University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

Identifying Effective Genes as Targets for RNA Interference in Sustainable Pest Control Strategies

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ARTICLE INFO

Article type:
Research Article

Article history:
Received:5 December 2024
Revised:9 January 2025
Accepted:20 January 2025

Keywords:
Insect,
Gene silencing,
Pest control,
RNAi,
Target genes.

A B S T R A C T

RNA interference (RNAi) is a powerful genetic tool that has revolutionized pest management by enabling targeted silencing of specific insect genes. By introducing double-stranded RNA (dsRNA) to target genes, RNAi can lead to phenotypic changes, reduced fitness, or even mortality in pest species, providing an alternative to chemical insecticides chemical insecticides. However, the efficacy of RNAi is highly variable across different insect orders and target genes. RNAi could target specific genes involved in an insect's critical biological processes, such as growth, immunity, digestion, reproduction, and metabolism. This study reviews the potential of RNAi-based pest management strategies by focusing on key genes from various insect orders based on the significance of these orders in pest management, including Hemiptera, Neuroptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera. We address the variability in RNAi efficacy across different insect species, influenced by factors such as RNA degradation, delivery challenges, and organ-specific responses to gene silencing. Specifically, genes involved in cuticle formation, molting, chitin metabolism, immune response, and nervous system function are identified as prime candidates for RNAi-mediated pest control. For example, genes like chitin synthase, chitinase, acetylcholinesterase, and sex-determining genes show significant potential in reducing pest fitness, inducing mortality, and disrupting reproduction. Overall, this research provides valuable insights into the potential of RNAi as a species-specific and environmentally sustainable approach to pest control, laying the groundwork for developing innovative pest management tools that could reduce reliance on chemical insecticides.

Introduction

RNA interference (RNAi) is a powerful post-transcriptional gene silencing mechanism that has been extensively studied in invertebrates since its initial discovery in model organisms such as the nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster. By introducing double-stranded RNA (dsRNA) that specifically targets a desired gene, researchers can effectively silence that gene and observe the resulting phenotypic changes in insects to study gene function (Fig. 1). This mechanism has paved the way for the development of new and particular approaches to pest management. RNAi can reduce fitness or induce mortality by selectively targeting key genes in pest species, providing an alternative to traditional chemical insecticides (Zhu & Palli, 2020; List et al., 2022).

DOI: https://doi.org/ 10.22111/JEP.2025.50424.1075
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Publisher: University of Sistan and Baluchestan

How to Cite: Amiri, A. (2025). Identifying Effective Genes as Targets for RNA Interference in Sustainable Pest Control Strategies. *Journal of Epigenetics*, 6(1), 1-16. https://doi.org/ 10.22111/JEP.2025.50424.1075

RNA interference (RNAi) efficacy can vary significantly among insect orders and target genes. While specific orders, such as Coleoptera, exhibit high sensitivity to RNAi, demonstrating strong and long-lasting effects, others, such as Lepidoptera and Neuroptera, may show variable or transient responses (Palli, 2023).

For instance, in Coleoptera, such as the red flour beetle, *Tribolium castaneum*, targeting conserved cellular processes like protein degradation has proven effective, as demonstrated by genome-wide RNAi screens that identified highly effective target genes leading to significant mortality rates (Cedden et al., 2024). In contrast, Lepidoptera and Neuroptera often show transient or less consistent responses to RNAi, which may be attributed to differences in their cellular uptake mechanisms and the stability of dsRNA within these species (Hanamasagar et al., 2024). This variability can be attributed to abundant RNases, leading to double-stranded RNA (dsRNA) degradation. Studies have shown that various insect orders, particularly Lepidoptera, exhibit low RNAi efficiency due to high levels of gut dsRNases, such as SedsRNase1 and SedsRNase2 in *Spodoptera exigua*, which were found to degrade dsRNA significantly (Li et al., 2024). Additionally, the efficiency of processing dsRNA into small interfering RNA (siRNA) in these insects is often limited (Palli, 2023). Other constraints, including challenges related to the delivery, stability, and uptake of dsRNA, also contribute to the variable responses observed. Researchers have investigated numerous strategies and methods to enhance the delivery efficiency of dsRNA (Whitten, 2019).

Timing also plays a critical role in the application of dsRNA. Utilizing dsRNA at early developmental stages demonstrates higher efficacy compared to later stages. This underscores the importance of early application of RNA insecticides (Al-Ayedh et al. 2016; Guan et al. 2017). Moreover, continuous application over an extended period has been shown to yield better results compared to a single application (Willow et al., 2021).

Targeting specific organs also influences efficacy. Specific organs should be considered when selecting target genes. For instance, specific immune-related genes exhibit high efficacy in the integument, Malpighian tubules, or fat bodies while showing lower efficacy in gut tissues (Grizanova et al., 2021; Park et al., 2015; Vatanparast & Kim, 2017).

In this study, several target genes from various insect orders, including Hemiptera, Neuroptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera, are examined for future pest control strategies utilizing RNAi genetic tools.

