

Arthropod genomics research in the United States

Department of Agriculture, Agricultural Research Service: Applications of RNA interference and CRISPR gene-editing technologies in pest control

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ABSTRACT

The Agricultural Research Service (ARS) is the intramural research agency of the United States Department of Agriculture (USDA) which addresses basic scientific questions and develops applied solutions to a range of agricultural problems, and in doing so protects national food security and supports international trade. The damage to agricultural commodities inflicted by insects and other arthropod pest species causes a reduction in producer output and profitability, thereby affecting product quality, such that the development of novel and effective arthropod control tactics remains a research challenge

at USDA ARS. Additionally, USDA ARS conducts research into arthropod control within urban settings, where damage to dwellings, and ornamental and shade plants are of concern to homeowners and businesses alike. These goals of controlling pests must be balanced with environmental concerns, including the protection of pollinators and other beneficial species. The recent development of RNA interference (RNAi) and gene-editing technologies, such as Clustered Regularly Interspaced Short Palindromic Repeats and associated protein (CRISPR/Cas), opened new avenues for the development of novel arthropod control measures. Future RNAi applications

have the potential to increase the specificity and efficacy of pesticide treatments, as well as their environmental sustainability. In addition, gene-editing technologies like CRISPR/Cas provide researchers the means to generate stable genetic modifications within arthropods that facilitate both basic exploratory research, and support efforts to suppress arthropod pest populations using gene drives and other strategies. In this paper, the current translational research being conducted at USDA ARS using the application of RNAi and gene-editing to control arthropod pest species is reviewed, which includes broad-scope research encompassing arthropod pests that impact field and orchard crops, ornamentals, urban landscapes, and livestock production. These efforts and achievements by USDA ARS are contributing to improvements in agricultural production that benefits producers, the agricultural industry, and consumers, both domestically and abroad.

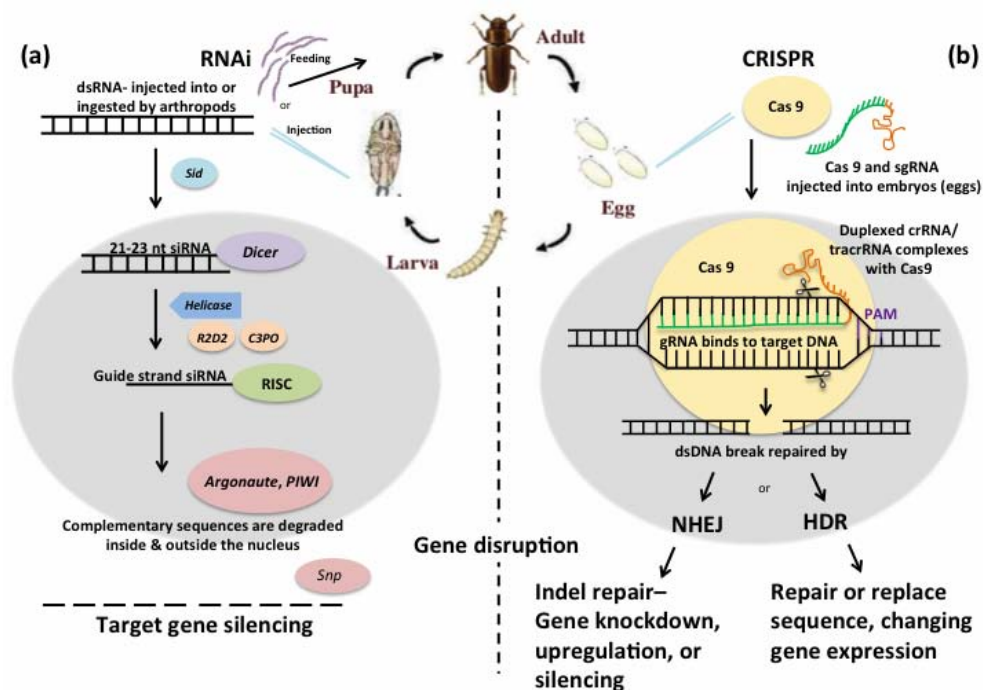
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1. Introduction

Significant challenges face agriculture in the 21st century wherein there is a need to supply quality agricultural products to support rapidly increasing global human populations without harming the environment. Furthermore, this must be accomplished while adapting to variable climatic conditions. By 2050, USDA estimates that production from the United States will contribute to feeding 9 to 10 billion people, worldwide. USDA ARS has always been highly committed to transforming agriculture in the United States to increase quality agricultural food availability, sustainability, and production capacity, while simultaneously lowering environmental impacts. Arthropod damage and disease transmission to plants, animals, and humans account for significant losses annually to agricultural production. Arthropods encompass a widely diverse and speciose branch of evolution that has adapted to inhabit seemingly every ecological niche, including genera that feed upon or cause damage to almost all agricultural commodities derived from plants and animals [1]. Arthropods are major vectors of plant and livestock disease agents and parasites, leading to infections that can dramatically reduce the efficiency of production or reduce commodity value. Similarly, insect feeding on crop plants reduces plant vigor and usually reduces the

marketability of plant products. Additionally, many arthropods are beneficial to agricultural production by pollinating crops, feeding upon invasive weeds, and controlling populations of pest insects. These beneficial insects are exemplified by the honey bee, *Apis mellifera*, a major pollinator across many different ecosystems [2]. This beneficial insect has experienced population reductions caused by Colony Collapse Disorder (CCD) and other factors [2, 3]. Therefore, managing the impact of arthropods, both beneficial and harmful, has been identified as a top agricultural research challenge for this century [4], and is being addressed by research conducted at the USDA ARS.

The reduction of feeding-damage to agriculturally important crop plants by pest arthropods remains a challenge in part due to the evolution of insecticide resistance to several different classes of chemical insecticides [5], and more recently, resistance to different *Bacillus thuringiensis* (Bt) protein toxins expressed by transgenic crop plants [6, 7]. Analogously, the exposure of *Rhipicephalus* ticks, the major vectors of southern cattle tick fever, to a range of commercial acaricides has led to resistance [8]. The development and judicious application of novel pest control technologies will speed ongoing efforts to counteract the development of insecticide resistance and ensure the stability of global crop production. Novel pest control applications have been spurred on by translational research that demonstrates that endogenous RNA transcripts of most eukaryotic organisms, as well as invading viral RNAs, are silenced *via* a sequence-specific cellular degradation mechanism. Specifically, the endogenous cellular RNA interference (RNAi) pathway utilizes short interfering RNAs (siRNAs) that function to hybridize by complementary base-pairing to bind and initiate a sequence-specific degradation pathway [9]. It is this interaction between the siRNA and target RNAs that provides the RNAi pathway a degree of specificity that allows the specific regulation of endogenous transcript levels and the degradation of invading viral-derived RNAs. As illustrated in figure 1, RNAi pathway-mediated degradation of RNA occurs through the use of a short guide RNA that is in association with the Argonaute protein of the RNA-Induced Silencing Complex (RISC) [10]. A wide array of encoded guide RNAs lead to their corresponding specificity towards the sense strand of targeted cellular RNAs, which subsequently initiates the degradation of these transcripts by the



Modified from: Lindsey C. Perkin, Sherry L. Adrianos, and Brenda Oppert.
Gene Disruption Technologies Have the Potential to Transform Stored Product Insect Pest Control. *Insects*, 2016 Sep; 7(3): 46.

Figure 1. Schematic of RNA interference (RNAi) (left panel) and CRISPR/Cas (right panel) technology in arthropods using red flour beetle *Tribolium castaneum* as an example. The gray oval represents the cell nucleus. On the RNAi side (a), double-stranded RNA (dsRNA) can be delivered by injection or by feeding. The dsRNA is incorporated into the cell *via* SID-1 orthologs. Dicers cleave the dsRNA into 21 nt pieces, and R2D2 and C3PO help load the RNAs into the silencing complex. Argonaute and PIWI endonucleases degrade the complementary RNA inside the nucleus while Snp (SNP) exonuclease degrades the complementary RNA outside of the nucleus. On the CRISPR side (b), Cas9 and a single guide RNA (sgRNA) are injected into eggs. Duplexed crRNA/tracrRNA complexes with Cas9 endonuclease, resulting in a precise dsDNA break. The break is repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR), which results in gene knockdown, upregulation, silencing, or changes in gene expression (Modified from Perkin, L. C., Adrianos, S. L. and Oppert, B. 2016, *Insects*, 7, 46).

endonuclease Dicer [11]. By these means, the RNAi pathway facilitates the effective post-transcriptional gene silencing of endogenous as well as invading RNAs, which respectively leads to the effective regulation of gene expression at the mRNA level [12] and protection against the harmful effects caused by infecting viruses [13].

