Chapter

Review of Insecticide Resistance and Its Underlying Mechanisms in *Tribolium castaneum*

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Abstract

The red flour beetle *Tribolium castaneum* has emerged as the genetically tractable model insect for population genetics, functional genomics, and evolutionary studies. This agricultural pest is notorious for its potential to severely damage stored products. *T. castaneum* has developed resistance to almost all insecticides. The reports of insecticide resistance from different parts of the world show that sustained insecticide usage has only aggravated the problem. As insecticides continue to be the mainstay of pest control programs, it is essential to identify the factors influencing insecticide resistance for implementing effective pest-management strategies. The development and progression of insecticide resistance in *T. castaneum* is thus an escalating global issue requiring immediate solutions. Several studies have investigated the multiple resistance mechanisms found in *T. castaneum*, such as reduced cuticular penetration, increased metabolic detoxification, and target-site insensitivity. The availability of Whole Genome Sequence and recent advances in Next Generation Sequencing technology has furthered a geneticist's grasp of resistance study in *Tribolium*. The strategic containment of this organism calls for an in-depth understanding of resistance development. The review mainly focuses on different kinds of resistance mechanisms and genes mediating insecticide resistance. Also, it exhaustively explores the *CYP450* gene superfamily in *Tribolium* to emphasize its role in governing resistance. The consolidated insights from this study will facilitate further research on identifying biological targets, thereby developing novel control strategies for effective insect control.

Keywords: Insecticide resistance, Resistance Mechanisms, Detoxification genes, *CYP450* gene superfamily, *Tribolium castaneum*

1. Introduction

The global population is expected to cross 9.1 billion by the year 2050 and food production is projected to rise to 70% to feed this growing population [1]. Many of the fastest-growing populations are in developing countries, several of which are already facing moderate or severe food insecurity and a shortfall in food supply. One in every six children suffer from hunger in developing countries [2] and the proportion of undernourishment has been steadily increasing since 2015 [3]. The increasing trend globally of food insecurity attests to the fact that severe food deprivation or hunger is a real threat, and this scenario nullifies the ambitious "zero hunger target" by 2030. The severity of "food insecurity" underscores the immense challenge in

attaining safe, nutritious, and sufficient food for all people [3]. Tackling problems of food insecurity demand intensive food production. However, increasing food production alone will not be a viable solution to achieve the "zero hunger target" by 2030 or for meeting the growing demand for food.

The pre-harvest and post-harvest issues combined with insect infestation represent a very strong limitation in optimal food production, causing mass losses of grains. After harvest, food grains undergo a series of processes such as threshing, cleaning, drying, storage, processing, and transportation before it reaches the consumer. It has been identified that food losses in the post-harvesting chain start at the time of harvest and continue up to food marketing at the consumer's end [4–6]. Grain losses may also take place due to technical limitations such as inadequate stock management facilities, improper packaging, and insufficient infrastructure.

In many countries, 15% of food grains are lost during or after harvest [7]. The Food and Agricultural Organization (FAO) estimated post-harvest grain loss at 40% and cereal loss at 30% in India [8]. The post-harvest losses account for on-farm, processing, and storage loss. Studies attribute massive grain loss in developing countries to manual operations in different stages of harvesting, which causes 15% loss on the field, 13–20% loss at processing, and 15–25% storage loss [9]. Several studies show that insects are the main contributor to storage loss in the food supply chain [10–12], which accounts for 10–20% of storage loss [13].

A diverse community of stored product species are associated with different environments where farmers store grains and cereals; from farm bins to processing facilities, to feed mills, to flour mills, to retailer stores [14–16]. Among this complex pest system, 600 species from Coleoptera and 70 species from Lepidoptera can cause substantial losses by eroding the quality of grains [17]. Coleoptera is the largest order of insects with over 250,000 described species and contains in its fold some of the most notorious stored grain pests. In these, *T. castaneum* requires special attention because of the significant harm that it can have on stored products. It attacks a large variety of stored and processed commodities and is the most harmful insect in the pest complex for its ability to inflict severe damage on stored products. Curtailing *Tribolium* infestations in the supply chain would be one critical step that can help strengthen food quality, reduce storage loss, and improve food security.

2. *Tribolium castaneum* and its damage

The red flour beetle, *Tribolium castaneum*, is an important model organism and a common inhabitant of milled cereal products, stored flour, and fungus-infested grain [18–21]. *T. castaneum* causes severe damage in flour mills and wherever dried foods and cereal products are stored or processed. They rank among the most harmful pests inhabiting grain storage facilities and processing facilities [16, 22, 23]. These insects frequently leave storage locations and migrate across heterogeneous landscapes on a daily, seasonal, or irregular basis to find new mates and resources [24]. The movement of grains from producers to consumers generates a complex network of grain storage and transportation that facilitates the dispersal of pests and pathogens associated with the grain [25]. The dispersal of the *T. castaneum,* through transportation and storage networks, allows them to find suitable habitats where they feed and reproduce, ultimately exploiting the resource patches of grain.

The adult females of *T. castaneum* lay eggs on the flour, complete their life cycle, and deplete the nutritional quality of grains over time. In the case of serious infestation, the flour becomes adulterated with a pungent odor, diminished in nutritional and market value [26, 27]. In addition to direct feeding, *T. castaneum* contaminates the food products through molting and excretion, which makes the

product commercially undesirable. Depending on the level of infestation, the grain can be rejected or downgraded [28]. Product deterioration can also result from the production of quinones secreted from glands on the thorax and abdomen [29–31] leading to significant loss of quality and economic loss. The customer demand for infestation-free flour/grain has increased widely, raising the stakes of *T. castaneum* management in grain storage facilities. In a way, consumer demand for infestation-free products has been a fillip to the use of insecticides such as organophosphates and pyrethroids during storage.

Thus, a wide variety of insecticides has been applied as a primary strategy for *Tribolium* control by targeting the insect's neurological sites, including voltage-gated ion channels and acetylcholine system, causing irreversible disruption of neurological function, resulting in insect mortality. It has brought down the infestation rate, ensured long-term protection of stored commodities, and is relatively convenient to apply [32, 33]. But the incessant application of insecticides in storage facilities has accelerated the development of insecticide resistance in *T. castaneum* and resulted in the formation of particular resistant alleles in succeeding generations. The occurrence of insecticide resistance in *T. castaneum* found in grains and cereals during storage and shipping was recorded in many countries. The first instance of insecticide resistance was reported in *Tribolium* between 1959 and the early 1960s [34, 35]. Halisack and Beeman [36] applied discriminating doses of malathion to *T. castaneum* populations collected from cereal storages in the US and detected 20-fold resistance in 31 of 36 *T. castaneum* populations. In Canada, 54 strains of *T. castaneum* showed resistance to malathion at an LC99.9 value of 0.012 mg a.i/cm^2 [37]. The populations of *T. castaneum* collected from flour mills in the USA were exposed to discriminating doses of malathion to measure their resistance status. Of 28 strains, 93% of the *T. castaneum* population tolerated the discriminating doses of malathion [38]. The resistance status of Egyptian populations of *T. castaneum* was studied using the filter paper bioassay method against three contact insecticides and populations of *T. castaneum* were found to be more resistant against pirimiphos-methyl [39]. *T. castaneum* resistance is extended to pyrethroid insecticides, which is one of the most widely-used classes of insecticides in food and fodder houses as it is effective on a wide range of insects, has high efficacy at the minimum dose, and low toxicity on mammals [40–42]. Cases of pyrethroid resistance have been detected in *T. castaneum* populations from Pilot-Scale Warehouses [43] and peanut storage warehouses [44]. Several cases of resistance have been reported in different populations of *T. castaneum* collected from different countries across the world such as Italy [45], United States of America [46–50], Africa [51], Serbia [52], Bangladesh [53], Philippines [54], Pakistan [55, 56], Iran [57] Australia [58]. The occurrence of insecticide resistance in *T. castaneum* has been reported against various fumigants-methyl bromide and phosphine [59–66], synthetic pyrethroids, e.g., cypermethrin, deltamethrin, cyfluthrin, fenvalerate, and permethrin [67, 68], organophosphates [47, 52, 69, 70].