Cuticle

- The growth and development of insects are significantly influenced by genes involved in cuticle formation, molting, and chitin metabolism. Maintaining a balance between chitin synthesis and degradation is crucial for the growth and molting of insects. Various enzymes heavily regulate and coordinate these processes (Moussian, 2010). Notably, these enzymes include chitin synthase, chitinase, and chitinase-deacetylase. Generally, studies indicate the potential of RNA interference (RNAi) as a tool for pest control by targeting key genes involved in cuticle formation, molting, and chitin metabolism (Saxena et al., 2022; Tariq et al., 2024; Zhou et al., 2024). For example, studies on the Colorado potato beetle revealed that silencing genes related to cuticle and ecdysteroid signaling significantly increased mortality and reduced reproductive success (Tariq et al., 2024). The most commonly used and successful dsRNAs targeting cuticle formation and metabolism are chitin synthase 1 (chs1), chitin synthase 2 (chs2), and chitinase (cht). Even at relatively low concentrations of dsRNA, significant mortality has been observed at various insect developmental stages. For instance, in the study on Leucinodes orbonalis, silencing of the chitin synthase A (CHSA) gene resulted in increased mortality during the larval stage, alongside extended larval periods and reduced fecundity (Saxena et al., 2022). Similarly, Aphis gossypii exhibited mortality rates of up to 59% in third-instar nymphs after just 72 hours of dsRNA-CHS1 feeding, indicating a strong correlation between chitin synthase knockdown and insect survival (Ullah et al., 2019). Furthermore, the Asian citrus psyllid (Diaphorina citri) displayed malformed phenotypes and increased mortality when its chitin synthase gene was silenced, underscoring the critical role of chitin synthesis in insect development (Lu et al., 2019). These

findings enhance our understanding of insect growth and provide valuable insights for developing RNA-based pest management strategies (Lu & Shen, 2024).

Chitin Synthase

The biosynthesis of insect chitin originates from trehalose, a primary hemolymph sugar, and during the conversion to chitin, chitin synthase genes, including chitin synthase 1 (chs1) and chitin synthase 2 (chs2), play crucial roles in regulating chitin production in the cuticle and the peritrophic matrix (PM), respectively (Moussian, 2010). The peritrophic matrix is a crucial extracellular structure found in most insects, serving multiple functions, including mechanical protection, pathogen defense, and digestive efficiency enhancement. Composed primarily of chitin and peritrophins, the PM forms a membranous sheath around food boluses, facilitating the separation of digestive enzymes from the midgut epithelium, thus preventing damage from digestive processes (Güney et al., 2024).

Chitinase

The cuticle and peritrophic matrix undergo periodic degradation and remodeling to ensure normal growth and development. In this process, chitinase enzymes play a pivotal role in breaking down chitin into soluble oligosaccharide forms. This degrades chitin and rejuvenates the peritrophic matrix, protecting the intestinal epithelium from toxins or pathogens (Moussian, 2010).

Chitinase-Deacetylase and Tanning

Chitinase-deacetylase (CDA) plays a vital role in chitin metabolism, converting rigid chitin into soluble and softer chitosan polymers by removing acetyl groups (Moussian, 2010). This deacetylation process increases solubility and reduces the density of chitin fibers. After each molt, newly formed soft cuticles undergo tanning and hardening to regain their protective functions. This involves the cross-linking of cuticular proteins (CPs) with chitin, facilitated by enzymes such as laccase (Lac), which is crucial for the pigmentation and sclerotization of the cuticle (Jiang et al., 2021; Wang et al., 2021; Ze et al., 2023).

Immune System

Insects lack the antibody-mediated immune defenses found in mammals. Instead, they possess an innate immune system as their primary defense against microbial pathogens (Kingsolver & Hardy, 2012). The key tissues involved in insect immunity include hemocytes (immune cells) and the fat body of larvae. The immune response encompasses pathogen recognition, cellular immune response, humoral immune response, and antimicrobial peptide (AMP) production. The cellular immune response involves hemocytes that perform phagocytosis, encapsulation, and release microbicidal compounds (Einhorn & Imler, 2019). In contrast, the humoral response is characterized by AMP secretion of AMPs into the hemolymph, disrupting microbial membranes and exhibiting broad-spectrum antimicrobial activity (Wu et al., 2018). These AMPs, which include defensins and cecropins, are crucial for immediate defense against bacteria and fungi, showcasing the evolutionary adaptation of insects to their pathogen-rich environments (Wu et al., 2018).

Pathogen Recognition

Insects recognize invading pathogens through pattern recognition proteins (PRPs), such as hemolysin, peptidoglycan recognition proteins (PGRPs), immune lectins (IMLs), and β-glucan recognition proteins (βGRPs). These proteins bind to pathogen-associated molecular patterns (PAMPs) to identify microbial molecules. Certain gene families, such as pathogen response (RePat), are associated with the immune response and are highly expressed in Bt-resistant strains. The pathogen response (RePat) gene family plays a crucial role in the immune response of insects, particularly in species like *Spodoptera frugiperda* and *Plutella xylostella*, which exhibit high expression levels in Bt-resistant strains. For instance, the gene SfREPAT38 in *S. frugiperda* is significantly upregulated in response to various microbial pathogens, including Bacillus thuringiensis. It is involved in mediating the Toll signaling pathway, essential for immune function (Wang et al., 2024).

Cellular Immunity

The cellular immune response includes phagocytosis, nodulation, encapsulation, and melanization. The spreading behavior of hemocytes is a critical cellular immune response

that facilitates interaction with pathogens or foreign objects. Melanization involves the activation of prophenoloxidase (proPo) to phenoloxidase (Po) in hemocytes, helping to restrict the response site to the level of invading bacteria and facilitating pathogen encapsulation. The plasma cell-spreading peptide (PSP) promotes the adhesion and dissemination of plasmatocytes on external surfaces.

