome Subunit Beta Type-5 specific to CPB, results in significant mortality effects on larval instars [152]. This mortality effect was found to significant decreases in foliar damage on potatoes (*Solanum tuberosum*) resulting from CPB feeding in greenhouse and field trials relative to control [151,152]. In field trials, weekly applications of Ledprona combined with an adjuvant resulted in plant protection similar to that provided by weekly applications of Spinosad and chlorantraniliprole [151].

Currently, the products described above provide evidence of the effectiveness of dsRNA against beetle pests. However, this evidence is limited to two beetle species within Chrysomelidae (WCR and CPB), and plant protection has not yet been shown when using dsRNA to manage a broad diversity of beetles varying in ecology. Aspects of insect ecology such as host plant/patch fidelity, host range, and mobility may affect the efficacy of dsRNA in providing plant-protection in the field.

In this study, we sought to evaluate whether foliar sprays of dsRNA targeting an actin gene in *Popillia japonica* would provide similar plant protection to rose plants against feeding damage caused by this species. This sprayable formulation has been developed by Dr. Reddy Palli's lab and has been shown to produce gene-silencing associated mortality in-lab and caged plant assays. For this, we set up two experiments in two locations to evaluate 1) whether actin

dsRNA provides residual protection to roses from *P. japonica* feeding damage and 2) whether weekly applications of dsRNA can provide plant protection to rose plants.

3.2 Methods

3.2.1 Plant protection experiment, Belle Mina, AL

The first field experiment was initiated on 31 May, 2023 with the objective of determining whether foliar application of dsRNA could provide residual plant protection against adult *P. japonica* plant damage on roses. Six replicates of potted (three-gallon trade) knockout roses (*Rosa* × *radrazz* 'knockout') were established across two locations at the TN Valley Research Center, Belle Mina, AL. At each site, a central point was established for the irrigation system. Three transects were established at each location starting 5 m from the central point with a 90-degree separation between transects. Four plants were randomly selected and placed every 5 m along each transect (**Figure 1 A&B**). Two Japanese beetle food lures were deployed between the transects (**Figure 1A**). Plants were subsequently assigned randomly to one of four treatments and treated for two weeks. Treatment groups consisted of Actin dsRNA +NuFilm® P adjuvant, GFP dsRNA + NuFilm® P adjuvant, bifenthrin (Talstar P, 7.9% bifenthrin, FMC, Philadelphia PA), and a non-treated control group (NTC) (**Table 2**). Before applications, 10 mL NuFilm adjuvant (Miller Chemical & Fertilizer, LLC, Hanover, PA) was added to both dsRNA treatment groups. Bifenthrin, a commonly used non-systemic pyrethroid insecticide, was used as a positive control in the field assays.

On the second application date, we collected preliminary data. The following parameters were assessed for each plant: Number of blooms, abundance of beetles on the blooms and foliage, bloom damage, and leaf damage. Damage was assessed using double blind visual ratings

and the average of these ratings calculated. For blooms, each flower was separately rated for damage and an average bloom damage for each plant was calculated.

Table 2. Treatment groups used in the experiment, along with the amount applied in the field and the application rate. The field application of dsRNA is presented as a range because the concentration varies between 2-5 mg dsRNA/mL heat-killed *E. coli*.

Treatment	**Active ingredient**	**Amount applied**	**Application rate**
Talstar P	Bifenthrin	175 mL	21.7 mL /378.54 L water
Actin dsRNA	Nucleotide duplex	165 mL	2.0-5.0 mg/mL
GFP dsRNA	Nucleotide duplex	165 mL	2.0-5.0 mg/mL

Foliar damage by *P. japonica* (skeletonization) on rose is distinct. Each plant was visually assessed and both observers estimated the whole plant's percent defoliation [253]. Roses having <10% defoliation were rated as 5% defoliated unless the feeding was isolated to one leaflet. In those instances, plants were assessed as 1%. Preliminary data was collected before adult emergence and were considered zero. Preliminary data was collected on 31 May, and applications were made on 31 May and 6 June. Data collection at this site was on 5 July.

3.2.2 Plant protection experiment, Auburn, AL

A second experiment was conducted in Auburn, AL with the objective of evaluating whether weekly applications of dsRNA would provide plant protection to roses against *P. japonica* feeding damage. We conducted this experiment using established plantings of knockout roses (*Rosa* × *radrazz* 'knockout') on the Auburn University campus.