In the Indian context, the first cases of insecticide resistance were reported in 1971 by Bhatia et al. [71] who found *T. castaneum* collected from the Food Corporation of India, Delhi, to be resistant to malathion. Since then, high frequencies of insecticide resistance were recorded in *T. castaneum* collected from different storage facilities across India. Saxena et al. [72] monitored the dicholorvos resistance status of 13 samples from warehouses of the Food Corporation of India located at Mirzapur and Allahabad. The results revealed that strains from Allahabad exhibited more than ten-fold resistance compared to the Mirzapur strain. The *T. castaneum* population collected from different types of storage premises in Punjab varied in malathion resistance and was measured at a maximum in the populations of beetles collected from a public warehouse in Ropar [73].

Similarly, malathion resistance level in Indian populations of *T. castaneum* collected from thirteen different seed centres was tested and high levels of resistance were found in the Coimbatore strain. Eleven strains differed in terms of resistance levels in the range of 1.18 to 24.53 folds [74]. Insecticide resistance in *T. castaneum* has been studied in most Indian states vis-à-vis different insecticides such as malathion [75], dichlorvos [72], deltamethrin [76, 77], cypermethrin [78]. This rapid increase of resistance against different insecticide classes in India jeopardizes effective pest management strategies. The situation has only worsened with the recurring use of the same insecticide in grain storage facilities, which exert strong selection pressure on *T. castaneum* and hence reduce the efficacy of insecticides. The foregoing results confirm that the development and progression of insecticide resistance in *T. castaneum* is widespread and requires immediate solutions. Since insecticides exist as the mainstay in pest control programs, identifying the factors influencing insecticide resistance is essential in devising new and effective pest management strategies. This review presents a comprehensive picture of different resistance mechanisms and genes governing insecticide resistance in *T. castaneum*.

3. Insecticide resistance mechanisms in *T. castaneum*

The emergence and spread of insecticide resistance in an insect population is a slow and gradual evolutionary process. Following the initial exposure to the insecticide, there is a latent period in which resistance genes are segregated and linked with other genes that contribute favorable conditions for resistance development. During the evolution of resistance under insecticide selection pressure, the target species show a noticeable increase of tolerance to the pesticide. In the next stage, insecticide resistance slowly develops, followed by a period of rapid development, during which many factors influence the selection of resistance to insecticides. Rapidly developing resistance results in explosive population growth of the pests in stored products that become almost impossible to control. It is challenging to detect the resistance mechanism because they emerge over evolutionary time. Many key factors such as intensive application of insecticides, control operations, mode of inheritance of resistance genes, change in fitness of individuals, and genetic background of insects influence resistance [79]. Despite species diversity and chemical diversity of insecticides, only three mechanisms are known to cause insecticide resistance in *T. castaneum:* i) Target site insensitivity, where changes in sensitivity of target site inhibit insecticide binding ii) Metabolic resistance, where the elevated quantity of enzymes lead to increased activities of metabolic detoxification iii) Lack of penetration, where cuticular thickening or cuticular modification prevents penetration of insecticides and render them bound to the target.

The advances in genomic research (e.g., transcriptomic sequencing and whole-genome sequencing) have made significant progress in understanding resistance mechanisms such as metabolic resistance, penetration resistance, and knockdown resistance in *T. castaneum*. An even more fascinating and rapidly advancing area of microbiome research that blends entomology with microbiology is the study of the potential of entire communities of bacteria, viruses, and fungi, that live within the insect hosts, to detoxify insecticides. Existing studies highlight candidate resistance mechanisms such as symbiont-mediated insecticide resistance in various insects and have documented the major bacterial taxa in the adaptation to detoxify xenobiotic compounds [80–83].

Researchers around the world have begun to evaluate the symbiotic associations in different pest populations, how they interact with their hosts and whether they have the potential to detoxify insecticides. Interestingly, bacterial symbionts have been

involved in insecticide degradation and resistance development in some insect pests, weeds, and nematodes. There are a growing number of reports where pest resistance to insecticides is not only due to the mechanisms within the pest genome but also due to the organisms in the microbiome community [84]. However, the microbial communities inhabiting *T. castaneum* and the unique intricate connection between symbionts and insecticide resistance have not yet been investigated. Many fundamental questions about the microbial shifts in response to insecticides and the functions of particular microorganisms in mediating resistance in *T. castaneum* remain unresolved.

3.1 Target site insensitivity

Insecticides such as organophosphates, carbamates, and pyrethroids produce neurotoxicity by inhibiting the enzyme acetylcholine esterase associated with the central nervous system [85–88]. These insecticides also affect other target sites such as voltage-gated sodium channels (VGSC) and gamma aminobutyric acid (GABA) receptors in the insect nervous system [89]. The DDT and pyrethroid insecticides primarily target VGSC in the nervous system [90]. Several potential insecticides such as cyclodienes and fipronil bind to the GABA receptor and block the receptor function [91]. Most commonly used insecticides primarily target different receptors on the nervous system (**Figure 1**).

Insecticide-resistant insects perform normal neurological functions despite the presence of insecticide because they have evolved insensitive acetylcholine receptors which provide resistance to organophosphate and carbamate insecticides. The reduced sensitivity of acetylcholinesterase to OP and carbamate insecticides has been studied in many resistant insect species of agricultural and veterinary importance [92–96]. The reduced target site sensitivity is a result of altered insecticide target molecules. There are mainly four types of target site insensitivity mechanisms observed in various insect species. These include a) Altered Acetylcholinesterase (AChE) resistance mechanism, which provides resistance to organophosphates and carbamates b) Knockdown resistance (*kdr*) mechanism which confers resistance to DDT and pyrethroids c) Reduced GABA receptor sensitivity mechanism, which causes resistance to phenylpyrazoles and cyclodienes and d) Altered nAChRs, which provide resistance to neonicotinoids [89, 97, 98].