Humoral Immunity

Following the recognition of PAMPs by pattern recognition receptors (PRRs), signaling pathways such as the Toll and Immune Deficiency (IMD) pathways are activated. PGRPs initiate a signaling cascade by phosphorylating and degrading proteins like cactus (in the Toll pathway) and Relish (in the IMD pathway), leading to the activation of NF-kB (nuclear factor kappa B) transcription factor. This activation promotes the expression of AMP-coding genes, aiding in pathogen clearance. The production of reactive oxygen species (ROS) is another defense mechanism used to damage fungal cells. After repeated exposure to fungal pathogens, insect metalloproteinase inhibitors (IMPI) enhance antifungal defense. Coordination between humoral and cellular immunity is essential for effective defense.

Various studies have investigated the effects of RNA interference (RNAi) on immune genes and pathogen responses, revealing their roles in pest control. Numerous studies have focused on preventing pathogen recognition, emphasizing specific genes' critical roles in insect immunity and pathogen response. RNA interference (RNAi) is crucial in preventing insect pathogen recognition by silencing specific immune-related genes, thereby modulating their immune responses. In the tobacco hornworm *Manduca sexta*, RNAi-mediated knockdown of recognition proteins such as Hemolin and Immulectin-2 significantly increased susceptibility to the bacterial pathogen Photorhabdus, indicating that these proteins are vital for effective immune recognition (Eleftherianos et al., 2006). The successful use of dsRNA targeting the hemline gene has been reported as an effective pest control strategy linked to immunity. In some cases, combining microorganisms and dsRNA has resulted in more potent pest control strategies (Jung et al., 2019; Hrithik et al., 2021; Wan et al., 2021). RNA interference is potent for disrupting insect immune responses, particularly in humoral and cellular immunity and pathogen recognition. By employing double-stranded RNA (dsRNA) to silence specific immune-related genes, researchers have demonstrated that this technique can significantly impair the insect's ability to recognize and respond to pathogens.

Digestion

The diverse proteases found in the digestive tract of insects reflect their adaptation to various ecological niches and food sources. Insects rely on these proteases to overcome dietary challenges, extract essential nutrients from their food, and defend against toxins and antinutritional compounds in their host plants. Proteases are essential for insect survival and adaptation, particularly their ecological niches, as they facilitate critical physiological processes such as digestion, development, and immune responses. Insects utilize diverse proteases, including digestive enzymes like trypsin and chymotrypsin, which are crucial for breaking down food and activating proteins necessary for various metabolic pathways (Jagdale et al., 2017). The adaptability of insects, such as the polyphagous pest *Spodoptera frugiperda*, is significantly influenced by their ability to express different protease isoforms in response to various host plants, allowing them to efficiently exploit diverse food sources (Hafeez et al., 2021).

Proteases

Proteases can be classified into three main types: serine proteases, cysteine proteases, and carboxypeptidases. Each type has unique properties and catalytic mechanisms that enable insects to digest dietary proteins effectively. Serine proteases are crucial for insect digestion, growth, and immune responses. They include enzymes such as trypsin and chymotrypsin, which hydrolyze proteins into peptides and smaller amino acids, facilitating efficient nutrient absorption (Holtof et al., 2019). Chymotrypsin-like peptidases also play roles in chitinolytic and proteolytic processes, aiding in the breakdown of old cuticles during molting.

Cysteine proteases are involved in degrading dietary proteins into smaller peptides and amino acids, promoting the absorption of essential

nutrients (Holtof et al., 2019). They perform various functions, including digestion, embryogenesis, molting, tissue remodeling during metamorphosis, detoxification of plant defensive proteins, and immune responses. Cysteine proteases play a critical role in insect physiology and survival by facilitating essential biological processes such as digestion, development, and immune response. These enzymes, particularly cathepsins, are involved in protein degradation, which is vital for nutrient absorption and energy production, especially in the larval stages of insects like *Tenebrio molitor* and *Tribolium castaneum*, where they are concentrated in the acidic anterior midgut (Martynov et al., 2015). Cysteine proteases are implicated in reproductive functions, as seen in the maize weevil *Sitophilus zeamais*, which are present in alimentary organs and germ cells, indicating their involvement in digestion and reproduction. Furthermore, these proteases contribute to immune defense mechanisms by degrading pathogens, thus enhancing survival rates under stress conditions (Jagdale et al., 2017).

Cathepsins, a type of cysteine protease, are primarily active and stable at an acidic pH, participating in protein degradation in lysosomes and other cellular processes. Carboxylesterases are crucial for detoxification by catalyzing the hydrolysis of various carboxylic esters and cholinesterases. Insects utilize carboxylesterases to metabolize and eliminate xenobiotic compounds such as insecticides, reducing their toxicity. They also aid neurogenesis by degrading specific compounds (Holtof et al., 2019). Various insect species exhibit specific adaptations by utilizing different proteases, which play crucial roles in their ecological niches. For instance, lepidopteran species, such as the castor silkworm (*Samia ricini*), utilize serine proteases like trypsin and chymotrypsin for digestion, which are essential for their dietary adaptations when transitioning between host plants, influencing their growth and fecundity (Kashung et al., 2023).

Osmoregulation

Osmoregulation is a vital physiological process for insects that feed on phloem sap, primarily due to the high osmotic pressure of the sap resulting from its high sugar content. Insects express various genes related to osmoregulation in their gut to maintain water balance and process ingested sugars effectively. These genes include gut sucrase−transglucosidase (GLU) for sugar digestion, gut aquaporin proteins (AQP) for water balance regulation, and sugar transporters (ST) to ensure appropriate sugar concentration and prevent osmotic stress (Campbell et al., 2008).