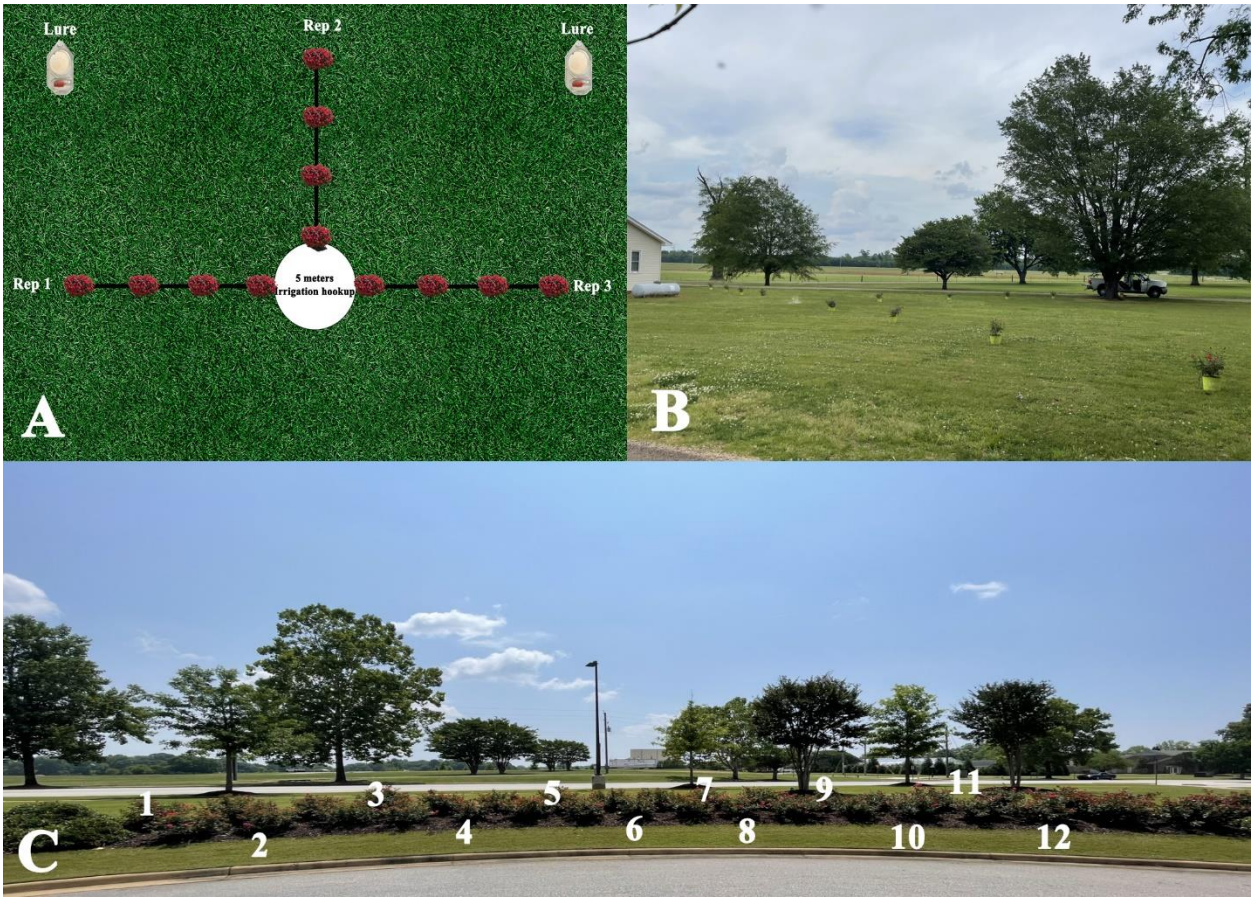


Figure 8. Figures depicting the experimental design and sites used for the field trials. A) A visual construction of the experiment in Belle Mina, AL using potted plants. B) Picture of one of the sites used in Belle Mina, showing the three transects used as replicates for the experiment. C) A picture showing the spacing of established rose plantings used for the experiment in Auburn, AL.

beetle feeding damage. We conducted this experiment using established plantings of knockout roses (*Rosa* × *radrazz* 'knockout') on the Auburn University campus. The average spacing between roses was 2.14 m, with all treated roses having at least 2 m spacing from one another. The rose plants were spaced enough that no plants were in contact with one another. There were two untreated plantings between each treatment group which served as a border **(Figure 1C)**. For this experiment, 5 replicate roses were used per treatment. The treatments and data collected were the same as outlined in section 3.2.1. Preliminary data was collected on June 21st, and data was collected each week prior to application for two weeks. Data collection was terminated at this site after two weeks because the beetle flight ceased.