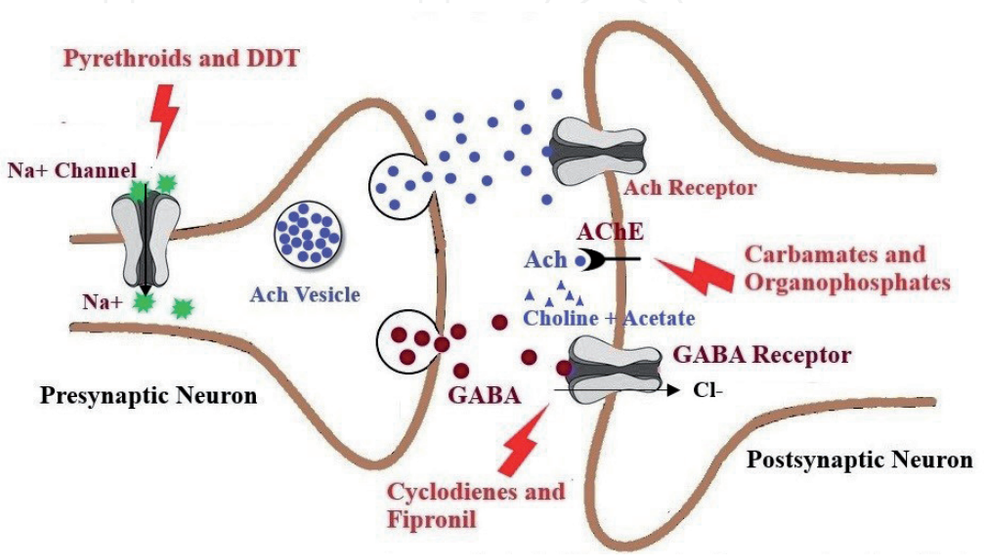

Figure 1.
Diagrammatic representation of pre and post synaptic neurons, showing the different target sites of most commonly used insecticide classes. Source: Adapted and modified from [85–91].

3.1.1 Altered acetylcholine esterase (AChE) resistance mechanism

Acetylcholinesterase (AchE) is a vital enzyme required for regulating the neurotransmitter acetylcholine (ACh). It terminates the synaptic transmission by hydrolyzing acetylcholine into acetate and choline at cholinergic synapses in insects [99]. The inhibition of AchE increases the concentration of the acetylcholine at the synaptic cleft, which leads to a prolonged binding of ACh to its postsynaptic receptor. The high quantity of acetylcholine at the postsynaptic receptor causes neuroexcitation and produces intoxication symptoms such as tremors, convulsions, and eventually paralysis-related death. This enzyme is a target site of organophosphates and carbamates insecticides, which are bound to a serine residue on the active site of AchEs and convert the AchEs into their non-functional form. This causes the accumulation of acetylcholine at the nerve endings and disrupts nerve activity, resulting in paralysis and the death of insects [100].

Several Organophosphorous compounds have been used to protect agricultural commodities from insect infestation. But most of the insects have developed resistance against these insecticides due to insensitive AchE. Target insensitivity of AchE to insecticides occurs through the mutations in the active site of "*Ace*" genes that encode acetylcholinesterase enzyme, which is the most common reason for conferring insecticide resistance. Because of the incessant application of insecticides, different mutations are induced in the *Ace* genes either singly or in combination, which reduces the sensitivity of AchE to the insecticides [101, 102]. The first major research in insects was conducted on *Drosophila melanogaster* that mapped the *Ace* locus at the molecular level and the genomic sequencing effort confirmed that *Ace* encodes the acetylcholinesterase enzyme [103]. The existence of the *Ace* gene and its genome structure has been identified in many insects such as *Drosophila melanogaster* [104], *Musca domestica* [105], *Anopheles gambiae* [106], *Lucilia cuprina* [107], *Tribolium castaneum* [108], *Pieris rapae* [109], *Sitobion avenae* [110], *Bemisia tabaci* [111], *Bombyx mori* [112], *Aphis gossypii* [113], *Plutella xylostella* [114], *Blattella germanica* [115], *Aedes aegypti* [116]. The gene sequence and genomic organization of *Ace* genes in different insects revealed that most insect species possess two *Ace* genes (*Ace* 1 and *Ace* 2) except *Drosophila melanogaster, M. domestica,* and *Lucilia cuprina*. The introduction of the point mutation in *Ace* genes through single amino acid substitution reduces the sensitivity of AChE to insecticides inhibition. These insensitive acetylcholinesterases impart resistance to carbamate and organophosphorus insecticides.

Recent evidence for resistance-conferring mutations in *Ace* genes has focussed on their involvement in insecticide resistance and their biochemical and physiological properties in different insects. Lu et al. [108] studied the genome organization, expression patterns, phylogenies, and three-dimensional models of two *Ace* genes in *T. castaneum* extensively to better understand the functional role of *Ace* genes and the molecular basis of insecticide resistance. The gene sequencing and comparative analysis of AChE1 and AchE2 genes (*Tcas Ace 1 & Tcas Ace 2*) in *Tribolium* revealed that both genes possess different features in the length of their genomic DNA, chromosome locations, and intron/exon organizations. Sequencing full-length cDNAs of AChE genes showed that AChE1 is distributed on chromosome 5 and AchE2 on chromosome 2. In addition, AChE1 consisted of one intron, whereas AchE2 consisted of five introns. Further, extensive protein simulation studies provided evidence that AChE1 has been associated with the hydrolysis of acetylcholine, whereas AChE2 has not been involved in the hydrolysis of acetylcholinesterase substrates. This novel finding prompted Lu et al. [108] to investigate the functional differences of two AChE genes in cholinergic, non-cholinergic neurotransmission, and insecticide resistance by gene-silencing in *T. castaneum*.

RNAi results of both *TcAce1* and *TcAce2* in *T. castaneum* larvae were consistent with the observations of protein modeling studies. Thus, the protein simulation studies of AchE coupled with RNAi experiments have proved that AChE1 is essential for cholinergic neurotransmission and is the target for anticholinesterase insecticides such as organophosphorous and carbamates, which disable the hydrolysis activity of AchE1and cause incapacitation. Whereas, AChE2 is not responsible for neurotransmission in *T. castaneum*. This study also suggested that genetic modifications of AchE1 are most likely responsible for the insensitivity of acetylcholinesterase to organophosphorus and carbamate insecticides. It is remarkable that target site insensitivity is due to different mutations (mainly point mutation) at the catalytic sites of AchEs and conferred resistance in *Drosophila melanogaster* [117], *M. domestica* [105], *Bactrocera oleae* [118], *Leptinotarsa decemlineata* [119], *Chilo auricilius* [120], *Apolygus lucorum* [121]. Sequencing of the gene encoding AchE has generated insights on different point mutations which causes the alteration of AchE genes and a decrease in the sensitivity of anti-cholinesterases insecticides inhibition. In addition, the efficacy of organophosphates and carbamates has been challenged by multiple mutations in the same AchEs of the insects [117, 122]. Thus, the point mutations and multiple mutations result in decreased hydrolytic efficiency of AchE and are associated with insecticide resistance.