Gene silencing involving RNA interference (RNAi) targeting digestion and osmoregulation genes in various insect species has shown promising potential as a pest control strategy. Studies targeting genes associated with proteases have yielded encouraging results (Guan et al., 2017; Vatanparast & Kim, 2017; Bento et al., 2020). Within digestion, chymotrypsin (chy2) and carboxylesterases have been identified as promising targets for RNAi-mediated gene silencing, particularly when combined with insecticide application. These findings provide valuable insights for developing highly efficient RNA-based insecticides for digestive processes.

Metamorphosis

Molting and metamorphosis in insects are primarily regulated by two key hormones: juvenile hormone (JH) and ecdysteroids (Truman & Riddiford, 2002). The balance between juvenile hormones and ecdysteroids plays a crucial role in determining the timing and progression of insect development. Ecdysteroids, produced by the prothoracic gland, promote molting and trigger metamorphosis by inducing the E93 transcription factor, which facilitates apoptosis in larval cells and remodeling structures such as the midgut. Conversely, JH, synthesized in the corpora allata, inhibits metamorphosis by maintaining larval characteristics and preventing premature developmental transitions (Bellés, 2020). The interplay between these hormones is crucial.

Juvenile Hormone (JH)

The regulatory mechanism of juvenile hormones allows insects to avoid metamorphosis and maintain a pre-metamorphic state. Juvenile hormones play a crucial role in preventing metamorphosis in insects by maintaining a juvenile state and inhibiting the action of the molting hormone, 20-hydroxyecdysone (20E). Juvenile hormone levels are high during early developmental stages, and their decline is necessary for the transition to metamorphosis, allowing morphogen signaling to dominate and drive the development of imaginal structures (Bellés, 2020). Juvenile hormone is synthesized in the corpora allata of the insect's brain and is transported to target cells via binding proteins. The biosynthesis of juvenile hormones consists of two

protected components. The synthesis and degradation of juvenile hormones require precise regulation during the various stages of growth (Truman & Riddiford, 2002).

Ecdysteroids

Ecdysteroids, particularly ecdysone, are steroid hormones synthesized in the prothoracic glands and play a vital role during metamorphosis. Ecdysone is converted into its active form, 20-hydroxyecdysone (20E), through the action of enzymes encoded by Halloween genes (Truman & Riddiford, 2002).

Neuropeptides

Neuropeptides such as allatotropin and allatostatin stimulate or inhibit juvenile hormone production, thereby regulating molting. Neuropeptides play a crucial role in the hormonal regulation of insect development by acting as key signaling molecules that influence various physiological processes. They regulate ecdysteroid and juvenile hormone production, essential for growth and metamorphosis and in processes such as oocyte development and ecdysis. Neuropeptides function as bioactive messengers that can act locally and systemically, modulating a wide array of functions, including metabolism, homeostasis, and behavior (Altstein & Nässel, 2010).

Several studies have investigated the effects of RNA interference (RNAi) targeting specific genes in hormonal regulation during insect growth. Overall, signaling pathways of juvenile hormones and ecdysteroid receptors (er) have been extensively studied. Among the most effective genes for RNA-based insecticides are ecr and kr-h1, which have been shown to inhibit molting and lead to high mortality rates, making them valuable candidates for pest control strategies (Xu et al., 2019; Bento et al., 2020; Liu et al., 2023).

Reproduction

The reproductive capacity of insects is a significant factor in their status as serious pests. High fecundity in insect pest species poses significant challenges for pest management due to their rapid population growth and potential for agricultural damage. For instance, the brown planthopper (*Nilaparvata lugens*) exhibits high fecundity linked to specific genes regulating vitellogenin expression, influencing egg production and survival rates (Qiu et al., 2016). Similarly, aphids can produce up to 10^11 descendants in a single season through parthenogenetic reproduction, allowing them to establish and dominate agricultural ecosystems quickly. The invasive nipa palm hispid beetle (*Octodonta nipae*) also demonstrates increased fecundity with multiple matings, further complicating control efforts as populations can surge rapidly under favorable conditions (Li et al., 2014). Understanding the mechanisms underlying the high fecundity of insects and developing strategies to suppress their reproductive capabilities have been key research areas in pest control (Smagghe et al., 2019).

Sex Pheromones

Sex pheromones are crucial in attracting the opposite sex within a species. Several genes that regulate insect reproduction have been identified, including vitellogenin and ribosomal proteins (rp). For instance, in the soldier bug *Podisus maculiventris*, vitellogenin constitutes a substantial portion of egg and hemolymph proteins, with its synthesis closely linked to dietary intake, influencing ovarian growth and reproductive output (Shapiro et al., 2000). Furthermore, the production of sex pheromones is regulated by a neuropeptide known as the pheromone biosynthesis activating neuropeptide (PBAN), which is synthesized in the subesophageal ganglion (Roy et al., 2018). Another neuropeptide, hyperthermic hormone (HTH), is important for regulating energy metabolism in fat bodies (Smagghe et al., 2019). Insects utilize neuropeptides such as PBAN (Pheromone Biosynthesis Activating Neuropeptide) and HTH (Hypertrehalosemic Hormone) to regulate reproduction and energy metabolism. PBAN, identified in various species, plays a crucial role in reproductive processes, including pheromone production, which is vital for mating and population dynamics (Choi & Meer, 2009). Additionally, PBAN influences physiological functions related to oviposition and hormonal balance, affecting reproductive success (Jiang et al., 2015).