RNAi-based suppression of mRNA termed “RNA knockdown” has garnered the attention of scientists and agricultural biotechnology companies, mainly due to the capability of RNAi to target and cause the degradation of specific transcripts that are essential for cellular and organismal survival [15]. Exploratory science has exploited RNAi-based transient mRNA knockdown as a laboratory tool for interrogating gene functions [16-18]. The control of some pest insect species is possible when cellular uptake of

RNAi molecules (dsRNAs; siRNAs) occurs following oral ingestion [19, 20]. This oral route of RNAi delivery led to the knockdown of target mRNA transcripts of a vATPase gene and caused mortality among larvae of the highly destructive corn pest, the western corn rootworm, *Diabrotica virgifera virgifera* [21]. The efficacy of oral-delivered dsRNA to *D. v. virgifera* larvae was also shown when insecticidal RNAi molecules were expressed by transgenic corn plants [22], for example the transgenic MON87411 that expressed insecticidal RNAi constructs that target the *D. v. virgifera Snf1* gene [23]. This transgenic RNAi causes a systemic silencing response that ultimately leads to the loss of cell integrity and high levels of larval *D. v. virgifera* mortality [24-26]. These and other research findings now open the possibility that RNAi

technologies could be harnessed as a novel class of insecticidal agents within the agricultural marketplace [19]. Control of target pests by RNAi is most effective when the insect species can directly ingest naked dsRNA [20], but oral delivery is not always effective among all arthropods [27]. Ongoing research is investigating methods to increase the efficacy among insects that are refractory to RNAi uptake [28]. Still other technological advances have increased the environmental stability of naked RNA molecules, which opens the potential for broadcast foliar RNAi applications [29].

While RNAi has been useful to study protein function in many insect species, the response is not robust in certain species, including such lepidopteran crop pests [27] as the codling moth [30]. Thus, alternative methods in the form of targeted genome-editing technologies are employed, including the application of zinc finger nucleases (ZFNs) [31], transcription activator-like effector nucleases (TALENs) [32, 33], and the CRISPR/Cas nuclease [34, 35]. The commonality among these methods is the generation of a nuclease-induced double-strand break (DSB) in genomic DNA which is acted upon by the ubiquitous DNA repair mechanisms, homology-directed repair (HDR) and non-homologous end-joining (NHEJ). NHEJ results in the disruption of DNA regions by insertion/deletion mutations (indels), whereas HDR introduces point mutations or integration of novel sequence *via* recombination between DNA at the cleavage site and an introduced donor sequence [36]. The CRISPR/Cas9 system has arguably become the more prevalent of these tools used for *in vivo* site-directed mutagenesis and is the focus here. The CRISPR system is involved in bacterial defense against invading viruses and plasmids, and functions by incorporating foreign DNA (protospacer) between endogenous CRISPR repeat sequences [37]. CRISPR repeat sequence-derived transcripts that include the integrated foreign protospacer DNA, are cleaved into CRISPR RNAs (crRNAs) that consist of the transcribed foreign DNA and a portion of the CRISPR repeat. Subsequent hybridization occurs between the crRNA and the *trans*-activating CRISPR RNA (tracrRNA) within the Cas9 nuclease protein complex [38]. The complementarity of the exogenous DNA-derived protospacer RNA provides sequence-specific targeting by the Cas9 nuclease, but only in instances where a protospacer adjacent motif (PAM) is present in

target DNA sequences. Adaptation of the *Streptococcus pyogenes* type II CRISPR system involves the simultaneous introduction of the Cas9 nuclease and a synthetic guide RNA (gRNA), where the latter is composed of a fused crRNA and tracrRNA that induces cleavage adjacent to a canonical 5'-NGG PAM [34, 39, 40]. These advances have led to successful genome editing within several lepidopteran species [41-49]. In most of these cases, gene mutation rates generated by injecting eggs with CRISPR/Cas9 components are relatively high (up to 90%), and these mutations are transmitted to progeny. The relative ease by which CRISPR/Cas genome editing generates gene knockouts provides a powerful tool for studying protein function in insects and other arthropods.

Indeed, the relatively recent emergence of RNAi and gene-editing has opened up new and exciting fields of investigative and translational research for arthropods. Research at USDA ARS is applying current RNAi and gene-editing methods, as well as developing promising new technologies based on these methods, that will arguably change the landscape of agricultural pest control by enhancing the efficacy, species-specificity, and environmental safety of pesticide treatments. The remainder of this review will focus on applied RNAi- and CRISPR-based research being conducted at USDA ARS that is contributing to the effective control of arthropod pests thereby benefitting stakeholders in the United States and worldwide.

2. RNA interference (RNAi) applications to elucidate arthropod gene function(s)

RNAi has been widely used to test gene function in a variety of organisms *via* reverse genetics [16, 17] including insects [18]. Much of the early arthropod RNAi work has been performed in the red flour beetle, *Tribolium castaneum*, a worldwide pest of stored grain products and a genetic model for Coleoptera and other stored-product pests at USDA ARS [Center for Grain and Animal Health Research, Stored Product Insect and Engineering Research Unit, Manhattan, KS]. A well-annotated and highly contiguous genome assembly for *T. castaneum* provides ideal resources for the investigation of gene function through RNAi knockdown, as demonstrated for several candidate genes using injected dsRNAs [50-52].

Candidate gene targets for stored-product pest control have included those in the *T. castaneum* gut which expresses the primary proteolytic enzymes cysteine and serine proteases [53]. Gene families encoding gut digestive proteases in *T. castaneum*, and other stored-product insects such as *Tenebrio molitor* mealworms, are highly duplicated, likely an adaptation to circumvent protease inhibitors present in grains and cereals [50, 54, 55]. These duplicated proteases are hypothesized to provide for more efficient hydrolysis of cereal proteins containing a large number of proline and glutamine amino acids [56-58]. A compensatory mechanism used by *T. castaneum* to overcome the deleterious effects of serine or cysteine protease inhibitors in cereal grains is the differential expression of inhibitor-insensitive proteases [55, 59, 60]. RNAi knockdown of the most highly expressed gut cysteine protease gene in *T. castaneum*, LOC659441, resulted in reduced transcript levels of the target as well as a cysteine protease paralog, and caused the differential expression of other cysteine and serine proteases in response. These results were analogous to compensatory responses to dietary protease inhibitors [61]. This research confirmed that dsRNA-based knockdown of LOC659441 could induce a feedback mechanism that reduces gut enzyme activity equivalent to protease inhibitors, such that digestion can be inhibited. The cysteine protease genes including cathepsin B, L, and O of *T. castaneum* may represent additional targets for RNAi-based pest control based on gut expression patterns [62].

Functions for the *T. castaneum* aspartate 1-decarboxylase (*ADC*), a gene involved in the cuticle-tanning pathway [63], were investigated at USDA ARS using a combination of RNAi knockdown and RNA-seq [64]. This dsRNA-based *ADC* transcript knockdown in larvae resulted in a black cuticle among *T. castaneum* adults instead of the wildtype red-brown. Corresponding RNA-seq data from knocked-down individuals confirmed a depletion of the *ADC* target transcript, and the differential expression of other genes including the significant up-regulation of the dopamine receptor 2 transcript. Adult *T. castaneum* beetles subjected to larval *ADC* knockdown (and increased dopamine receptor 2 expression) moved shorter distances and were slower compared to control beetles. These RNAi experiments and RNA-seq validation conducted at

USDA ARS uncovered a previously unknown connection between gene pathways, and may prove valuable for manipulating insect behavior.

RNAi also confirmed that the *cadherin* protein functions as a receptor for the Bt Cry3Aa toxin in *T. molitor* [65]. This function was demonstrated by the effective knockdown of *cadherin* transcripts following *cadherin* dsRNA injection into one-month old *T. molitor* larvae, and subsequent reduced mortality among Cry3Aa-challenged individuals as compared to controls. These studies demonstrate that RNAi is a useful tool to correlate genetic and biological function. The addition of differential gene expression analyses using RNA-seq data derived from treatment and control individuals can not only confirm target gene knockdown, but also demonstrate unanticipated impacts on gene-gene interactions and regulatory pathways.

3. Development and application of arthropod control measures (molecular biopesticides)

3.1. Arthropods of agricultural importance

3.1.1. Arthropods of cultivated crops

Greater than 10,000 arthropod species infest food, fiber, and biofuel crops worldwide, though only a small fraction are considered major pests [66]. Members of the insect orders Coleoptera, Heteroptera, Lepidoptera, and Thysanura, and arthropods such as mites in the subclass Acari are responsible for the majority of economic damage. For example, in the United States insect and mite pests of corn, cotton, soybean, and vegetable crops (e.g. potato, tomato, and eggplant) alone incur crop damages and management costs that exceed tens of billions of U.S. dollars, annually [67-71]. Traditional control measures based on foliar insecticide applications as well as transgenic Bt toxin have failed in many instances due to the evolution of insecticide resistance (see Introduction), such that research into alternative control methods has led to the development of technologies employing RNAi. Efforts spearheaded at USDA ARS focusing on the major orders of field crop pests are described below.

Lepidopteran pests: RNAi-based methods for the control of noctuid moth pests of cultivated crops is being carried out at USDA ARS labs [Southern Insect Management Research Unit (SIMRU), Stoneville, MS; Horticultural Crops Research Unit, Corvallis,

OR; Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, FL; Invasive Insect Biocontrol and Behavior Laboratory (IIBBL), Beltsville, MD; Biological Control of Insects Research Unit (BCIRU), Columbia, MO; Natural Products Utilization Research Unit, University City, MS; and Plant Science and Entomology Research Unit, Manhattan, KS]. Heliothine moths, particularly *Helicoverpa* and *Heliothis* species, are among the world's most damaging to major cultivated crops [72]. ARS research has focused on identifying potential RNAi targets and dsRNA-processing pathways in *H. virescens* (tobacco budworm) and *H. zea* (corn earworm) [73-75]. Pheromone biosynthesis activating neuropeptide (*PBAN*), first identified in *H. zea* at USDA ARS [76] represents a potential RNAi target [77] due to its regulation of multiple biological processes, including sex pheromone biosynthesis. Direct injection of *PBAN* dsRNA into female *H. zea* pupae induced mortality [78, 79], whereas dsRNA of the *PBAN* receptor (*PBAN-R*) resulted in decreased adult mortality and reduced sex pheromone production [80]. These results indicated that mortality was primarily from the failure of adults to emerge rather than developmental impacts, and *PBAN-R* signaling did not appear to be involved [78, 80].