3.1.2 Knockdown resistance (kdr) mechanism

Over the years, Pyrethroids have come to be the most sought-after class of insecticides for pest control in commercial and household environments because of their affordable and durable qualities [123]. However, their utility has been limited by the widespread development of insecticide resistance in many major pests. Pyrethroids are synthetic derivatives of pyrethrin, and the pyrethroids were classified into two groups namely class I and class II based on their physical characteristics and knockdown effect against insects. Class I pyrethroids contain a basic structure of cyclopropane carboxylic ester. These compounds include permethrin, resmethrin, phenothrin, bifenthrin, allethrin, tefluthrin, and tetramethrin. Class II pyrethroids contain a cyano group and these compounds include cypermethrin, deltamethrin, cyhalothrin, fenvalerate, cyfluthrin, fenpropathrin, flumethrin. The toxicity of pyrethroids was found to be 2250 times higher in insects than mammals due to their increased sodium channel sensitivity, lower body temperature, and smaller structure [124]. When an insect is intoxicated with non-cyano pyrethroids (class I), it produces strong excitatory action and tremors on the nervous system. The cyano pyrethroids trigger a quite different action, which includes salivation and choreoathetosis. It has been suggested that poisoning symptoms differ based on the cyano or non-cyano pyrethroids [125]. The pyrethrin and pyrethroid insecticides primarily target the VGSC in the nervous system. Pyrethroids and DDT produce their toxicity by binding onto the voltage-gated channels in axonal membranes, altering their gating properties, and the channels remain open for a long time. This causes a prolonged sodium influx, thereby depolarizing the axonal membrane and stimulating the neurons to produce repetitive discharges, finally resulting in paralysis [90, 126]. In insects, modification of voltage-gated sodium channel structure by point mutation or substitution causes insensitivity and reduces the binding affinity of the insecticides to protein.

Knockdown resistance (*kdr*) is one of the major mechanisms involved in resistance to all pyrethroids, pyrethrins, DDT, and its analogs [127]. The *kdr* resistance was first identified in the house fly [126]. Since then, *kdr* has been described in several insects against pyrethroids and an organochlorine class of insecticides [128]. Knockdown resistance occurs due to different point mutations in voltage-gated

sodium channels. These sodium channels are composed of a larger α-subunit (260 kDa) and smaller β-subunits (30–40 kDa). The pore-forming α-subunit has four homologous domains (I – IV), and each domain possesses six transmembrane helices (S1 – S6). The domains are joined together to form a central aqueous pore and the pore is lined by S5, S6 linkers, and S5, S6 helices. In each domain, the S4 segment is involved in voltage sensing, and a positively charged amino acid residue is embedded in every third position [129, 130]. In mammals, the sodium channel encodes nine genes [131] whereas, in insects, the sodium channel encodes only a single gene known as *para* [132, 133]. However, *para* undergoes an extensive alternative splicing process to increase the heterogeneity and functional diversity of sodium channel [133, 134]. These distinct variants of the *para* sodium channel in insects produce different levels of sensitivity to pyrethroids and DDT. The different amino acid substitutions in the para sodium channel variants of nerve membranes have been demonstrated in *M. domestica* [135, 136], *Blatella germanica* [137, 138], *Ctenocephalides felis* [139], *Drosophila melanogaster* [140] that render the loss of sensitivity to pyrethroids. The secondary mutation designated as *super kdr* has been identified mostly in the domain II region of the sodium channel and reported in *B. tabaci* [141], *Haematobia irritans* [142], *Plutella xylostella* [143] which confers enhanced resistance to pyrethroids. The occurrence of the *kdr* mutations in voltage-gated sodium channels limits the efficacy of pyrethroids and it remains a threat to the control of *T. castaneum* [144]. This has been earlier reinforced by the functional expression studies of voltage-gated sodium channel paralytic A gene ($TcNa_v$) of *T. castaneum.* RNAi-induced knockdown results reveal that the $TcNa_v$ gene of *T. castaneum* is a potential candidate to target for the future control of *T. castaneum* and lends support for the use of RNAi as a viable method for controlling this insect [139]. The results of this study provide convincing evidence which shows that pyrethroid resistance in *T. castaneum* correlated with the presence of point mutations in the sodium channel *para* gene of insect nervous membrane. Thus, the identification of *kdr* mutations provides insights into the resistance mechanism in *T. castaneum* and has also proven critical for designing new insecticides for insect control.

3.1.3 Reduced GABA receptor sensitivity mechanism

The GABA ionotrophic receptor of the neuron membrane is formed by the oligomerization of five subunits around a central pore and each subunit possesses a large N terminal domain and four membrane-spanning domains (M1–M4). Several potential insecticides such as cyclodienes and fipronil stick to the M2 membrane-spanning a region of GABA receptor as competitive inhibitors, which prevent the chloride uptake of the GABA ion channel. The inhibition of GABA stimulated chloride uptake enhances the firing of nerve impulses in insects, which initiates lethal effects on insects [145]. However, the mutations or modifications in the molecular structure of the GABA ion channel influence the activity of insecticides. The mutated GABA receptor becomes insensitive to the insecticides at varying levels and this insensitivity has the potential to increase resistance in an insect species.

Such a resistant modification was first characterized in the GABA receptor subunit gene *Rdl* ('Resistance to Dieldrin gene') of *Drosophila* by using positional cloning approach [146, 147] and polymerase chain reaction [148]. The resistance-associated mutation studies in resistant *D. melanogaster* strains showed that the amino acid alanine^{302} in GABA gated chloride channel encoded by *Rdl* was replaced by serine. This single amino acid replacement was found to be associated with resistance in *D. melanogaster* and exhibited 4000 fold resistance against *cyclodiene* [149]. The cyclodiene resistance was conferred by replacing a single alanine at the second membrane-spanning region M2 with serine or glycine, which readily

prevents direct binding of drugs and allosterically modifies the conformation of the Rdl receptor. Further studies on point mutation (Ala^{302} to Ser) in the GABA gated chloride channel gene *Rdl* in different insect orders, namely Diptera, Coleoptera, and Dictyoptera, conclude the fact that mutations associated with single base-pair replacement are highly conserved [150]. The remarkable conserved nature of single Ala > Ser mutation in the GABA receptor was confirmed in *D. simulans, T. castaneum,* and *B. germanica* by amplifying the resistance-associated *Rdl* sequences and the same replacement of Ala > Ser as found in *D. melanogaster* was observed. This finding raises the question as to whether this resistance-associated substitution arises once and then disseminates globally or if resistance arises independently in different populations. To address this, Andreev et al. [151] collected 141 strains of *Tribolium* globally and screened for dieldrin resistance. Of the 141 strains, 23 homozygous resistant strains and 6 susceptible strains were chosen for *Rdl* sequencing and mutational differences were compared among them. Phylogenetic analysis provided strong evidence of multiple independent origins of dieldrin-associated mutation, which suggests that resistance surfaced independently in 23 resistant *Tribolium* strains. *T. castaneum* genome sequencing effort facilitated the characterization of GABA gated ion channels and the post-translational modification of the *Rdl* gene. The post-translational modification such as alternative splicing have been identified in exons 3 and 6 of the *T.cas Rdl* gene and observed the three splice variants for exons 3. This information on *Rdl* isoforms of *T.cas* helped to investigate their contribution in modifying the tolerance to insecticides [152]. These results suggest that variant isoforms of the *Rdl* gene define insensitivity to cyclodiene and have gone on to develop resistance to cyclodiene in *T. castaneum* through alternative splicing of exon3 and exon6. This is a significant factor enhancing cyclodiene insensitive transcripts in *T. castaneum*.