Insect sex pheromone RNA interference (RNAi) strategies have emerged as innovative methods for pest control, mainly targeting sex-specific traits in insects. RNAi can disrupt the maturation of larvae into adults by silencing genes associated with sex determination, as demonstrated in the diamondback moth, where small RNA sequences target the male-determining gene PxyMasc (Harvey-Samuel et al., 2022). Additionally, the use of double-stranded RNA (dsRNA) targeting pheromone biosynthesis activating neuropeptide

(PBAN) genes has shown promise in controlling pest populations by affecting growth and development, leading to increased mortality in species like the corn earworm and tobacco budworm (Choi & Meer, 2019).

Vitellogenin

Vitellogenin, a yolk precursor protein in insect eggs, serves as a marker for measuring fertility. It is produced in the fat bodies, secreted into the hemolymph, and transported to developing oocytes via the vitellogenin receptor (VgR). Multiple signaling pathways regulate vitellogenin expression, including insulin, rapamycin (TOR) target, juvenile hormone pathways, and 20-hydroxyecdysone (Smagghe et al., 2019). Additionally, the regulation of spermatogenesis involves the activity of various protein kinases and specific genes. Testis-specific serine/threonine kinases (TSSK), tectins, and Bol (Boll) proteins play vital roles in the development and differentiation of sperm (Sohail et al., 2019; Zhai, 2023).

RNAi has been extensively utilized to investigate its role in the reproductive processes of various insect species. For example, in the alligator weed flea beetle *Agasicles hygrophila*, three distinct vitellogenin genes were identified, with their expression in the fat body being crucial for ovary development and egg-laying capacity; RNA interference targeting these genes resulted in reduced reproductive success (Zhang et al., 2019).

In summary, genes associated with vitellogenin and its receptors have proven to be highly effective targets for controlling reproductive processes in insects. Treatments with dsRNA targeting these genes have been shown to delay egg development, induce sterility in insects, and result in the formation of inactive ovaries, offering a potential approach for reducing progeny numbers and controlling insect populations (Sohail et al., 2019; Wei et al., 2021; Zhai, 2023).

Response to Stress

Diapause is a physiological state adopted by insects to endure harsh winter conditions. This state involves significant changes in gene expression that lead to various physiological and behavioral modifications. During diapause, insects conserve nutrient reserves, reduce metabolic rates, and halt or slow down growth. These adaptations enhance tolerance to environmental stresses, including low temperatures (Storey & Storey, 2012).

Insect diapause serves a critical ecological function by allowing species to survive unfavorable environmental conditions, with significant variability observed across different species. Diapause can manifest as a developmental arrest during adverse seasons, such as winter or summer, and is influenced by various factors, including temperature, photoperiod, and food availability (Denlinger, 2023). The timing and duration of diapause can vary widely, with some species exhibiting prolonged diapause as a bet-hedging strategy in unpredictable environments. Additionally, the physiological mechanisms underlying diapause differ among species, with univoltine and semivoltine life cycles showcasing distinct adaptations to seasonal changes (Numata & Shintani, 2022). This variability reflects evolutionary responses to ecological pressures and highlights the potential for rapid shifts in diapause strategies in response to climate change (Denlinger, 2023).

Low-Temperature Stress

In response to low temperatures, insects have developed rapid cold hardening (RCH) mechanisms. This mechanism involves exposure to temperatures slightly above freezing, resulting in various physiological and biochemical changes, including synthesizing cryoprotectants such as glycerol and sorbitol. These compounds help protect cells by lowering the freezing point of liquids and preventing ice crystal formation (Storey & Storey, 2012). During rapid cold hardening (RCH), insects undergo significant physiological and biochemical changes that enhance their cold tolerance. Key adaptations include alterations in fatty acid composition, with increased levels of unsaturated fatty acids, such as linoleic acid, which improve membrane fluidity and stability under cold stress (Overgaard et al., 2006). Additionally, the accumulation of cryoprotectants like glucose and trehalose is observed, which helps to mitigate cellular damage during freezing conditions (Zhang et al., 2024). Heat shock proteins (HSPs) are crucial in stabilizing proteins and membranes to prevent denaturation under stress.

Oxidative Stress

In addition to cold stress, insects also face oxidative stress due to reactive oxygen species (ROS) produced during cellular metabolism (Storey & Storey, 2012). Insects generate reactive oxygen species (ROS) from various environmental

factors, including abiotic stressors such as desiccation, heat, and starvation, which have been shown to significantly elevate ROS levels in *Drosophila melanogaster* larvae (Bomble & Nath, 2024). Exposure to pollutants like heavy metals, ultraviolet light, and pesticides further exacerbates ROS production, leading to oxidative damage to cellular components such as lipids, proteins, and DNA (Limón Pacheco et al., 2017).

Insect's enzymatic antioxidant defense systems include superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). These enzymes work together to neutralize ROS and maintain redox balance. SOD catalyzes the conversion of superoxide anion (O2-) to hydrogen peroxide (H2O2). At the same time, catalase decomposes hydrogen peroxide into water (H2O) and molecular oxygen (O2) to reduce widespread damage caused by highly reactive hydroxyl radicals (•HO). Metallothioneins (MTs) also contribute to detoxifying unnecessary trace elements and protecting cells from oxidative stress.