3.2.3 Statistical analyses

All graphs were constructed using Graphpad Prism 8.4.2. All data analyses were performed using R statistical software (R Core Team (2023)). Leaf damage at the Belle Mina site was square-root transformed and analyzed using a linear regression analysis. The reported estimates for average leaf damage are back-transformed estimates. Beetle abundance at the Belle Mina site was analyzed using a generalized linear model with a negative binomial distribution to account for overdispersion in the data. Bloom damage and bloom number at the Belle Mina site was not analyzed because blooms were absent on half (12/24) of the experimental units.

Linear regressions were performed to test whether treatment, week, or their interaction predicted leaf and bloom damage at the Auburn site. We used generalized linear models to analyze bloom number and beetle abundance. For bloom number, we fit the data to a negative binomial distribution to account for overdispersion in the data with replicate considered as a random factor. Non-statistical significance (ns) was considered when $P > 0.05$.

3.3 Results

3.3.1 Plant protection experiment, Belle Mina, AL

After one-month post-treatment, few differences were observed between treatments at the Belle Mina site (**Figure 3**). The overall linear regression model for average percent leaf damage was significant (R^2= 0.342, DF=3, 20 F=3.465, P=0.046). Roses treated with bifenthrin resulted in a significant (23.34± 16.58%, P= 0.011) decrease in average leaf damage relative to the non-treated control roses. Roses treated with bifenthrin resulted in significant decreases in average leaf damage relative to actin dsRNA (8.70± 16.6%, P=0.041) and GFP dsRNA (13.83± 20.94, P=0.012) No other treatment group significantly differed from one another.

Data for rose damage and the number of roses were not analyzed as 50% (12/24) of the experimental roses did not have blooms. Roses that were not treated resulted in the numerically greatest mean percent bloom damage (26.67 ± 10.93), followed by GFP dsRNA (17.50± 7.50), actin dsRNA (16.25± 6.88), and bifenthrin (12.33± 5.36). Numerically, bifenthrin had the greatest number of blooms (4.33± 3.23), followed by GFP dsRNA (2.167± 1.52), non-treated control group (1.167± 0.65), and actin dsRNA (1.00± 0.45). Bloom damage was highly skewed. Out of 52 total blooms observed at this site, 20 of them occurred on a single bifenthrin treated rose bush.

Significant differences in the abundances of beetles on the rose plants were observed between treated and non-treated roses. Relative to the non-treated control roses, actin dsRNA treated roses had 0.14 (0.025-0.806: 95% CL, P=0.02) times the average number of beetles on the rose plants.