In addition, genome sequencing advancements have facilitated the detection of multiple *Rdl* genes in Lepidopteran genomes [153, 154] and *Acyrthosiphon pisum* genome [155]. The multiple origins of the resistance-associated mutation have implications for understanding the evolution of resistance, the spread of resistance, and its management. Interestingly, the polymerase reaction amplified the same region in different resistant strains of insects *T. castaneum* [138], *D. simulans* [156], *B. germanica* [157] and confirmed that this single base pair substitution conferred the cyclodiene resistance. Another replacement at residue $A^{296}S$ (equivalent to position 301 in *D. melanogaster*) was reported in *Anopheles arabiensis* and *A. gambiae* [158] was found to be associated with higher levels of dieldrin resistance. Similarly, *Rdl* mutation with the replacement of Ala^{302} with Serine conferred 237-fold fipronil resistance in *Nilaparvata lugens* [159]. The single base pair substitution (Ala to Ser/Gly/Asn) in the *Rdl* receptor at the site analogues to 301 in *Drosophila* has also been identified in *Laodelphax striatellus* [160, 161], *Anopheles funestus* [162]. These findings suggest that point mutation and post translational modification of the *Rdl* gene are significant evolutionary phenomena in insects and modulate insecticides' binding/sensitivity, which makes insects resistant to most natural and synthetic insecticides.

3.2 Metabolic resistance

The most common resistance mechanism in insects is the metabolic detoxification mechanism, enabling the insect to degrade or sequester the insecticides faster before releasing their toxic effect. This resistance mechanism allows insects to overproduce the enzymes mainly cytochrome *P450* monooxygenases (*CYP450s*), carboxylesterases (*CarEs*), and glutathione S transferases (*GSTs*), to thwart the toxic effects of insecticides. This advantage helps to evolve resistance in *T. castaneum*

populations for all main classes of insecticides currently used for stored product pest control such as organophosphates, carbamates, and pyrethroids [163–165]. The comprehensive genomic and transcriptomic analysis has led to the identification of the key genes encoding detoxifying enzymes such as *CYP450s*, *GSTs*, and *CarEs*. These genes are being frequently associated, over the past few decades, with the rise of insecticide resistance in *T. castaneum* through overexpression, copy number variation or gene duplication, coding sequence mutations, or as a combined effect of these mechanisms.

Several studies showed that resistant insects possess generally higher levels of *P450* dependent monooxygenases, and have high catalytic activity towards the toxicant [166, 167]. Some studies showed that amplification of transferase gene exerts insecticide sequestration/detoxification of many different endogenous and xenobiotic substances including insecticides [168–170]. The studies of the mechanisms of metabolic resistance to carbamates, organophosphates, and pyrethroids have revealed the role of esterases (especially carboxylesterases) in resistant insect species [171–176]. These enzymes are capable of sequestering insecticide substrates through two principal mechanisms 1) Overexpression of one or more esterases and 2) Mutations in gene encoding esterase [171].

3.2.1 Cytochrome P450s (CYP450s)

Recent years have witnessed the rapid evolution of insecticide resistance due to their continuous exposure. However, the resistance mechanism in insects is not fully understood, and the evolution of resistance to insecticides in *T. castaneum* populations threatens the long-term future of the food storage system. The exposure of *T. castaneum* to insecticides triggers a complex defense response that includes genes that encode key Cytochrome *P450* monooxygenase detoxification enzymes. These metabolic systems are involved in the inactivation of xenobiotic compounds such as pesticides and drugs [177]. The availability of whole-genome sequence and well-functioning RNAi proves *T. castaneum* as a powerful model system for studying insecticide resistance and functional genetics [178]. Whole-genome sequencing of *T. castaneum* identified 133 functional *CYP* genes and 10 *CYP* pseudogenes [179]. These 143 genes belong to four clans (clan1, clan2, clan3 and mitochondrial clan), 26 families, and 59 subfamilies. Nine new families were identified including *CYP3* clan families *CYP345, 346, 347*, and *348* and mitochondrial family *CYP353*; *CYP4* clan families *CYP349, 350, 351*, and *352* and mitochondrial family *CYP353*. To identify the phylogenetic and evolutionary relationships of *T. castaneum CYPs* with *CYP*omes of other insects, four phylogenetic trees were constructed and a remarkable 1:1 orthology of *CYP2* and mitochondrial clans of *T. castaneum* with *D. melanogaster, A. gambiae,* and *A. mellifera* insect genomes was observed, suggesting functional conservation of these *CYPs* [180]. The number of *P450* genes in *T. castaneum* is much larger than *D. melanogaster* and *A. gambiae* but considerably lower than *C. quinquefasciatus* and *A. aegypti*. The large number of *CYP* genes in *T. castaneum* provides excellent protection against xenobiotics and other insecticides via an enzymatic detoxification mechanism [178]. Among 143 *CYP* genes, 99 *T. castaneum CYPs* were mapped on 9 chromosomes, 87 of which were located on six chromosomes LG3, LG4, LG5, LG6, LG8, and LG9. No *CYP* gene was mapped on the LG1 = X chromosome. The distribution and location of another 44 *CYPs* on the chromosome remain unknown. This confirms that several genes are under gene duplication events and that they descended from a common ancestral *P450* gene [180, 181].

Increased detoxification by cytochrome *P450s* has been considered to be the major mechanism involved in insecticide resistance of *T. castaneum*. *CYP450* gene

CYP6BQ9 showed 200-fold overexpression in the deltamethrin-resistant *QTC279* strain of *T. castaneum* and this upregulation suggests that *CYP6BQ9* has a significant impact on *T. castaneum* to metabolize deltamethrin [144]. Functional genomic and qRT-PCR based methods revealed that the high expression of *CYP6BQ9* in the brain might enhance the ability of the brain cells to catalyze deltamethrin and provide the defenses to protect the target site [144]. Additionally, RNAi-mediated knockdown of possible transcription factors was performed to understand the mechanism of the overexpression of the *CYP6BQ9* gene. Out of the 7 transcription factors tested, CncC and Maf transcription factors have been identified as key regulators for the activation of *CYP6BQ* genes and responsible for deltamethrin resistance in *T. castaneum* [182]. In another study, RNA sequencing, RNAi knockdown, and qRT-PCR data showed the involvement of CncC in the regulation of expression of multiple detoxification genes involved in phase I (*P450s*) and phase II (*GSTs*), and Phase III (ABC transporters) detoxification mechanisms in pyrethroid resistance strain of *T. castaneum* [183]. Both studies suggest that transcription factor CncC is required for the induction of genes coding for proteins involved in xenobiotic degradation. Many studies in flies and beetles have reported CncC regulation of expression of genes coding for proteins involved in phase I (*P450s*) and phase II (*GSTs*) detoxification mechanisms [184, 185].