USDA ARS researchers have also injected cytochrome p450 gene *CYP321B1* dsRNA into 5th instar *S. litura* (oriental leaf worm) larvae [81] and validated that transcript knockdown in the midgut and fatbody led to increased susceptibility towards chlorpyrifos (organophosphate), cypermethrin (pyrethroid), and methomyl (carbamate) insecticides. Due to the importance of chitin in forming the epidermal cuticle of insects, chitin isoforms have been proposed as potential RNAi targets [75].

Difficulties remain regarding the efficacy of RNAi among Lepidoptera (see Introduction). Silencing the tryptophan oxygenase (*TO*) by microinjection of dsRNA into *Plodia interpunctella* (Indian mealmoth) embryos [82] [USDA ARS Grain Marketing and Production Research Center, Manhattan, KS] resulted in only 2.9% knockdown (reduced levels of expression) and 1.9% knockout in spite of the high *TO* dsRNA concentrations used (~1 µg/µl), suggesting the potential lack of a dsRNA-processing mechanism. The RNAi and microRNA (miRNA)-processing pathway machineries are

present in many lepidopteran pests; this was shown for orthologs of the siRNA and piRNA pathway components *dicer-2*, *argonaute-2*, *argonaute-3*, and *aubergine* genes, as well as the miRNA-processing pathway genes *exportin-5*, *Drosha*, *DGCR8*, *loquacious*, *dicer-1*, and *argonaute-1*, that were identified in the *H. virescens* transcriptome. Other transcripts such as SID-1 and lipophorin that may facilitate uptake of dsRNA were also present [74]. However, the microinjection of *H. virescens* eggs with approximately 5 nl of 50 ng/µl membrane-bound alkaline phosphatase (*mALP*) dsRNA resulted in knockdown of mALP in 60% of 3rd instar larvae, but feeding the same mALP dsRNA at up to 4 µg/µl yielded inconsistent results. In contrast, transcripts of a chitinase gene were depleted by 63-64% in *Ostrinia nubilalis* (European corn borer) (Family: Crambidae) after feeding 10 µg of dsRNA [83] [Plant Science and Entomology Research Unit, USDA ARS, Manhattan, KS]. Similarly, collaborative research between USDA ARS SIMRU and the Department of Entomology, Louisiana State University reported the knockdown of three aminopeptidase genes in *Diatraea saccharalis* (sugarcane borer) that resulted in increased tolerance to the Bt Cry1Ab toxin [84].

Hemipteran pests: Mirid bugs (Heteroptera: Miridae) are polyphagous pests of economically important cultivated food, fiber, and seed crops [85]. Though they have historically been considered secondary pests, the wide-spread adoption of transgenic crops expressing *Bt* toxins by producers has led to a reduction in applied chemical-based control measures and a concomitant increase in mirid infestations. Additionally, reduced efficacy of control with traditional insecticidal chemistries has been reported in field populations of these insects [86-89]. These events resulted in the elevation of mirids to principal pest status in cotton and related crops [90, 91], and has sparked a renewed interest in the identification of novel targets for potential RNAi-based mirid control measures.

Although challenges persist in the development of RNAi methods for hemipteran control [92, 93], it has been applied successfully to elucidate *in vivo* gene function among mirid bugs. Transcripts targeted for knockdown to date have typically been involved in the critical biological processes of development, olfaction, or feeding. For example,

at USDA ARS [Pest Management and Biocontrol Research Unit, Maricopa, AZ] the injection of dsRNAs targeting the cytochrome P450 Halloween gene, Spookiest (*CYP307B1*), into *Lygus hesperus* (western tarnished plant bug) caused arrested nymphal development at the 5th instar, wherein stunted reproductive tissue development was observed [94]. In contrast, injected siRNAs targeting the A and B forms of the ecdysone (molting hormone) receptor extended developmental times between instars in *Apolygus lucorum*, and reduced the weight of 5th instar nymphs and increased mortality [95, 96]. Injecting dsRNAs corresponding to different coding regions of the *A. lucorum* juvenile hormone epoxide hydrolase, an enzyme linked to juvenile hormone degradation, likewise reduced survival and impaired molting [97].

Genes involved in mirid olfaction have also been targeted by RNAi, where siRNA injection-mediated knockdown of the olfactory receptor co-receptor (*ORCO*) in newly emerged *A. lucorum* adults resulted in significant dampened electrophysiological responses to a common plant volatile and a putative sex pheromone component [98]. Analogously, electrophysiological responses of *Adelphocoris lineolatus* (alfalfa plant bug) antennae to multiple compounds were likewise reduced following the targeted knockdown of an odorant-binding protein *via* injected siRNAs [99]. Genes involved in feeding have also been used as RNAi targets. Mirids use a cell rupture or lacerate-and-flush method of feeding in which proteolytic digestive enzymes are secreted into the ruptured plant cellular matter [100, 101]. Targeted knockdown in *A. lucorum* of two polygalacturonases which facilitate plant cell wall degradation and are the principal effectors of plant damage during feeding, *via* injected siRNAs reduced the level of damage in cotton flower buds exposed to salivary gland extracts [102]. Other mirid genes have been targeted for knockdown, including the *L. lineolaris* (tarnished plant bug) gene inhibitor of apoptosis [Biological Control of Pests Research Unit, Stoneville, MS] which causes an increase in mortality of both nymphs and adults [103], and in *A. suturalis* metathoracic gland desaturase that presumably is involved in sex pheromone biosynthesis [104], and an *A. lucorum* subunit of mitochondrial complex I (*NADH*: ubiquinone oxidoreductase) [105]. This latter gene was targeted using an engineered tobacco rattle virus to drive

dsRNA production in cotton leaves, which caused significantly higher *A. lucorum* mortality on leaves with the modified virus compared to wildtype virus alone.

3.1.2. Arthropods of specialty crops

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) was recently introduced into North America where it is an invasive agricultural pest of high-value, specialty, row, and staple crops, as well as an indoor nuisance pest. Since *H. halys* feeds by alternate salivation and ingestion with slow movement of stylets in a lacerate-and-flush feeding manner, scientists at USDA ARS [Invasive Insect Biocontrol and Behavior Laboratory (IIBBL), Beltsville, MD; U.S. Horticultural Research Laboratory (USHRL), Ft Pierce, FL] developed a new vegetable-mediated delivery method for dsRNA delivery that induces an RNAi response [106]. An analysis of transcriptomes from nymphal instars (2nd and 4th) and adults (female and male) identified transcripts differentially expressed among developmental stages and/or sexes, respectively [107]. Transcriptome analyses also identified genes associated with immune response to septic puncture, novel microbial entities such as a *Nosema* species present exclusively in imagoes and having a male-preferential expression pattern, as well as a novel iflavirus [108]. RNAi-mediated silencing transcripts occupying central locations in biosynthetic pathways that may be controlled by other precursors in the same pathway were targeted. For example, the depletion of juvenile hormone acid methyltransferase (*JHAMT*), which encodes a key enzyme in juvenile hormone biosynthesis, significantly diminished expression of farnesyl diphosphate synthase (*FPPS*) within the same pathway. Analogously, the silencing of *FPPS* significantly reduced the expression of *JHAMT*, and knockdown of both *JHAMT* and *FPPS* in combination exhibited greater effects. Additionally, depletion of *JHAMT* arrested molting from nymph to adult stages, eventually resulting in mortality of all the test insects [Ghosh and Gundersen-Rindal, 2017 (submitted)]. These RNAi-mediated depletion experiments against *H. halys* demonstrated effects on development and survival, and showed that gene-specific dsRNAs have the potential to be delivered orally and deployed in the environment as molecular biopesticides. The extension of this technology to other invasive insect pests is an ongoing research focus at ARS.

In a parallel project aimed at the harlequin bug, *Murgantia histrionica* (Hemiptera: Pentatomidae), a voracious pest of mustard and cole crops, USDA ARS [IIBBL] scientists analyzed various gene families involved in detoxifying xenobiotic compounds, which include chemical insecticides [109]. Knockdown of such genes may not eliminate the need for insecticide applications, but can potentially decrease the application rates needed for effective insect control and thus mitigate their negative environmental impacts. USDA ARS continues to interrogate transcriptomes of agriculturally relevant hemipteran pests, including bagrada bug, *Bagrada hilaris*; kudzu bug, *Megacopta cribraria* and neotropical brown stink bug, *Euschistus heros*. In addition to characterizing gene expression patterns, sampling over such a wide array of taxa will help to inform the specificity of gene target selection while minimizing off-target risks.