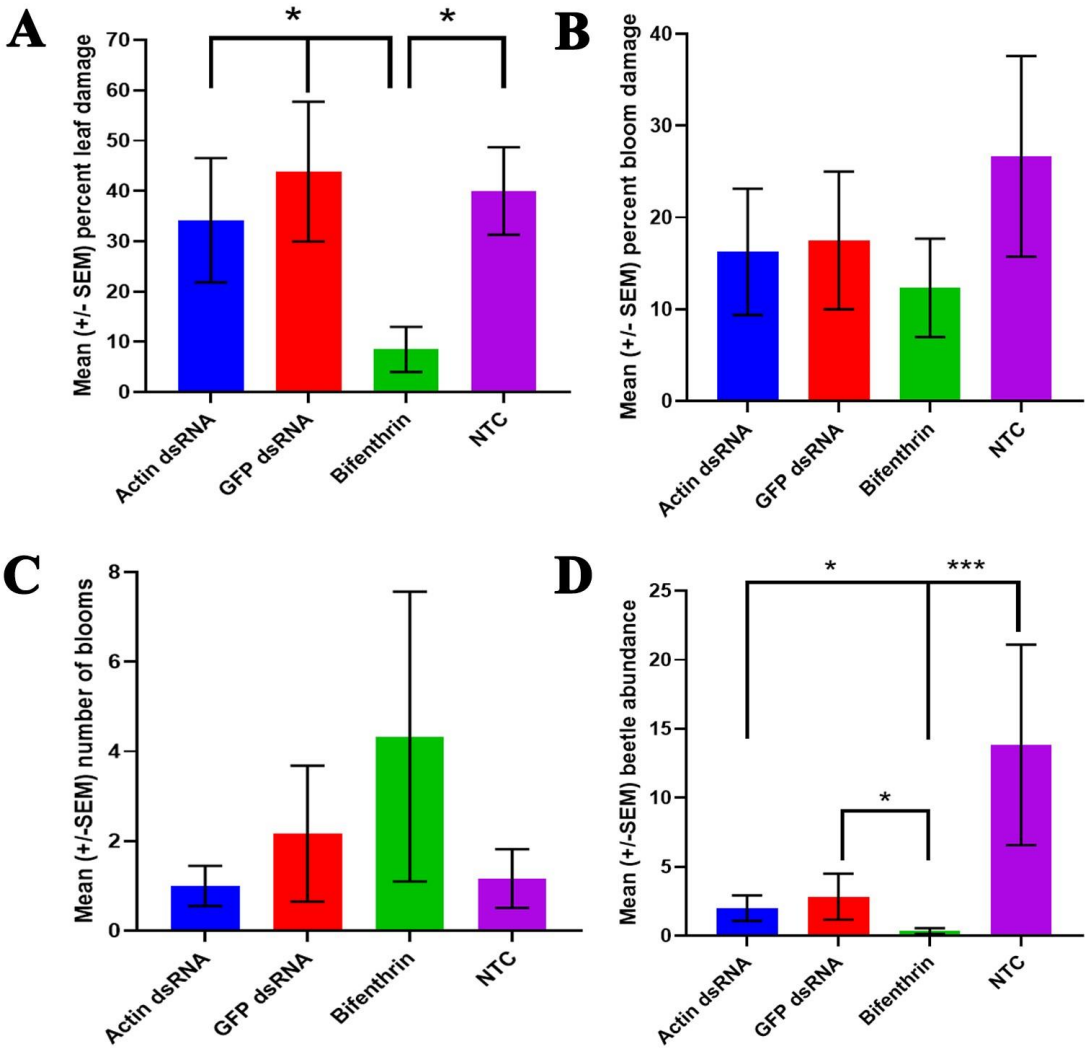


Figure 9. Results from the plant protection trial in Belle Mina. Graphs depicting percent leaf damage (A), percent bloom damage (B), number of blooms (C), and beetle abundance (D) one-month post-application of treatments. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ Non-statistical significance (ns) was considered when $P > 0.05$.

Bifenthrin treated roses resulted in 0.024 (0.002-0.182: 95% CL, P<0.001) times the average beetles observed on non-treated control roses. GFP dsRNA treated roses resulted in an average beetle abundance 0.21 (0.037-1.115: 95% CL) times that of non-treated roses, which was not statistically different from non-treated control roses (P=0.056). Bifenthrin treated roses had an average beetle abundance 0.117 (0.011-0.931: 95% CL, P=0.048) times that of GFP dsRNA treated roses. Actin dsRNA treated rose plants did not statistically differ from Bifenthrin nor GFP dsRNA in average beetle abundance.

3.3.2 Plant protection experiment, Auburn, AL

Overall, the amount of damage recorded on the blooms (4.77± 0.60) and the leaves (9.58± 1.09) was low (**Figure 4 A&B**). The overall linear regression model for leaf damage was significant (R^2= 0.317, DF=11, 48 F=2.029, P=0.046). No treatments resulted in significant differences in average leaf damage relative to the non-treated control group. Roses treated with bifenthrin resulted in a (4.6 ± 5.45%, P= 0.096) decrease in average leaf damage relative to the non-treated control roses. Average leaf damage significantly decreased with time. Average leaf damage significantly decreased by 5.8 ± 5.45% (P= 0.038) in week 1 and week 2 relative to preliminary counts regardless of treatment.

The overall linear regression model for bloom damage was significant (R^2=0.2575, DF=11, 48 F=2.029, P=0.046). However, no statistical differences in average bloom damage occurred between treatments and times. Rose plants treated with bifenthrin resulted in a numeric decrease (7.0 ± 10.083%) in bloom damage which is not statistically different from the non-treated roses (P=0.17).