CYP4BN6 and *CYP6BQ11* expression was induced in *T. castaneum* by dichlorvos and carbofuran and a higher level of expression of these two genes in late pupal and adult stages was detected. Furthermore, RNA interference (RNAi) mediated knockdown repressed the expression and increased the susceptibility of *Tribolium* to these two insecticides, suggesting that *CYP4BN6* and *CYP6BQ11* genes play an important role in developing resistance. More significantly, in addition to the findings mentioned above, expression of both *TcCYP4BN6* and *TcCYP6BQ11* was reduced by *latrophilin* (*lph*) gene knockout, indicating that these two *CYP* genes are controlled by the *lph* gene responsible for the susceptibility of the beetles to insecticides [186]. Cytochrome *P450s* are a supergene family of metabolic enzymes and the upregulation of *CYP* genes mediated by different insecticides has been extensively studied in *T. castaneum* [163]. Liang et al. [163] found that three (*CYP4G7*, *CYP4BR3,* and *CYP345A1)* out of the eight selected *CYP* genes (*CYP4G7*, *CYP4Q4*, *CYP4BR3*, *CYP12H1*, *CYP6BK11*, *CYP9D4*, *CYP9Z5,* and *CYP345A1*) showed high expression when the insects were exposed to four insecticides- cypermethrin, permethrin, cyhalothrin, lambda imidacloprid. Also, selected genes from *CYP6* and *CYP9* families did not exhibit any insecticide mediated overexpression in this study, although the genes from these families are known to confer resistance to a wide range of insecticides and metabolic detoxification [144, 182]. Specifically, they found that the upregulation of a specific gene can be influenced by insecticide concentration, developmental stage of insects, and exposure duration. They also suggested that the overexpression of *CYP* genes was affected by relatively low concentrations of insecticides, and increasing insecticide concentration did not show any significant upregulation, possibly due to increased toxic stress to the insects. In addition, tissue-specific expression patterns of *CYP* genes revealed that 7 out of 8 *CYP* genes were significantly upregulated in insect detoxification tissues including malpighian tubules, midgut, and fat bodies. The possible role of *CYP450* genes in phosphine resistant strain of *T. castaneum* has been studied previously and two *CYP* genes (*CYP4Q4* and *CYP4Q7*) are overexpressed in the midgut of permethrin resistant *T. castaneum* strain [187]. Two *CYP450* genes *CYP4Q4* and *CYP4Q7* identified in a pyrethroid-resistant strain of *T. castaneum* showed some level of upregulation which indicates that overexpression of *CYP450* genes is an important factor governing insecticide resistance in *Tribolium* [188]. The previous studies have shown that *T. castaneum* has developed insecticide resistance to 33 active ingredients [189]

and genomic sequence analysis revealed an expansion of members of *CYP* families belonging to metabolic detoxification enzymes [179]. Zhu et al. [178] characterized the expression and induction of *CYP6BQ* gene cluster in deltamethrin resistant strain of *T. castaneum*, revealing that 10 out of these 12 genes were significantly upregulated in resistant strain than in the Lab-S susceptible strain. Moreover, the tissue-specific expression pattern of genes within the *CYP6BQ* cluster found that four genes (*CYP6BQ9, CYP6BQ5, CYP6BQ2, CYP6BQ4*) and three genes (*CYP6BQ11, CYP6BQ2, CYP6BQ4*) were significantly upregulated (>100 fold) in the tissues of the head and midgut respectively. All these studies have shown that overexpression of a specific *CYP* gene can be influenced by the type of insecticide, toxicity of insecticide, concentration of insecticide, exposure duration, and physiological status of insects.

3.2.2 Glutathione S tranferase (GSTs)

The glutathione S tranferase (*GSTs*) is a superfamily of multifunctional enzymes involved in insecticide resistance [190]. These enzymes metabolize the insecticides by conjugation reaction with reduced glutathione to hydrophobic xenobiotics and produce water-soluble metabolites that are easily excreted. Insect's *GSTs* were classified based on their location within the cell- cytosolic, microsomal, and mitochondrial [191, 192], and these *GSTs* are members of Delta, Epsilon, Sigma, Theta, Omega, and Zeta protein classes in arthropods [193]. Cytosolic *GSTs* possess a carboxyl (C)-terminal α-helical domain and an amino (N)-terminal α/β-domain joined by a variable linker region. The N terminal region is comprised of a highly conserved G site, which binds reduced GSH, and the C terminal domain consists of a highly variable H site that interacts with hydrophilic substrates. This hypervariability characteristic of the H site allows *GSTs* to metabolize various hydrophobic residues [194]. Sequencing the insect's genome provided an opportunity to identify and characterize the *GSTs* on a genome-wide scale [192, 195, 196]. This provides a platform for a better understanding of the evolution of insecticide resistance in arthropods. Using the genome sequence of *T. castaneum,* 36 putative cytosolic *GSTs* and 5 microsomal *GSTs* were discovered [192]. Among the 41 *GSTs*, thirty-eight *GSTs* were located on 4 chromosomes and the remaining three *GSTs* were mapped to other 3 of the 10 *T. castaneum* chromosomes. *T. castaneum* possesses the 3 Delta *GST* genes and 19 Epsilon *GSTs* gene, which were the fewer and higher *GST* genes than in *Drosophila, Anopheles, Apis, Bombyx, and Acyrthosiphon* [192, 197]. The expansion of the Epsilon class in *T. castaneum* indicates that they are frequently involved in high duplication events than the other four *GST* subclasses (Omega, Theta, Zeta, Sigma) and are fairly variable between different species and conserved within the species. And the four *GST* subclasses of Omega, Theta, Zeta, and Sigma of *T. castaneum* possess three, one, one, and seven genes respectively [192]. The previous studies reported that the Epsilon class of *GSTs* encoding enzymes are responsible for degrading certain insecticides such as DDT and pyrethroids in *Aedes aegypti* [198] and *Anopheles gambiae* [199]. The detoxification ability of the Epsilon class of *GSTs* in different insects suggested that *T. castaneum* maintains higher insecticide resistance and such tolerance may be due to the presence of expanded epsilon *GSTs* [192]. In addition to the epsilon class of *GST* mediated detoxification, the delta class of *GSTs* in *T. castaneum* was engaged in resisting poisonous chemicals and developing resistance to certain kinds of insecticides [200].

To gain insights on the regulatory, functional, and biological significance of *GST* delta 1 *T. castaneum* (*TcGSTd1*), Chen et al. [200] merged the RNA-sequencing technology and RNAi of control and RNAi treated larvae (*ds-TcGSTd1*) of *T. castaneum*. The results from this study established that *TcGSTd1* took part not only in the

detoxification process but was also involved in insect fitness, survival, reproduction, and development. Further, Song et al. [201] conducted functional research on three deltas *GSTs* of *T. castaneum* (*TcGSTd1*, *TcGSTd2*, and *TcGSTd3*) to identify their role in insecticide degradation, metamorphosis, and physiology. The three delta *GSTs* of *T. castaneum* with their full-length sequences were identified and further characterized by cloning and sequencing. In this study, the expression levels of three delta *GSTs* were consistent across all developmental stages, implying that they may act as housekeeping genes and play an important role in the metamorphosis of *T. castaneum*. The expression profiling experiments revealed greater expression of *TcGSTd3* and *TcGSTd2* and lower expression of *TcGSTd1* after exposure to phoxim and lambda-cyhalothrin. Interestingly, the expression of *TcGSTd2* and *TcGSTd3* significantly increased by phoxim treatment than with the lambda-cyhalothrin. The results from this study imply that under elevated *TcGSTd2* and *TcGSTd3* activity conditions, *T. castaneum* can detoxify phoxim activation products, leading to resistance development. Similarly, elevated levels of *GSTs* have been reported to be associated with insecticide metabolism and producing resistance in many insects [171, 198, 202, 203]. In addition, resistance was induced by gene duplication within the structural *GST* genes which changes their substrate specificity [204]. Thus, the knowledge of *GST* mediated detoxification mechanism helps to detect resistance at an early stage, to remove the particular insecticide before the resistance alleles become fixed in the populations, and to design an effective molecule of insecticide.