The application of RNAi to target the aforementioned genes has effectively disrupted insect behavioral adaptations and defense mechanisms, ultimately leading to pest control. Most studies have focused on genes that enable organisms to resist extreme environments. For instance, the injection or feeding of dsRNA targeting hsp70 significantly reduced heat and cold tolerance in various insects, resulting in increased mortality under extreme temperature conditions. The effectiveness of double-stranded RNA (dsRNA) targeting heat shock proteins (HSPs), particularly Hsp70, in insects such as the sweet potato whitefly (*Bemisia tabaci*) has been demonstrated through various studies. Feeding dsRNA targeting hsp70 resulted in a significant knockdown of mRNA levels and increased mortality under thermal stress conditions, indicating the critical role of Hsp70 in insect survival during heat shock (Shim et al., 2015). Additionally, dsRNA targeting HSPs has proven effective in preventing insects from thriving in challenging environments, such as snow and frost (Ji et al., 2020; Zhang et al., 2022). Other research has utilized genes related to oxidative stress. These studies demonstrate RNAi's potential to enhance sensitivity to adverse environmental conditions.

Nervous System

The nervous system plays a crucial role in the physiology of insects and includes several genes with specific functions. The nervous system in insects plays a crucial role in behavior, sensory perception, and environmental interaction. It consists of a central nervous system (CNS), comprising the brain and ventral nerve cord, and a peripheral nervous system that innervates muscles and sensory receptors, facilitating rapid information transfer through electrical impulses (Klowden, 2008). Sensory transduction occurs via specialized cells, such as those in compound eyes, converting environmental stimuli into neural signals, enabling insects to respond effectively to their surroundings. The sensory input and motor output integration is further enhanced by the diverse neurotransmitters and neuromodulators involved in synaptic transmission, allowing for sophisticated behavioral adaptations (Klowden, 2008; Ben-Shahar, 2015).

Acetylcholinesterase (AChE) is essential for terminating signal transmission by hydrolyzing excess acetylcholine (ACh). The inactivation of AChE leads to the accumulation of ACh in the intercellular space, resulting in the continuous stimulation of target cell receptors. This disruption of neurotransmission can cause various complications, including muscle paralysis, convulsions, spasms, and ultimately the death of the pest. Inactivation of acetylcholinesterase (AChE) in insects leads to an accumulation of acetylcholine (ACh) at cholinergic synapses, resulting in hyperstimulation of nicotinic and muscarinic receptors, which disrupts normal neurotransmission and can cause severe physiological effects. Organophosphorus and carbamate insecticides inhibit AChE by acylating its active site, preventing ACh hydrolysis, and leading to ACh levels increasing significantly up to 260% in some cases (Čolović et al., 2013). This excess ACh causes continuous stimulation of neural pathways, ultimately leading to muscle paralysis and convulsions due to receptor fatigue and overstimulation (Richardson, 2018). Additionally, sub-lethal doses of these neurotoxic agents can alter insect behavior and motor function, indicating that even non-lethal exposures can profoundly affect survival and ecological interactions (Williamson et al., 2013).

Insects typically possess two forms of AChE, AChE1 and AChE2, encoded by ace1 and ace2, respectively (Pang, 2014). Ryanodine receptors (RyRs), calcium-induced calcium release channels, play essential roles in muscle

contraction, neurotransmission, and calcium signaling across different cell types. In insects, RyRs are regulated by calmodulin (CaM), which enhances their sensitivity to insecticides, indicating a potential target for pest control strategies (Lin et al., 2022). Dysregulation of RyRs can lead to severe muscle disorders and cognitive dysfunction, highlighting their importance in health and disease across species (Zahradníková et al., 2024).

Several studies have investigated the application of RNAi to target specific genes related to the nervous system in various insect species. For instance, in the corn earworm and tobacco budworm, RNAi targeting the pheromone biosynthesis activating neuropeptide (PBAN) gene resulted in delayed larval growth and increased mortality, highlighting its potential for pest control (Choi & Meer, 2019). In *Drosophila*, a resource of transgenic RNAi lines has been developed to analyze gene functions in the nervous system, allowing for the targeted silencing of genes involved in critical processes such as axon pathway development (Ni et al., 2009). Treatment with dsRNA targeting the ace1 gene has decreased growth, reduced weight, impaired locomotor ability, and population decline. Overall, these studies collectively demonstrate the potential of RNAi technology for insect pest control by targeting genes associated with the nervous system. Inhibiting AChE is important in pest management, reducing survival rates and developmental disruptions (Malik et al., 2016; Majidiani et al., 2019).

Sex Determination

Successful mating is regulated by multiple factors before and after the actual mating event. Many factors, including hormonal regulation, sensory inputs, and genetic coordination, influence successful mating in insects. Female reproduction is primarily regulated by juvenile hormones and ecdysteroids, which control vitellogenesis and are influenced by nutritional signaling pathways (Roy et al., 2018). Male insects, such as *Drosophila melanogaster*, exhibit behavioral plasticity in mating duration based on sexual experience and environmental cues, optimizing their reproductive investment (Lee et al., 2022). Post-mating responses in females, triggered by seminal fluid proteins and octopamine, further enhance reproductive success by modulating physiological changes and gene expression (Guan et al., 2023). Additionally, the genetic coordination of sperm morphology and seminal fluid proteins plays a critical role in male reproductive success, affecting both sperm competition and female remating rates (Galvin et al., 2021). Both male and female insects must perform their roles effectively. Regulation of insect mating involves various genes associated with sexual development and sexual dimorphism.