3.1.3. Arthropod vectors of virus-borne diseases in crop plants

The whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is one of the most notorious insect vectors of plant viruses. RNAi-based management strategies to control this pest are being developed by USDA ARS scientists [USDA ARS Vegetable Laboratory, Charleston, SC and Agricultural Research Station, Salinas, CA]. The whitefly is capable of transmitting hundreds of pathogenic viruses within the genera *Begomovirus*, *Crinivirus*, *Carlavirus*, *Ipomovirus*, and *Torrodovirus* to a wide range of agronomic and specialty crops (bean, cassava, cotton, cucurbits, pepper, sweet potato and tomato) grown in tropical and temperate regions throughout the world. *B. tabaci* consists of a cryptic species complex, each with varying host preferences and genetic differences [110]. USDA ARS scientists in collaboration with the Boyce Thompson Institute in Ithaca, NY developed the first genome draft sequence for the widely studied *B. tabaci* cryptic species MEAM1 (formerly B biotype) [111], which was followed by the draft genome of the closely related *B. tabaci* cryptic species, MED1 (Q biotype) by Chinese and USDA ARS scientists [112]. A global transcriptomic response to feeding on virus-infected versus virus-free plants by *B. tabaci* revealed differences in temporal gene expression patterns based on the

virus-infection, tomato plants infected with *Tomato chlorosis virus* (ToCV) [113] compared to *Tomato yellow leaf curl virus* (TYLCV) (Hasegawa *et al.*, unpublished). This information will assist scientists in understanding how the complex interactions between *B. tabaci* and their host plants facilitate or inhibit virus transmission. In addition, transcriptome data are being used to discover new targets to potentially induce *B. tabaci* mortality for RNAi-based control tactics. dsRNAs have been designed to silence critical single- and multi-gene targets in the whitefly using artificial diets and other types of feeding assay methods that test the efficacy of RNAi constructs in controlling adult whiteflies [114]. Genes involved in *B. tabaci* nymphal development are also being targeted using leaf-mediated delivery systems [115]. These RNAi constructs are being ring-tested at two USDA ARS and one collaborator location [Charleston SC and Salinas CA, and International Institute for Tropical Agriculture, Tanzania]. These efforts include selection of constructs for the effective control of *B. tabaci* MEAM1 and *B. tabaci* SSA1, the primary vectors of virus transmission to cassava in Sub-Saharan Africa. This strategy is aimed to reduce crop feeding damage and disease vectoring by *B. tabaci* populations across a wide range of crops. Some RNAi constructs have shown high levels of effectiveness against *B. tabaci* on tomato and cassava, and are being evaluated in agriculture systems using topical sprays, hydroponics, and transgenic plant expression. Furthermore, constructs for stable dsRNA expression are currently being developed for tomato and cassava transformation.

3.1.4. Arthropods of forest and landscape

The gypsy moth, *Lymantria dispar* (Lepidoptera: Erebididae), is one of the most destructive arthropod pests of hardwood forests and urban greenspaces worldwide. In North America alone, the costs of forest damage and control of gypsy moth are estimated at \$3.2 billion annually [116], and enhancing the control of this insect is a goal at USDA ARS (IIBBL). Lepidopteran pests have often been refractory to systemic RNAi, and for oral delivery specific midgut-associated targets were selected from among highly expressed midgut genes perturbed by *B. thuringiensis* infection in 3rd instar *L. dispar* transcriptome data [108]. Several gene candidates were also included from pupal

ovary tissue-derived *L. dispar* cell line IPLB-Ld652Y, historically used to study insect-virus interactions [117-120]. dsRNAs produced by bacterial plasmid expression or by *in vitro* transcription were delivered orally on artificial diet in larval feeding assays. Bacterial-expressed dsRNAs were found to significantly deplete expression of the targeted genes when fed to larvae, and ~60% reduction in body mass was observed following knockdown of two gene targets of an unknown function known as locus 365 and 28365 [121]. Adult female *L. dispar* resulting from development of larvae treated with locus 365- and 28365-specific dsRNAs had 50% reduction in egg mass compared to controls, or 60% when the two dsRNAs were stacked [121]. These experiments demonstrated that the depletion of novel gene targets, individually or in combination, caused knockdown of target transcripts in a sequence-specific manner and negatively impacted normal *L. dispar* physiology. Consequently, these specific RNAi-based molecular biopesticides may prove to be useful tools for specific control of pest arthropod populations.

The Asian longhorned beetle, *Anoplophora glabripennis*, is a coleopteran wood borer, and a significant global forest pest that has become established in North America, attacking both healthy and stressed orchard, ornamental and forest tree species. ARS-led genome sequencing [122] resulted in the identification of several gene families capable of degrading plant cell walls and detoxifying plant defensive chemicals. Functional characterization led to the identification of genes with the abilities to degrade cellulose, xylan, pectin, and xyloglucan, the main components of plant cell walls. Comparative genomic analyses involving 14 additional insect species revealed that many genes involved in plant cell wall digestion were horizontally-transferred from bacteria and/or fungi. RNAi-based targeting of these genes linked to key digestive and detoxification processes could ultimately provide novel tools for pest management against *A. glabripennis* and are currently under development.

3.1.5. Arthropods of urban areas

The effective control of urban insect pests usually requires the application of chemical pesticides, but economical, effective, and environmentally-safe management strategies are often lacking. RNAi-based control methods have recently been experimentally developed to target several urban

insect pests, including ants, termites, and cockroaches [123]. The red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), which infests more than 130 million hectares in thirteen Southern States in the United States and Puerto Rico, is one of the most difficult urban pests to control, and is spreading rapidly (https://www.aphis.usda.gov/plant_health/plant_pest_info/fireants/downloads/fireant.pdf). Current control methods rely heavily on chemical insecticides used in toxic baits or mound drenches, and these could hopefully be replaced or complemented by RNAi- or biologically-based control alternatives. RNAi has been explored at USDA ARS to control the fire ant using a variety of targets, including egg formation [124], neuropeptide hormone [78] and its receptor [125], glycoproteins [126], pheromone production [125, 127], and chemosensors [128]. These RNAi treatments cause various genotypic and phenotypic impacts in queens, worker adults, and/or larvae during developmental and adult stages. Since fire ants are social, the larvae require nurse workers to feed them, by regurgitating foodstuffs obtained from foraging and/or reserve workers. Experiments indicate that dsRNAs targeting *PBAN*, fed to nurse workers remain active following the subsequent regurgitation and transfer to larvae, as shown by larval mortality data (Figure 2) [78, 129]. RNAi-based control tactics

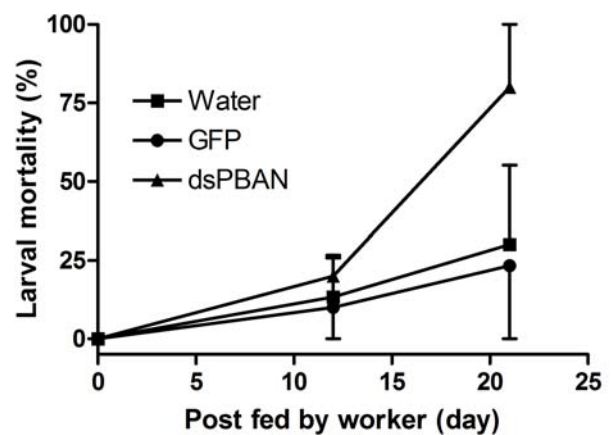


Figure 2. Mortality of fire ant worker larvae. Nurse workers were fed the fire ant PBAN dsRNA (1 $\mu\text{g}/\mu\text{L}$), GFP dsRNA (1 $\mu\text{g}/\mu\text{L}$), or water with 10% sucrose solution. Accumulated mortality was recorded 12- and 21-days post treatment. This figure was prepared by government employees as part of their official duties (Reproduced from Choi, M.-Y., Vander Meer, R. K., Coy, M. and Scharf, M. E. 2012, *Journal of Insect Physiology*, 58, 1159).

have also been investigated for the carpenter ant, *Camponotus floridanus*, where feeding dsRNAs targeting a peptidoglycan recognition protein gene led to the successful knockdown in the body of worker ants [130]. These examples show that RNAi induced by dsRNA feeding is effective in ants. Because food-based baits are readily available for social insects, effective control of fire ants and other urban arthropod pests may be achieved through RNAi-based methods using specific dsRNA-laced baits.

The Formosan subterranean termite, *Coptotermes formosanus*, is one of the most destructive pests in the United States, costing consumers over one billion dollars annually in home infestation prevention, remediation, and damage repair costs [131]. Termites are the only eusocial insects with caste-differentiated phenotypes such as worker, forager and soldier. RNAi-based methods for termite control were first investigated using two hexamerin genes, *Hex-1* and *Hex-2*, that are known to participate in the regulation of caste polyphenism in the eastern subterranean termite, *Reticulitermes flavipes* [132]. Hexamerin silencing resulted in significant downstream impacts on multiple members of a JH-responsive gene network which are involved in eusocial behavior [133-137]. RNAi targeting of several cytochrome P450 genes revealed that *Cyp15F1* has a role in JH-dependent termite caste differentiation [138-140]. Additionally, gram-negative bacteria-binding proteins (*GNBPs*), produced in response to a naturally encountered pathogen in subterranean termites, represents a potential RNAi target that could disrupt termite antifungal and pathogen defenses [141]. Functional studies also suggest that insulin signaling [142] and cellulose degradation [143] mechanisms may also represent potential gene targets for RNAi-based control of termites.