No significant differences in beetle abundance were observed between treatment groups. In week 2, average beetle abundance was 0.25 (0.06-1.01: 95% CL, P=0.058) times the average abundance observed during preliminary counts regardless of treatment. The number of blooms significantly differed between time points, not between treatments. The average number of blooms recorded in week 1 and week 2 were 4.73 (3.15- 7.38: 95% CL, P=0.027, P<0.001) and 7.69 (5.21- 11.85: 95%CL, P<0.001) times the average bloom numbers observed during preliminary data collection, respectively.

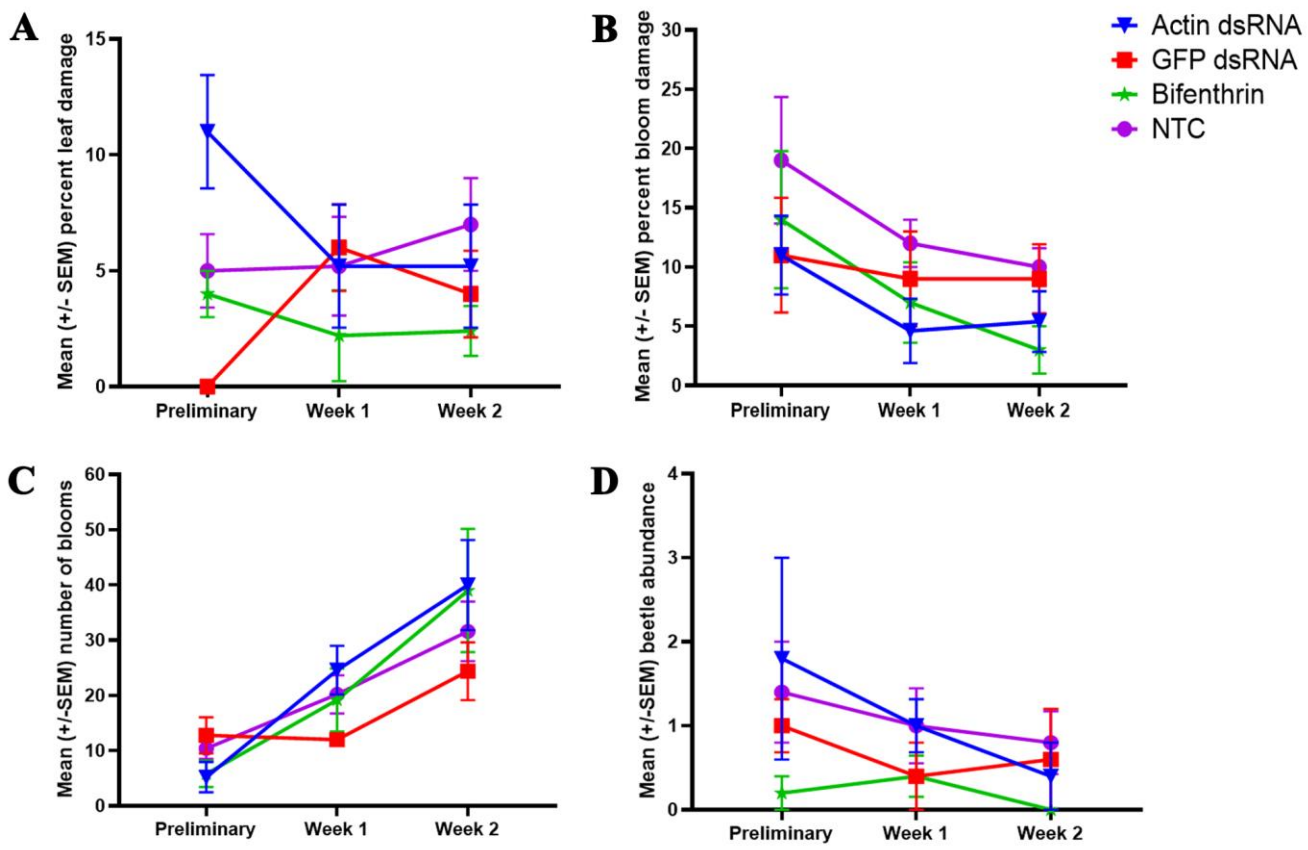


Figure 10. Results from the plant protection trial in Auburn. Graphs depicting mean percent leaf damage (A), mean percent bloom damage (B), mean number of blooms (C), and mean beetle abundance (D) over a three-week period.