3.2.3 Carboxyl Esterases (CarEs)

Carboxylesterases are ubiquitous enzymes involved in the detoxification of ester-containing xenobiotics. They are members of the esterase family of enzymes and have been isolated from all living organisms. As their name suggests, they are involved in hydrolysis reactions and convert the carboxyl esters into carboxylic acid and alcohol. Hydrolysis of the ester bond includes hydrolysis of a diverse range of phospho, thio, carboxylic, and other ester substrates. For carboxylesterases, the hydrolysis reaction is accomplished by 2 steps- first, the nucleophilic attack of oxygen of a serine residue on the carbonyl group of the substrate, removing the alcohol product, and generating relatively stable acyl enzymes. Second, a water molecule acts as an intermediary and makes a nucleophilic attack to remove the acid product of the reaction and produce the free enzyme. This reaction mechanism causes insecticide resistance in many insect species. As a key component of the detoxification mechanism, esterases have focused on the research of xenobiotic metabolism and resistance. The expression of carboxylesterases was significantly upregulated in the organophosphorous resistant *Aphis gossypii* strain than the susceptible strain [205]. Elevated carboxylesterase activity and carboxylesterase expression were identified in the pyrethroid-resistant strain of *Musca domestica* [206] and the tolerance to cypermethrin in *Musca domestica* was induced by high *CarE* enzyme activity. Similarly, the elevation of *CarE* activity in OP resistant and susceptible *Nilaparvata lugens strain* and the increased expression suggests that *CarE* mRNA was related to OP resistance in *Nilaparvata lugens* [207].

The occurrence of multiple mechanisms in an insect develops a very high level of resistance and in the case of *T. castaneum,* resistance was highest against pirimiphos-methyl and bifenthrin. It is interesting to observe that *T. castaneum* used two genetic strategies to adapt to these insecticides attack 1) a pool of Laccase2 enzyme ensured the protection by synthesizing the thicker cuticle which prevented the entry of insecticide into the insect body 2) a pool of esterases and lipases contributed the protection by hydrolysing or sequestering which rendered the insecticides ineffective [208]. Similarly, the functional role of two carboxylesterase

genes of *T. castaneum* (*Tcest4* or *Tcest6*) were investigated by RNAi and identified their interaction with *Latrophilin* [165]. *Latrophilin (lph)* is an adhesion G-protein-coupled receptor that is essentially involved in the physiological process and cellular detoxification process although one member of *lph* existed in *T. castaneum* [209, 210]. The induction of *Tcest4* and *Tcest6* gene expression after treatment with carbofuran or dichlorvos insecticides revealed their detoxification ability and the RNAi of *Tcest4* and *Tcest6* further confirmed that it had a vital role in insecticide resistance. In addition, the study suggested that *lph* has a vital role in regulating the activity of *Tcest4* and *Tcest6* [165]. All these studies revealed the detoxification ability of carboxylesterases towards toxicants and the induction characteristics of some carboxylesterases could be used as biomarkers to assess the resistance against certain xenobiotics.

3.3 Reduced cuticular penetration resistance mechanism

Reduced penetration resistance is uncommon and little is known about its workings in insects. Reduced penetration is also called cuticle resistance that reduces the dose of the insecticide reaching into the insect's body and in all probability strongly associated with insecticide resistance. Normally contact insecticide penetrates through the insect cuticle and reaches the target site for action [172]. The cuticle is composed mainly of two different components, chitin, and cuticular protein, and the three functional layers of the cuticle consist of the outermost envelope, protein-rich epicuticle, and chitin-rich procuticle [211, 212]. Cuticular barriers develop resistance in insects by altering the cuticular thickness or by changing the cuticular composition [213, 214] or remodeling the cuticle by the high occurrence of cuticular proteins. The overexpression of laccases and ABC transporters has been reported to be involved in the compositional change of cuticle, which increases insects' tolerance to insecticides in the environment. Arkane et al. [215] revealed the association between the cuticle tanning and the expression profile of *T. castaneum* Laccase2 (*TcLac2*) from pupation to adult eclosion. This study unambiguously demonstrated that RNAi of *TcLac2* affected the cuticle tanning and produced the cuticle to be unpigmented. The dysfunctional *TcLac2* affected not only the adult and pupal cuticle but also the larval cuticle of *T. castaneum*. The suppressed *TcLac2* produces white and more flexible cuticles in pupal or newly molted adults of *T. castaneum*, facilitating the entry of insecticides into its body, and the high occurrence of expressed *TcLac2* mediates the compositional change of cuticle, which reduces the penetration of insecticides. Consistent with this interpretation, a pool of laccases was expressed highly in *T. castaneum* that served as protection against pirimiphos-methyl and bifenthrin [208]. Besides the overexpression of laccase, cuticular penetration resistance has been reported in combination with the upregulated activity of ABC transporters and *CYP450* genes [178, 208]. Typically, a combination of two or more mechanisms contributes significantly strong resistance than a single resistance mechanism [177]. In addition, several cuticular proteins (*TcCPR18*, *TcCPR4*, and *TcCPR27*) were identified from *T. castaneum* and found to be associated with the formation of the rigid cuticle [216]. However, their exact role in the cuticular penetration mechanism remains elusive. Thus, future functional characterization of cuticular proteins could provide a strong foundation for identifying the major players involved in the cuticular resistance mechanism, enabling the development of new resistance management strategies.

Researchers have been previously using bioassays, genetic and biochemical techniques to study the resistance mechanisms in *T. castaneum*. The application of whole-genome and transcriptome sequencing platforms provided a larger repository of gene resources for further investigations of resistance mechanisms in

T. castaneum. Known mechanisms that confer insecticide resistance in *T. castaneum* include 1) target site insensitivity 2) metabolic resistance 3) reduced cuticular penetration. These different forms of resistance mechanisms have been identified to act in compounding layers to degrade or sequester the insecticides faster before releasing their toxic effect. (**Figure 2**). In pest model beetle *T. castaneum,* different biochemical and molecular mechanisms were investigated against different insecticide classes (**Table 1**).