3.2. Arthropods of veterinary/medical importance (pests and disease vectors)

3.2.1. Arthropods affecting livestock

Arthropod pests are a significant threat to livestock health worldwide causing severe impacts on the efficiency of animal production, meat and hide quality, and food safety and security. Arthropod pests either directly damage their hosts or transmit (vector) diseases, some of which are zoonotic

(<http://onehealthinitiative.com/>). The costs of flies and ticks in livestock production systems, with respect to control and reduced animal health is in the billions of dollars annually in the United States alone [144]. USDA ARS conducts basic and applied research at multiple locations that is directed at reducing losses in animal production, and protecting animal and human health. Challenges facing traditional control methods include the development of insecticide resistance in many arthropod populations as well as concerns about residual pesticides [145, 146], such that alternative methods are being explored. USDA ARS research laboratories use RNAi and gene-editing technologies for functional genomics, vaccine development, to explore gene targets for development into novel pest control strategies, and to understand vector-pathogen interactions. USDA ARS laboratories [Knippling-Bushland U.S. Livestock Insects Research Laboratory, Cattle Fever Tick Research Laboratory, and Veterinary Pest Genomics Center, Kerrville TX, Screwworm Production Plant (Panama), and Center for Medical and Veterinary Entomology (CMAVE), Gainesville FL] use gene-editing and RNAi technologies in basic and applied research directed to control ticks (*Rhipicephalus microplus*, *R. annulatus* and *Amblyomma americanum*), mosquitoes (*Aedes aegypti*, *Ae. albopictus*), biting flies (*Haematobia irritans*, *Stomoxys calcitrans*, *Cochliomyia hominivorax* and *Phlebotomus papatasi*), screwworm (*C. hominivorax*), and house flies (*Musca domestica*). Efforts are underway to develop tick vaccines, transgenic male-only strains for screwworm eradication, and molecular genomic and bioinformatics tools for the purpose of improving animal protection and health [1].

R. microplus ticks were eradicated from the United States, but are endemic in Mexico and constitute a significant threat to United States cattle producers. A critical component of the Cattle Fever Tick Eradication Program aimed to prevent the reintroduction and reestablishment of *R. microplus* relies upon the dipping of imported cattle into tanks of the organophosphate acaricide, coumaphos. Acaricide resistance among ticks is increasingly widespread and often incompletely understood mechanistically [147]. Functional genomics using RNAi aided in the characterization of multiple acetylcholinesterase genes expressed in *R. microplus*

(BmAChE1, BmAChE2 and BmAChE3), and demonstrated that simultaneous silencing of all three BmAChEs produced significant tick mortality, whereas silencing of one or two showed no observable effects on tick survival [148-150]. These studies therefore demonstrated that the enzymes have functional complementarity *in vivo* [148-151], and led to a greater understanding of tick cholinergic system complexities [148-150, 152, 153]. Gene silencing by microinjection of dsRNA is possible in *R. microplus*, but is relatively slow and labor-intensive [153, 154]. Use of multiple short dsRNAs aimed at silencing the same target has been recommended to minimize off-target effects and validate the phenotypic effects of RNAi [155]. However, not all small dsRNAs directed at silencing a target gene are equally efficacious. An *in vitro* dual luciferase reporter system was developed for *R. microplus* cell culture which facilitates the efficiency of high throughput evaluation and selection of highly efficacious RNAi molecules; this further enables the rapid evaluation and selection of RNAi constructs, thus substantially reducing costs and time [154, 156].

RNAi has proven useful in studying dipteran pests of livestock as well. Researchers at USDA ARS [CMAVE] have applied effective ribosomal targets from mosquitoes to the house fly, *M. domestica*, which proved effective in preventing oviposition [Sanscrainte, N. D. *et al.*, 2017, submitted] and miRNAs in the hematophagous stable fly, *S. calcitrans*, have also been shown to be potentially effective targets for RNAi-based control [157]. A collaboration between USDA ARS [Arthropod-Borne Animal Diseases Research Unit, Manhattan, KS] and Kansas State University scientists used RNAi for the first time in the biting midge, *Culicoides sonorensis* (Diptera: Ceratopogonidae), an important vector of orbiviruses that cause hemorrhagic diseases in susceptible ruminants [158]. RNAi was used to confirm the function of genes involved in apoptosis that had previously been identified in a *de novo* transcriptome [159]. Midges injected with dsRNA targeting transcripts of the apoptosis 1 regulator, *IAP1*, (*dsIAP1*) showed significantly increased mortality compared to controls. Further, *dsIAP1* injected into the hemocoel silenced target genes in the midgut. The *dsIAP1*-induced mortality was attributed to a loss of midgut tissue integrity and increased caspase activity. RNAi

knockdown of the initiator caspase *DRONC* by co-injection partially attenuated the *IAP1*-knockdown phenotype, resulting in increased survival and moderate restoration of gut integrity [160]. These studies demonstrated a functional RNAi pathway in *C. sonorensis* and a technique that can be used as a tool to identify antiviral responses in the midge. The ultimate goal of these research programs is to elucidate genes underlying midge orbivirus vector competence using RNAi.

3.2.2. Invasive/biting arthropods

Shortly after the discovery of the RNAi mechanism, experiments were initiated to combat arthropod-borne virus transmission by mosquitoes [161], and to silence genes in the salivary glands of *Anopheles* mosquitoes with the goal of targeting malaria parasites [162]. Researchers realized the potential benefits of using RNAi as a species-specific agent to control disease-vectoring mosquitoes [163]. USDA ARS joined with the U.S. Department of Defense (Deployed Warfighter Protection Program) in 2007 for a research program investigating the feasibility of exogenous RNAi use to control adult mosquito populations. USDA ARS [CMAVE] researchers used genomic resources to identify putative critical target genes [164, 165], and partnered with private sector corporations to produce large quantities of dsRNA. Over 40 of these dsRNA constructs were screened against potential targets [166, 167], wherein the knockdown of two ribosomal transcripts (Becnel patent pending) resulted in the near elimination of mosquito ovarian provisioning and oviposition (Figure 3), and the effect was sustained through several blood meals [167].

3.3. Methods for delivery of dsRNAs to induce RNAi in arthropods

3.3.1. Delivery to agricultural pest arthropods

Regulation of harmful and conventional pesticides due to resistance and damage to the environment has reduced the use of many pesticides in the United States [169]. Although only a few molecules of dsRNA are essential to elicit a systemic RNAi response [12], the method used for delivery into arthropod cells is of utmost importance. To date, effective dsRNA delivery into insects has included microinjection [170, 171], ingestion [21, 106, 121], soaking/bathing [51, 168], and the use of carriers such as liposomes [172]. Microinjection of dsRNA

was used for the seminal experiments in the nematode *Caenorhabditis elegans*, followed by knockdown in expression of the frizzled genes in the fruit fly, *Drosophila melanogaster* [9, 173]. The use of direct dsRNA microinjection has become the standard for both fundamental and functional studies in arthropods, such as *A. mellifera* [174, 175], *Acyrtosiphon pisum* [176], *Blattella germanica* [177, 178], *H. halys* [179], and certain lepidopteran insects [27]. The advantages of microinjection includes accuracy and precision of dose for quantitative studies, but septic punctures have the potential to elicit increased expression of immune-related genes due to injection trauma [107] and furthermore injection is not a practical option for use in the delivery of biopesticides into the environment.

Systemic RNAi response to orally ingested dsRNA was first achieved in *C. elegans* [180, 181] and was followed by RNAi induced-mortality in *D. v. virgifera* through oral ingestion of dsRNA [21]. Even though lepidopteran insects are more refractory to RNAi, instances of dsRNA-mediated RNAi have been observed [27]. For example, injection of dsRNA targeting the *S. litura* midgut aminopeptidase gene (*slapn*) led to transcript knockdown and susceptibility to the *Bt* Cry1C toxin [182]. Recently, *in vitro* synthesized and bacterial-expressed dsRNAs stimulated an RNAi response and caused mortality when fed *per os* to gypsy moth (*L. dispar*) larvae [121]. Oral dsRNA delivery *via* solution or droplet feeding was effective in light brown apple moth (*Epiphyas postvittana*) [183], larval diamondback moth (*Plutella xylostella*) [184], honey bee (*A. mellifera*) [185, 186], and the Asian citrus psyllid (*Diaphorina citri*) [187]. Feeding of dsRNA *via* blood meal to tsetse fly, *Glossina morsitans morsitans* [188], or by soaking into paper disks for delivery into the termite *Reticulitermes flavipes* [137] has also been effective. USDA ARS research led to one of the first field trials with an RNAi product and demonstrated that the health of a beneficial insect, *A. mellifera*, could be improved when fed a dsRNA trigger to suppress a viral pathogen [186]. The dsRNA product (Remebee™-IAPV, Beeologics, LLC, Miami, FL), targeted the *Israeli acute paralysis virus* which had been shown to cause bee decline [185]. Delivery of the dsRNA within ~60% sucrose solutions

was incorporated into the standard beekeeper practice of feeding hives during the winter months. Subsequent studies demonstrated that bees fed diets containing dsRNAs targeting parasite genes reduced infections of the fungal endoparasite *Nosema*, [189] and the mite ectoparasite *Varroa*, [190] without any harmful effects on the bees.