3.4 Conclusions and discussion

The field experiments sought to evaluate whether foliar sprays of dsRNA targeting *Popillia japonica* actin would provide plant protection against *P. japonica* adult feeding damage. Bifenthrin was the only treatment that showed trends for decreased plant damage, particularly leaf damage, in both experiments. At the Belle Mina site, where beetle density was high, bifenthrin treated roses had leaf damage significantly less than all other treatments. Interestingly, we also observed that all treated roses resulted in decreases in the abundance of beetles on the plants relative to non-treated roses in Belle Mina. However, for actin dsRNA treated roses, the decrease in beetle abundance did not translate to lower average plant damage. This suggests that the inert ingredients may affect the ability of the beetles to pick up volatile cues emitted by the roses, but likely does not deter feeding of beetles once on the plant.

At the Auburn site, beetle pressure was very low, and no differences could be observed between control plants and any of the treatments. The maximum recorded number of beetles on a single rose plant was six and no beetles were observed on many of the plants, regardless of time point. It is hard to determine from the Auburn experiment whether weekly applications could provide plant protection in areas where overall beetle pressure is low. Future work should repeat this study over multiple years to determine whether foliar sprays of dsRNA could provide plant protection in regions with low beetle density, such as central and southern Alabama.

Overall, the results of our experiments suggest that foliar applications of actin dsRNA will not provide pant protection against feeding damage caused by adult *P. japonica*. Previous experiments have found that field applications of an exogenous dsRNA product, Ledprona, can provide plant protection to potatoes against damage caused by CPB [151,152]. However, there are key differences between the ecology of CPB and *P. japonica* that may provide insights into why

plant protection was not acquired in the field despite the mortality effects observed in lab assays and caged greenhouse assays.

First, CBP adults have a high host-plant fidelity and typically remain multiple days feeding within a patch [254]. Thus, individuals residing in a treated patch have a high probability of consuming a lethal dose of dsRNA. On the other hand, *P. japonica* retention time is much more ephemeral, and the rate of patch emigration and immigration is high [255,256] . Females will typically occupy a host plant in the morning and depart to oviposit eggs around mid-day, while males typically leave in the evening [255]. Males feeding habits are variable relative to females and they are often excluded in feeding assays. In natural settings, they devote a great deal of their time and energy competing for a limited number of females and mate guarding [255,257]. Patch emigration may have a huge, overlooked impact on the effectiveness of the dsRNA via individuals consuming a sublethal dose in a single feeding bout and emigrating to a new patch. In other beetle systems, chronic ingestion of dsRNA resulted in significant decrease in survivorship at a 10-fold lower concentration relative to acute feeding on higher concentrations of the same dsRNA [160]. This aspect of *P. japonica* adult ecology could not only contribute to the lack of effectiveness in the field but could also contribute to population resistance over time.

Although *P. japonica* retention time on a host is short, many individuals can aggregate at feeding sites during the day. Sexually mature *P. japonica* females cease producing a sex pheromone after their first mating event [258]. Adult beetles thereafter exhibit a strong positive chemotaxis response to a suite of plant volatiles that are released by the plant in response to feeding damage [259,260]. This could explain why we observed no differences in leaf and bloom damage at the Belle Mina site, where beetle density was relatively high. Initial feeding damage elicits the release of plant volatiles, recruiting new individuals. A significant amount of plant

damage could accumulate before the product produces a lethal phenotype. This may be partly a consequence of the lag-time observed between consumption of dsRNA and lethal effects resulting from systemic RNAi, which can take several days (often 6-10 d post exposure) [174,261]. This lag effect has also been observed for Ledprona, however, the susceptibility of CPB to the product decreases with developmental maturation and is likely most effective in early larval instars that feed much less relative to later life stages [151]. This suggests that although actin dsRNA may work effectively to cull a proportion of the population, the effects of volatiles on recruitment of new individuals may lessen the effectiveness of dsRNA as a plant protectant against adult *P. japonica*.

In summary, this work has pointed out the necessity to consider the ecology of the insect when trying to construct an effective RNAi management plan. The feasibility of plant protection conferred by dsRNA may be limited to species that have low patch turnover, weak mobility, are specialists, and have life stages that share a common host. With species that show none of these traits, such as *P. japonica*, objectives for RNAi-based management that focuses on local extirpation of the population over time will likely be more feasible relative to providing plant protection.

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