In insects, sex determination is controlled by a set of genes that code for RNA-binding proteins, which recognize and bind to specific pre-mRNAs to control the inclusion or exclusion of specific exons in sex-specific transcripts. This alternative splicing produces different protein isoforms that contribute to sexual dimorphism. Insects utilize RNA-binding proteins (RBPs) to regulate sex determination and alternative splicing (AS), mainly through the doublesex (dsx) gene, which produces male and female isoforms essential for sexual differentiation. In *Drosophila*, the Nab2 RBP promotes female-specific splicing of the Sex-lethal (Sxl) gene by preventing m6A hypermethylation, thereby ensuring proper sex-specific expression (Jalloh et al., 2020). Similarly, the SR-rich protein SRm160 influences the AS of the transformer gene, a key player in the dsx splicing cascade, highlighting the role of RBPs in modulating sex-specific splicing patterns (Qiu et al., 2019). In the silkworm, BmIMP, a male-specific RBP, enhances the binding activity of BmPSI to the splicing silencer CE1, promoting male-specific splicing of Bmdsx (Suzuki et al., 2010). Collectively, these findings illustrate a complex interplay of RBPs that fine-tune AS mechanisms, ultimately shaping sexual development in insects (Zheng et al., 2019).

Various RNAi experiments have been conducted on sexual development, fertility, and reproductive traits in different insect species. For example, in mosquitoes, RNAi targeting the gamma-glutamyl transpeptidase (GGT) gene has demonstrated a female-specific lethality, facilitating sex separation in species such as *Aedes aegypti* and *Anopheles gambiae*, thereby enhancing pest control strategies (Mysore et al., 2022). Furthermore, RNAi targeting the rotund gene in the ground beetle Carabus maiyasanus revealed its critical role in genital morphology, affecting reproductive isolation mechanisms (Sota et al., 2018). Lastly, in *Drosophila simulans*, endogenous RNAi has been shown to suppress selfish genetic elements that disrupt

spermatogenesis, highlighting its importance in maintaining reproductive viability (Vedanayagam et al., 2022).

The transformer (tra) and doublesex (dsx) genes are pivotal in the sex determination pathways of various insect species, including *Drosophila* and agricultural pests like *Ceratitis capitata* and *Bactrocera* species. The tra gene functions as a splicing regulator, promoting female-specific splicing of dsx through interactions with transformer-2 (tra2) proteins, which are also crucial for this process (Perrotta et al., 2023).

In summary, dsx and tra are highly effective genes in insect sex determination. When dsRNA targeting these genes is administered to female insects via injection or feeding, it disrupts sex determination and impacts their egg-laying ability. Collectively, these experiments emphasize the potential of RNAi as a powerful tool for manipulating reproductive processes in insects (Zhuo et al., 2018; Castellanos et al., 2019).

Metabolism

Metabolism is crucial in pest control strategies, mainly targeting specific insect metabolic pathways. Lipid metabolism is essential for various insect functions, and disrupting this process using lipid biosynthesis inhibitors, such as spirodiclofen, has shown promise in managing pest populations (Toprak et al., 2024). Additionally, hydrolytic enzymes like chitinases and lipases are integral to insect metabolism and can be exploited to degrade insect cuticles, facilitating infection by entomopathogenic organisms (Ahmad & Rasool, 2021). Furthermore, cytochrome P450 enzymes are pivotal in the oxidative metabolism of xenobiotics, including insecticides, and understanding their role can lead to the development of selective insecticides that overcome resistance mechanisms (Katsavou et al., 2022). Chitin metabolism also targets biorational insecticides, as compounds like benzoylphenyl ureas disrupt chitin synthesis, which is essential for insect growth and development (Doucet & Retnakaran, 2012). Cellular metabolism is crucial for producing ATP and efficient energy transfer to support cellular processes such as biosynthesis, active transport, muscle contraction, and signal transduction. Various genes involved in metabolism serve as potential RNAi strategies for pest control.

ATP Production

In insects, particularly in flying species like Drosophila, ATP production exhibits unique adaptations compared to other organisms, primarily due to their high energy demands. Insects rely heavily on mitochondrial oxidative phosphorylation for ATP synthesis, with studies indicating that about 95% of ATP is generated this way (Yang et al., 2014). *Drosophila*, for instance, demonstrates a distinct respiratory adaptation characterized by tightly packed respiratory chain complexes and elevated concentrations of electron carriers, which enhance electron transfer efficiency without forming supercomplexes, a feature common in mammals (Shimada et al., 2018). Additionally, the ATP synthase in insects has evolved through a positive selection of mitochondrial genes, reflecting adaptations to increased energy requirements associated with flight (Yang et al., 2014). Furthermore, daily variations in ATP production have been observed, with peaks occurring in the morning, suggesting a temporal regulation of energy metabolism that may be influenced by environmental factors (Shinhmar et al., 2022).