Oral delivery to arthropods by ingestion of plants has potential as an effective method of delivery of selected insecticidal dsRNA(s) that target essential arthropod genes. Plant nuclear or chloroplast genomes can be altered to create transgenic plants that express and produce insecticidal dsRNAs for specific arthropod gene silencing, and could represent a viable method for crop protection [191, 192]. Oral delivery to arthropods by ingestion of insecticidal dsRNAs through feeding on non-transgenic plants has been explored as well. For example, researchers at USDA ARS [USHRL] demonstrated that citrus trees can absorb dsRNA through roots, *via* trunk injections, and foliar sprays [193, 194] (Figure 4a). The applied dsRNA was detected throughout the tree canopy, as well as within the phloem-feeding psyllid, *D. citri*, and the xylem-feeding glassy-winged sharpshooter leafhopper, *Homalodisca vitripennis*. Furthermore *D. citri* and *H. vitripennis* mortality increased when these pests feed on trees treated with dsRNAs against corresponding arginine kinase transcripts [171, 193]. USDA ARS researchers [IIBBL, Beltsville MD; USHRL, Ft. Pierce, FL] also devised a vegetable-mediated delivery method using green beans immersed in a solution of dsRNAs targeting *JHAMT* and vitellogenin (*Vg*) of the phloem-feeding *H. halys* [106] (Figure 4b). Depletion of *JHAMT* transcripts significantly retarded nymphal development to adult and ultimately caused mortality [Ghosh and Gundersen-Rindal, 2017 (submitted)]. This demonstrated that broadcast (topical) applications of dsRNAs are transported into the xylem and phloem, and are subsequently capable of producing a systemic RNAi response in plant-feeding hemipterans or other stem-dwelling pests [21, 106, 195, 196]. Furthermore, this non-transgenic plant delivery approach can last for weeks to months after application depending on initial concentrations of dsRNA used for treatment [106, 193]. The limitations of dsRNA delivery by ingestion or injection include the rapid degradation in arthropod salivary secretions and haemolymph by putative

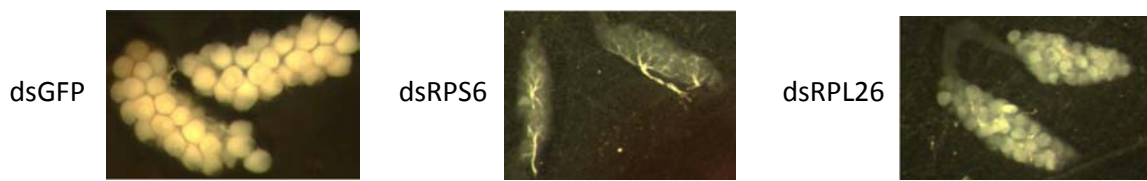


Figure 3. Ribosomal transcript targeting with dsRNA reduces oocyte maturation after a blood meal. Ovarian dissection at 36 hours after a blood meal showed differences in egg provisioning. Provisioning of eggs was proceeding as expected [168] for dsGFP-injected mosquitoes. By comparison, developing eggs in dsRPS6-injected and dsRPL26-injected organisms were delayed or reduced. DsRPS6 appeared to produce greater inhibition of egg maturation (Reproduced from Estep, A. S., Sanscrainte, N. D. and Becnel, J. J. 2016, *Journal of Insect Physiology*, 90, 17; <https://doi.org/10.1016/j.jinsphys.2016.05.001>; <https://s100.copyright.com/AppDispatchServlet?publisherName=ELS&contentID=S0022191016301056&orderBeanReset=true>).

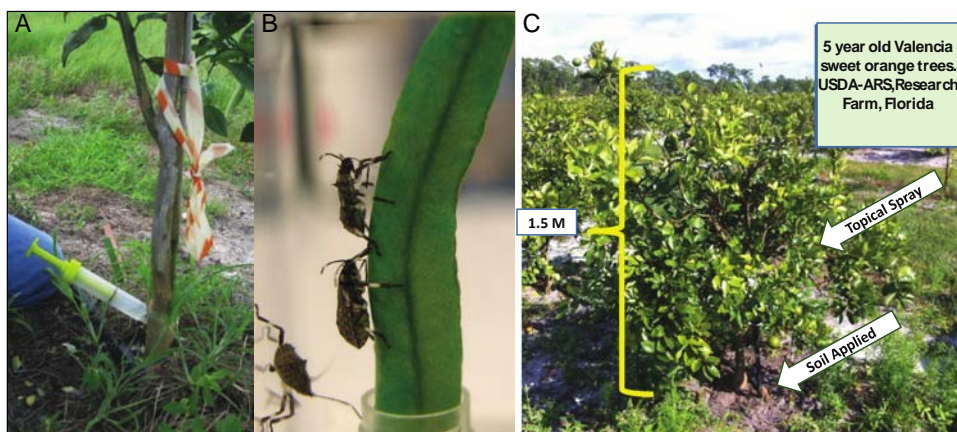


Figure 4. Methods for delivery of gene-specific dsRNA into plant tissue for insect feeding. (A) Tree trunk injection of a dsRNA solution specific to the Asian citrus psyllid, into citrus trees (Reproduced from Hunter, W. B., Hail, D., Tipping, C., Paldi, N. and Bextine, B. R. 2010, *Symposium Proceedings, Pierce's Disease Research Symposium, CDFA*, 24-27. https://www.cdfa.ca.gov/pdcp/Documents/Proceedings/2010_Proc.pdf); (B) a vegetable-based delivery system using a green bean segment soaked in dsRNA solution specific to the brown marmorated stink bug (Reproduced from Ghosh, S. K. B., Hunter, W. B., Park, A. L. and Gundersen-Rindal, D. E. 2017, *PLoS ONE*, 12, e0171861); and (C) soil applied to root zone directly and Topical Foliar sprays of trees and other plants also delivered dsRNA into plants and insects for up to 60 days post treatment.

RNases, as well as gut pH [28, 197-200]. The addition of polyethylene glycol (PEG) increased the efficacy of injected siRNA inhibiting hydrolysis by serum nucleases and absorption by serum proteins [201, 202]. For example, a complex of a 2% PEG and a high concentration of dsRNA efficiently silenced gypsy moth transcripts by oral delivery, but the drawback is the high dsRNA concentrations required to induce RNAi, presumably needed to overcome the negative effects associated with gut pH and nucleases [27, 121]. Concentrations of dsRNA for oral delivery must therefore be optimized for the target insect and different physiological environments.

Soaking insects or their cells with dsRNA in extracellular medium is promising for large-scale applications. Soaking was utilized to silence the *pos-1* gene in *C. elegans* [203], and by USDA ARS researchers [Honey Bee Breeding, Genetics, and Physiology Research Laboratory, Baton Rouge LA] to silence the *Am18w*-encoding Toll-like receptor in *A. mellifera* [51]. Soaking generally does not induce a robust RNAi response in comparison to microinjection, but can be used efficiently for insects cells [168, 203]. Clemens and colleagues (2000) used soaking to deliver dsRNA into *Drosophila* S2 tissue culture cells to inhibit Downstream-of-Raf1

(*DSOR1*) (mitogen-activated protein kinase kinase, MAPKK) subsequently preventing activation of the pathway [168].

Delivery of siRNA and dsRNA using carriers such as nanoparticles and liposomes imparts stability, and increases the efficacy of the delivered dsRNA [28, 172, 204-206]. A comprehensive review of patents of a new class of nanoparticle-based delivery vehicles for nucleic acids that can be used in both *in vitro* and *in vivo* therapeutic applications was outlined [207]. Since then, a study in *A. mellifera* demonstrated that knockdown of a targeted gene by an aerosolized siRNA-perfluorocarbon nanoparticle emulsion was possible *via* absorption through spiracles [205]. Using nanoparticles as a vehicle for dsRNA delivery has drawbacks such as poor solubility, hydrophobicity, or limited bioaccumulation [202], but the suitable choice of an appropriate polymer may outweigh these disadvantages.

RNAi is highly adaptable to agricultural systems where a short-term treatment duration is desired, or where other methods cannot easily be applied [22, 200, 208-210]. Aspects of RNAi such as the high degree of specificity and rapid degradation in the environment [106, 211] suggests that it may be an environmentally-sound approach to pest and pathogen management [196], but these properties also make effective RNAi formulations and treatments a challenge [22, 27, 28, 196, 212, 213]. The key to development of RNAi products is efficient delivery, efficacious trigger activity, and significant time of suppression, which require an understanding of the biology of the target pest or pathogen along with knowledge of agricultural industry practices. This provides for the development of suitable delivery methods and formulations [106, 121, 194, 206, 212, 214]. Further incorporation of protectants, whether bound by charge (peptide +/-nucleotide -), nanoparticle encapsulation, [44, 204, 215-217], or chemically bound to dsRNA can provide a product that can resist rapid degradation in arthropods when orally ingested, thereby improving penetration of the gut lining and facilitating entry into gut cells [29, 206, 218]. Research has demonstrated that RNAi treatments can be combined with attractants such as food, pheromone, or lights, and encapsulated in nanoparticles of various material composition, lipids, chitosan, virus, or directly applied, thus providing numerous approaches to deliver

control tactics aimed to manage arthropod pests or pathogens affecting livestock and agroecosystems.

3.3.2. Delivery to biting arthropod pests

In contrast to the wide variety of methods developed for the delivery of dsRNA to plant-feeding arthropod pests described above, corresponding delivery strategies for biting/blood-feeding arthropods are limited. Microinjection is well utilized and is the standard dsRNA delivery method for laboratory functional studies and for validating the efficacy of a particular gene target in blood-feeding insects. However, oral or topical delivery methods are required for practical biting arthropod vector control. Several groups have reported that the delivery of dsRNA constructs to larval mosquitoes using different novel strategies, such as microalgal or yeast *in vivo* expression systems for delivery [204, 219, 220]. USDA ARS researchers are currently working to develop methods and tools to assess the efficient delivery of dsRNAs as adult mosquito-specific pesticides. Difficulties with translocation of large hydrophilic nucleic acid polymers directly through a hydrophobic insect cuticle remain a challenge for dsRNA-based control of biting arthropod disease vectors [164, 165]. However, identification of effective carriers to assist in the oral uptake by adult mosquitoes is the focus of current research. Delivery of RNAi-inducing dsRNA to arthropod vectors *via* a sugar meal is an avenue of research that shows some success. Limited transcript knockdown was demonstrated by ARS scientists against adult *Aedes aegypti* mosquitoes by targeting vacuolar *ATPase* with a dsRNA-containing sucrose solution [166]. Successes in feeding complexed dsRNA to larval mosquitoes are currently being tested in adults to determine if a variety of chitosan or cationic agents would assist in uptake of a sugar meal. To address many of these issues a team of ARS scientists is developing antisense oligonucleotide-based gene-targeting technologies similar to RNAi, which show the capability to suppress targeted transcripts in tick, mite, and biting fly pests of livestock by oral delivery from treated blood meals (Hunter *et al.*, unpublished).

4. Beneficial arthropods

Much of the previous discussion involved protecting beneficial arthropods from the standpoint of “non-targets”. In this section, the use of RNAi research

to improve the health of beneficials is discussed. Beneficial arthropods perform valuable ecological services that enhance agricultural production. For example, *A. mellifera* and other pollinators are responsible for the pollination of nearly one-third of all crops and are vital to the success of food production, worldwide [221]. Predatory beetles, dragonflies, flies, lacewings, true bugs, and parasitic wasps are natural enemies which feed on herbivorous insects or mites, thus assisting in the suppression of agricultural pest arthropod populations within Integrated Pest Management (IPM) programs. Thus, supporting the health of beneficial insects from parasites and pathogens, including viruses, bacteria, fungi, and microsporidia parasites [222], as well as providing environmental conditions that are conducive to the survival of beneficial insects can enhance the efficiency of agricultural production. RNAi also is now being explored as a novel approach to support the health of beneficial arthropods through enhanced control of parasites and pathogens that can do beneficial insects harm. In addition, RNAi has also been widely used to study gene function to understand the biology of beneficial insects and their host-pathogen interactions, and are actively being investigated at USDA ARS.

RNA or DNA viruses and microsporidia cause many of the diseases that afflict beneficial insects, such as the *deformed wing virus* (DWV) and *Nosema* in *A. mellifera* populations. Research at USDA ARS [Bee Research Laboratory (BRL) Beltsville, MD] demonstrated that *A. mellifera* can ingest dsRNAs or siRNA that are complementary to DWV, *Israeli acute paralysis virus* (IAPV), and Chinese sacbrood virus (CSBV), and subsequently leads to the significant reduction in virus titres within infected bees [223, 224]. Additionally, research led by USDA ARS BRL showed that RNAi targeting the internal ribosomal entry site (IRES) or the virus-encoded protein, viral suppressor of RNA silencing (VSR), of IAPV leads to significant reductions in virus titres, and that the nucleotide conservation among VSR regions of multiple viruses suggest possible cross suppression by therapeutic RNAi molecules [223, 224].

Nosema ceranae is an intracellular microsporidian parasite of *A. mellifera*, and affects honey bee health in many ways and often has been implicated in colony declines. USDA ARS BRL was involved

in the sequencing of the *N. ceranae* genome [225]. The primary site of *N. ceranae* infection is in the *A. mellifera* midgut, and RNAi targeting the *N. ceranae* ADP/ATP transporter showed some success in reducing the number of spores found in the honey bee gut [189]. Interestingly, it is also possible to silence genes of the ectoparasitic mite, *Varroa destructor*, which is the single most detrimental pest of *A. mellifera* by causing damage by direct feeding and the vectoring of multiple viruses. Research demonstrated the significant reduction in the number of *Varroa* mites within honeybee colonies by suppressing a number of *Varroa* housekeeping transcript targets *via* RNAi [190]. These methods being developed at USDA ARS are aimed to specifically target pathogens and thus support the health of beneficial insects.

5. Application of genome editing CRISPR/Cas technologies to arthropod control

Novel technologies aimed to control the damage caused by arthropod pest species in agriculture, being developed at USDA ARS also involve the application of gene-editing. The advantage of gene-editing resides in the fact that it results in stable and heritable changes in the genome of arthropods, which contrasts with the more transient nature of RNAi-based insect control and its limitations (exposure, uptake, and dosage issues). Additionally, gene-editing generates permanent alterations within arthropod genomes that assist in basic research aimed to elucidate biochemical functions or applications in novel control tactics. USDA ARS research conducted within these areas directed towards future insect control technologies are highlighted within the following subsections.

5.1. Applying gene-editing technologies to understand chemoperception in orchard pests

The relative ease of CRISPR/Cas9 genome editing for generating gene knockouts provides a powerful tool for studying gene functions in Lepidoptera (see Introduction). Functional genomics research at USDA ARS [Temperate Tree Fruit and Vegetable Research Unit (TTFVRU) Wapato, WA] is directed towards the identification of genes and gene products that can be used in targeted RNAi-based control of the codling moth, *C. pomonella*, a worldwide insect pest of apple, pear and walnuts. The USDA ARS TTFVRU, in collaboration with

academic colleagues, focuses on understanding the lepidopteran chemosensory system [226-231], which serves as an interface for the insect and its chemical environment [232]. Semiochemicals, including pear ester, a plant-derived kairomone, and codlemone, the main sex pheromone component, have greatly enhanced codling moth-monitoring and control in the orchard [233]. Additionally, the use of codlemone for mating disruption within IPM strategies has greatly reduced the amount of insecticide required to achieve effective control of this pest [233].

Odorant receptors (ORs) are the key detectors for volatile compounds in an insect's environment, and activation of ORs can lead to behavioral responses. Through analysis of transcriptome data, ORs expressed in *C. pomonella* antennae have been identified [231, 234], and at USDA ARS TTFRVU these OR genes are being targeted by CRISPR/Cas9 genome editing in attempts to identify associated behavioral changes. The *C. pomonella* OR1 gene (*CpomORI*) is the most highly expressed transcript in the antennae of adult males, and is hypothesized to be a receptor for codlemone [231]. Editing of the *CpomORI* using CRISPR/Cas9 resulted in the non-response of 17% of edited adult *C. pomonella* G0 adult males, arising from embryos initially treated with the CRISPR/Cas9 system, to high doses of codlemone in flight tunnel bioassays. In attempts to obtain more robust responses (or lack of response), G0 edited males and females were mated to generate progeny for flight tunnel testing. Unexpectedly, edited females produced fewer eggs and the eggs were inviable, which provided a putative physiological role for *CpomORI* in females [49]. This suggests that caution may need to be exercised within gene-editing experiments to account for unexpected impacts on cellular systems and insect physiology.

5.2. Creation of a temperature-sensitive sex-determination mutation by gene-editing

The sterile insect technique (SIT) has proven to be one of the most effective biologically-based population-suppression methods to control insect pests that impact agriculture and human health [235]. The SIT is based upon the premise that the release of an overwhelming number of sterile males into an insect population over successive generations will render females effectively non-reproductive,

and thus lead to the suppression or eradication of the targeted pest species [236]. Technical challenges of the SIT require the capability to mass-rear and release large numbers of sterile, though sexually active, pharate adult males. For species where sex separation is not possible, females may be sterilized and released along with males, although this alternative is more costly and inefficient, especially in some instances where females may require higher radiation doses. To address these inefficiencies, it has been a long-term goal of USDA ARS to use genetic manipulations to create sexing strains where females are eliminated and the remaining males are sterile [237].

The sex-determination gene pathway has been elucidated in *D. melanogaster*, wherein null mutations for two genes result in the default state of chromosomal XX females developing as sterile phenotypic males. Functional cognates of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes have been identified in the medfly (*Ceratitidis capitata*) [238], the caribfly (*Anastrepha suspensa*) [239], and other *Anastrepha* species [240]. Notably, the functions of *ds-tra* and *ds-tra-2* have been validated by RNAi in medfly and caribfly. Chemical-induced conditional temperature-sensitive mutants of *tra-2*, *tra-2^{ts}*, are expressed in *D. melanogaster* at permissive temperatures below 18 °C, but not at temperatures of 29 °C or above [241, 242]. Thus, insect growth at the restrictive temperature results in XX chromosomal females developing as phenotypic males (or pseudo-males) that are sterile, along with XY chromosomal males that develop normally but are also sterile.

At USDA ARS [CMAVE] work is progressing to develop temperature-sensitive sexing strains of fruit flies [237]. To test the proof of principle that a *tra-2* knock-out may result in sterile XX and/or XY individuals among tephritids, CRISPR/Cas9 homologous recombination was used in the spotted wing drosophilia, *D. suzukii*, to replace the native *Ds-tra-2* gene with a mutated sequence along with an insertion of the *IE1hr5-DsRed* marker gene [243, 244]. The resulting missense point mutation resulted in a Proline to Serine substitution known to cause the *tra-2^{ts2}* mutation in *D. melanogaster* [245]. Homozygous *Ds-tra-2^{ts2}* gene-edited mutant females developed normally at 16-20 °C, but as sterile intersexuals at 26 °C having a predominantly male phenotype. These chromosomal XX females

have male foreleg sex combs, posterior tergite pigmentation, male genital structures, and wing spot pigmentation. In contrast, the XY *Ds-tra-2^{ts2}* flies developed as normal, albeit sterile, males at 26 °C. The downstream influence of *Ds-tra-2* protein on terminal sexual differentiation was also evident by temperature shifts during adulthood, in that either activated or suppressed female-specific *Ds-Yolk protein 1* transcription in XX; *Ds-tra-2^{ts2}* pseudo-males shifted to either 16 °C or 29 °C after eclosion, respectively, that was similar to *tra-2^{ts2}* in *D. melanogaster* [246].