The electron transport chain (ETC) is a vital component of cellular respiration in eukaryotic cells' inner mitochondrial membrane. It consists of protein complexes and coenzymes that transfer electrons to create a proton gradient for ATP synthesis. Nicotinamide adenine dinucleotide (NADH), subunits ND1 and ND2 in the NADH dehydrogenase complex, cytochrome P450 reductase (CPR), and glycerol-3-phosphate dehydrogenase (GPDH) are critical in this process. In insects, the mitochondrial subunits ND1 and ND2 are integral components of the NADH dehydrogenase complex, also known as complex I, which plays a crucial role in the electron transport chain (ETC). ND1, a core subunit, is essential for the assembly and enzymatic function of complex I, influencing the binding of ubiquinone and the proton channel, thereby affecting oxidative phosphorylation and cellular energy production (Lin et al., 2024). Similarly, ND2, while less studied, is implicated in maintaining mitochondrial function and energy metabolism, as evidenced by its involvement in spermatid elongation processes in Drosophila, where it supports mitochondrial integrity and membrane potential (Yu et al., 2023).

ATPase, specifically vacuolar-type ATPase (V-ATPase), is responsible for ATP synthesis by transferring ions across membranes, playing an

essential role as a proton pump in organelles, maintaining pH gradients in the gut, and regulating fluid secretion in Malpighian tubules.

Arginine Kinase

Arginine kinase is a phosphotransferase enzyme that plays a vital role in the cellular energy metabolism of invertebrates. It facilitates the transfer of phosphate from ATP to arginine, producing phosphoarginine and ADP. Invertebrates possess a unique biosynthetic pathway for phosphoarginine. Invertebrates, such as mollusks, utilize a distinct biosynthetic pathway for phosphoarginine (pArg) that differs significantly from vertebrate pathways. Invertebrates synthesize pArg through a mechanism involving specific kinases, such as arginine kinase, which catalyzes arginine phosphorylation using ATP. This process is well-documented in crustaceans and fish. In contrast, vertebrates primarily rely on phosphocreatine as a high-energy phosphate reservoir, with pArg playing a lesser role in energy metabolism. Additionally, the regulation of ion transporters, such as the Na+/Ca2+ exchanger, showcases different metabolic pathways in invertebrates, where pArg influences exchanger activity independently of ATP, unlike in vertebrates where ATP and phosphatidylinositol derivatives are crucial (DiPolo et al., 2004).

Detoxification Enzymes

Insects utilize a variety of detoxification enzymes, particularly cytochrome P450 monooxygenases (P450s) and glutathione S-transferases (GSTs), to metabolize typical insecticides and phytochemicals, which is crucial for their ecological adaptation and survival. P450s are known for metabolizing a wide range of xenobiotics, including synthetic insecticides and natural plant chemicals, often through synergistic interactions that enhance resistance mechanisms (Lu et al., 2021). For instance, studies on Helicoverpa armigera demonstrated that knocking out specific P450 gene clusters significantly increased mortality rates when exposed to various toxins, highlighting their essential role in detoxification (Wang et al., 2018). Additionally, GSTs, such as SlGSTE1 in Spodoptera litura, have been shown to effectively bind and detoxify multiple insecticides and heavy metals, indicating their importance in mitigating oxidative stress and enhancing insect resilience against environmental toxins (Chen, 2020). This enzymatic detoxification not only aids individual insects but also influences ecological dynamics by affecting pest populations and their interactions with plant hosts.

Detoxification enzymes are fundamental to insect metabolism, particularly in eliminating phytochemicals and insecticides. These enzymes include general esterases (GEST), cytochrome P450 monooxygenases (CYPs), and glutathione S-transferases (GST). They assist in detoxifying chemical insecticides and natural phytochemicals by converting them into less toxic forms for excretion or degradation (Nauen et al., 2022).

Numerous studies have highlighted the efficacy of RNAi in targeting specific genes involved in metabolism and insecticide sensitivity. These findings emphasize the importance of ATP-rebuilding enzymes, energy transfer, and detoxification in insect physiology and their potential as targets for pest control strategies. Among these genes, the most successful and widely accepted metabolic genes for pest management are the V-ATPase gene, especially V-ATPase A and ABC-ca. Inhibiting these genes through RNAi has shown promising results in pest control (Cao et al., 2018; Liu et al., 2019; Jin et al., 2021).

Conclusion

With the growing understanding of gene function and the widespread use of RNA sequencing (RNA-seq), researchers are now constructing extensive screening libraries, validating target genes for RNAi, and identifying potential candidates for pest management. The application of RNAi in pest control encompasses lethal effects, behavioral changes, reduced reproductive fitness, and decreased population size in an area. These research efforts have provided us with abundant genetic resources for their application. Notable 'star' genes include hb, chs1, lac2, cht, per, cyc, ecr, kr-h1, dsx, tra, vitellogenin, vgR, carE, V-ATPase, abc-ca, obp4, orco, ace, hemolin, hsp, and iap. These genes serve as valuable resources and pave the way for developing effective and environmentally sustainable strategies for pest management.

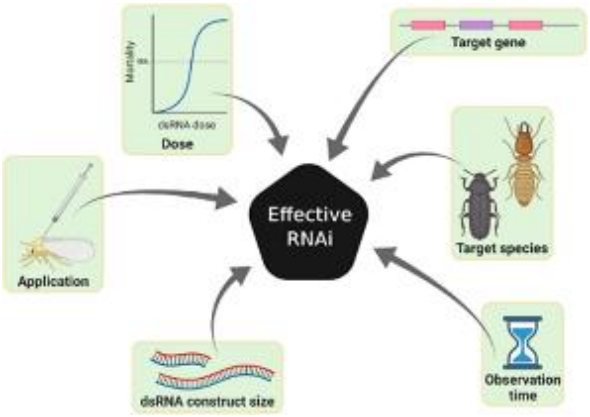


Fig. 1. A schematic summary of insect RNAi (List et al., 2022).

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