Experiments at USDA ARS demonstrated the ability to conditionally regulate sexual differentiation and fertility by *Ds-tra-2* gene-editing, and provided proof of principle for the use of editing in applications that create sterile males-only strains for SIT. This could include other insects that have a conserved *tra-2* cognate sequence and function, which currently include tephritid, lepidopteran, and hymenopteran species [240]. Beyond the applications for conditional gene expression, gene-editing also has the potential to create a broad range of temperature-dependent conditional alleles for fundamental genomic analyses.

5.3. Targeted mutagenesis to develop novel genetic sexing strains in tephritid pests

The currently mass-produced SIT genetic sexing strains (GSS), such as the Vienna-7 and Vienna-8 strain of *C. capitata* and the Tapachula-7 strain of *A. ludens*, were created *via* random mutagenesis using ethyl methanesulfonate (EMS) treatment [247, 248]. GSS traits are based on gamma irradiation-induced balanced translocations between the autosomal gene(s) causal of the desired phenotype and the Y chromosome, with examples generated in *Bactrocera dorsalis* and *B. cucurbitae* [247, 249-251]. EMS and irradiation treatments are effective at generating the desired sex-linked and conditional lethal mutations, but also cause background mutations throughout the genome that are potentially detrimental to individual fitness [252]. Future work in developing classical GSS-based SIT strains will require targeted mutagenesis which can be achieved using standard transgenic techniques such as piggyBac transformation, which was demonstrated in *C. capitata* and *A. suspensa* [253, 254]. Additionally, CRISPR/Cas gene-editing techniques are currently being explored in

Tephritidae. To accomplish this, USDA ARS [Tropical Crop and Commodity Protection Research Unit, Hilo, HI] generated *B. cucurbitae* genomic resources necessary for future work in the development of SIT programs [251]. This includes gene annotation, placement of the assembly into a chromosomal context, and comparative genomics with model insect systems. The genome and gene set generated through various techniques are made available through the online web-tools that serve as a resource for CRISPR guide RNA design. Overall, this provided a strong foundational resource for functional genomic studies in this previously understudied species and other tephritid fruit flies. Genes associated with GSS traits in existing strains are being characterized using genomic methods, and upon validation in wild type strains using various knock-out techniques, are transferable to new species upon identification of one-to-one functional orthologs. The subsequent generation of balanced translocations between the autosome and Y chromosome, and the establishment of sex-linkage can be accomplished using gene-editing technologies. Through the work of USDA ARS, orthologs have been assigned between tephritid species and the model species *D. melanogaster*, which opens the potential for applying the entire suite of conditional lethal genes identified in *D. melanogaster* for use in targeted mutagenesis of tephritid pest species.

6. Non-target considerations

The efficiency of United States agricultural production is continually being improved by the development of novel and more efficacious arthropod control methods, but this requires the use of sustainable practices and methods that ensure ecological safety and environmental stewardship. Risks to humans and other vertebrates by pest-specific products are anticipated to be low [193, 255]. USDA ARS has contributed to the evaluation of potential and realized risks posed for non-target organisms exposed to toxic insecticidal compounds. Recently, research at USDA ARS [Corn Insects & Crop Genetics Research Unit (CICGRU), Ames, IA] has focused on the susceptibility of non-target arthropods with respect to transgenic Bt crops [256, 257]. These efforts acknowledge that maintaining a diverse and balanced agroecosystem supports populations of pollinators and natural

enemies that positively benefit crop production [258]. Environmental risk assessments for non-target invertebrates include aspects of insecticidal toxicities and potential routes of exposure [259, 260] and the effects of chronic as well as acute exposures [261, 262]. Risks toward non-target arthropods from traditional chemical insecticides and Bt crops have also been investigated by USDA ARS CICGRU and collaborators within a framework that includes initial bioassay assessments of toxin susceptibilities across a set of “indicator species” [263, 264]. This USDA ARS research provided criteria for the selection of these “indicator species” and provided methods for evaluating abundance within the relevant agroecosystem [265]. This research has led to the current set of safeguards implemented by the agricultural biotechnology industry that aim to protect populations of beneficial insects.

Potential risk to non-target arthropods by insecticidal RNAi encompasses aspects of RNAi exposure, uptake, and intracellular liability to mRNA knockdown. Specifically, temporal and spatial aspects of delivery dictate arthropod exposure risks, and environmental RNAi uptake into cells varies across targeted and non-targeted species, such that realized intracellular silencing of transcripts (both unintended silencing of orthologous transcripts and off-target effects) could be highly variable. These uncertainties create a challenge for understanding environmental exposures, uptake pathways, and levels required to evoke unintended effects, as well as for developing an ecological risk assessment framework [266]. Since the cellular RNAi pathway appears to be conserved across insects, the potential for adverse responses by non-target arthropods remains relatively unknown.

Tiered testing of the western corn rootworm *D. v. virgifera* *DvSnf7* RNAi by industry research groups demonstrated that mortality is induced among species in the subfamily Galerucinae in the order Coleoptera (beetles), but showed no observable effects on the growth or development of a small number of hemipteran (true bugs), hymenopteran (bees and ants) and lepidopteran insects (moths and butterflies; [267]). Similar results demonstrated that a *D. v. virgifera* vATPase RNAi had no effect on the levels of orthologous transcripts within the *A. mellifera* [268]. USDA ARS has provided

methods for evaluating non-target exposures that use molecular markers for cell stress response mechanisms as bioindicators that assist in developing effective safeguards [269]. Furthermore, USDA ARS [Biological Control of Insects Research Unit, Stoneville, MS] has investigated methods to predict putative molecular off-targets of insecticidal RNAi molecules, such as the *DvSnf7* RNAi, within transcripts from the beneficial lady beetle, *Coleomegilla maculata* [270], and such genomic sequences for non-target arthropods provide resources for computational prediction of potential unintended impacts of ingested RNAi on non-target species. This research suggests the risk assessments based on sequence homology may become feasible for insecticidal RNAi, but recent literature demonstrated that a soil collembolan fed dsRNA based on the gene used in MON 87411 exhibited no adverse effects despite of predicted sequence homologies [271]. This likely indicates that aspects of degradation, absorption, and temporal expression of transcripts may also need to be considered when assessing risk. Thus, these putative risks predicted at the molecular levels must be combined with corroborating empirical bioassay data. RNAi technologies hold great promise for developing safe and highly specific insecticidal agents, but sound research-based assessments of any risks to non-target species will undoubtedly be a key component of the continued environmental stewardship practiced within the U.S. agricultural industry.

7. Conclusion

The past use of nonselective broadcast chemical insecticide approaches to arthropod control has contributed to environmental damage and the development of resistance traits in arthropod populations. Additionally, IPM practices and application of biological control measures suffer from difficulties in timing of applications and high management costs. Thus, new methods for management of agricultural pest and disease-vectoring arthropods are needed. At the same time protection of beneficial insects responsible for pollination and biological control is essential for sustainable agricultural production and environmental stewardship. In light of these challenges, agriculture and farming have become increasingly data- and technology-driven. Molecular biotechnologies allow

genetic improvement, regulation, and modification across a wide range of arthropods and organisms, and have become increasingly important tools for understanding arthropod biology and gene functions. In order to understand insect biology and fully utilize gene-editing technologies for the improvement of agricultural production, an increase in the number and scope of arthropod genome, transcriptome, and proteome resources are needed for pest, disease vector, and beneficial arthropods. To these ends, USDA ARS has championed arthropod genome and community-based annotation projects as well as data curation and maintenance: the i5K Workspace residing within the U.S. National Agriculture Library is a repository for a variety of arthropod genome sequence data, assembly, and annotation projects (i5K Initiative). The arthropod reference sequences and associated functional gene annotations provided by these projects are tremendous resources for the broader research community [1, 272]. Advances in systems approaches to genomic data analyses and Big Data infrastructure have been made through USDA ARS investments in SCINet, the internal computational hub of the agency. SCINet resources encompass large computational capacities, tailored applications and bioinformatics pipelines, personnel training opportunities, and high-speed data transfer capabilities that will enable the delivery of USDA ARS technologies to many stakeholders.

In addition to increasing arthropod genome availability, extensive experimental and predictive computational research is needed to increase the precision and specificity of RNAi-targeting among arthropods. In future development of technology-based molecular biopesticides, sustainable formulations that provide protection of dsRNAs (or other products) from degradation and efficient entry and absorption in arthropods are needed, as well as improved delivery methods [29, 44, 204, 206, 215-218]. Regulatory and biosafety issues associated with manipulating arthropod genomes through gene-editing technologies must also be addressed in order to safely use these technologies in the environment. Agricultural production provides humans access to a stable, plentiful, and nutritious supply of food, and research that leads to improvements in this endeavor is pivotal for present and future efforts to promote human health. Arthropod control using RNAi-based knockdown

and CRISPR gene-editing approaches has the capacity to improve agriculture in the United States and globally by lowering management costs, increasing production efficiency, enhancing food quality, and ensuring the stability of the food supply. The technologies outlined within this review, as well as yet unknown future advances, are set to have major positive impacts on plant, animal, and human health. USDA ARS remains committed to providing substantial improvements in agricultural practices that benefit American consumers, farmers, and the agricultural industry. The basic/translational and applied agricultural research conducted therein has and will continue to contribute practical applications that solve critical agricultural problems of national and international importance.

DISCLAIMER

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA ARS for its use. USDA is an equal opportunity provider and employer.

CONFLICT OF INTEREST STATEMENT

Authors claim no conflicts of interest.

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