3.4 Symbiont mediated insecticide resistance mechanism

Recent years have seen a sharp increase in the study of insect microbiome, its crucial role in metabolic detoxification, and modulation of host immune responses. In some insect hosts, the symbiotic association appears to be causal for insecticide degradation, whereas, in others, studies suggest that it is mediated by physiological trade-offs [225]. In addition, the relationship between microbial community and insecticide resistance differs greatly and is context-dependent [225]. Several studies have established a causal connection between the fitness-enhancing symbionts and insecticide resistance in the bean bug, *Riptortus pedestris* [226], *Bactrocera dorsalis* [81],

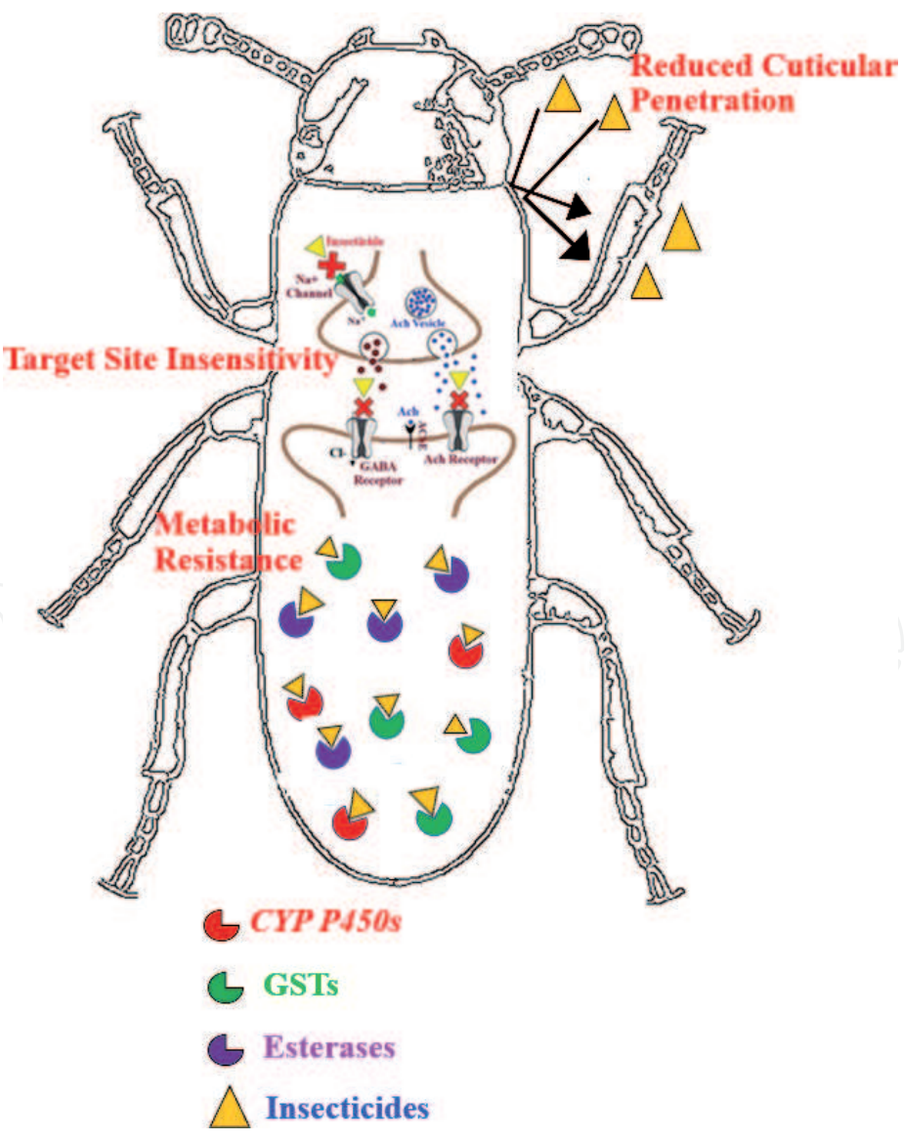

Figure 2.
Overview of types of resistance mechanisms in T. castaneum. Source: Adapted and modified from [108, 144, 151, 177, 215].

Sl. No.	Insecticide	Type of Resistance	Mechanism	References
1	Cyclodiene	Point mutations in the gene Resistance to dieldrin (*Rdl*)	Target site insensitivity	[138, 151]
2	Pirimiphos-methyl and bifenthrin	Elevated activity of lipases, esterases, and laccase2	Combination of reduced cuticular penetrance and metabolic detoxification	[208]
3	Dichlorvos, malathion, carbaryl, and carbofuran	Mutations in *AchE* gene	Reduced insensitivity of AChE	[108]
4	Malathion	Elevated activity of carboxylesterase	Metabolic detoxification	[217–220]
5	Malathion	Elevated activity of glutathione transferase	Metabolic detoxification	[221]
6	Deltamethrin	Elevated activity of *CYP6BQ9*	Metabolic detoxification	[144]
7	Phosphine	Elevated activity of *CYP346B1*, *CYP346B2*, and *CYP346B3*	Metabolic detoxification	[222, 223]
8	Phosphine	Elevated activity of *CYP4Q7*, *CYP4Q4*	Metabolic detoxification	[187]
9	Permethrin	Elevated activity of *CYP4Q4* and *CYP4Q7*	Metabolic detoxification	[187]
10	Cypermethrin, Permethrin and Cyhalothrin	Elevated activity of *CYP4G7* and *CYP345A1*	Metabolic detoxification	[163]
11	Phosphine	Elevated activity of *CYP345A1* and *CYP345A2*	Metabolic detoxification	[164]
12	Dichlorvos and carbofuran	Elevated activity of *CYP4BN6* and *CYP6BQ11*	Metabolic detoxification	[186]
13	Carbofuran or dichlorvos	Induction of carboxylesterase	Metabolic detoxification	[165]
14	Pirimiphos-methyl	Reduced cuticular penetration	Cuticular penetration resistance mechanism	[224]
15	Phoxim	Elevated activity of *GSTd2* and *GSTd3*	Metabolic detoxification	[201]

Table 1.
Studies performed in T. castaneum *showing different resistance mechanisms.*

Anopheles stephensi [227], *Lasioderma serricorne* [228], *Spodoptera frugiperda* [229], *Plutella xylostella* [230]. Among these studies, symbionts like *Burkholderia*, *Citrobacter freundii*, *Bacillus thuringiensis*, *Enterococcus mundtii,* and several gut bacteria provide physiological and evolutionary modifications to their insect host, thereby enhancing protection in a significant portion of insect pest taxa.

Increased access to a rapidly advancing metagenomic approach facilitated the understanding of the role of microbial communities in insecticide resistance, and several studies hint at this association. The initial research that isolated the bacteria and fungi from different life stages of *Tribolium confusum* (closely related to *T. castaneum*) dates back 60 years. It provided important clues on insect fecundity and pupation rate

when grown in microbe-free flour. Momir Futo [231] studied the immune priming phenomenon (a form of immune memory) in *T. castaneum* and investigated its high survival response when orally exposed to bacterial components of *B. thuringiensis bv. Tenebrionis*. The evidence suggests that microbiota plays a significant role in the activation of immune priming and produces various immunological responses to enhance the protection of this species. A recent study systematically characterized the microbiome of *T. castaneum* across life stages and sexes and observed microbiome-mediated fitness benefits such as increased fecundity, increased offspring survival, and long lifespan. They also observed a correlation between wheat flour microbiome and host beetle microbiome [232]. All these studies have established different aspects of microbiome-derived fitness in *T. castaneum*. However, the microbial communities inhabiting *T. castaneum* and the unique intricate connection between symbionts and insecticide resistance have not been investigated thus far. A high-throughput metagenome sequencing approach is required to reveal the link between microbiota and insecticide resistance. Discovering this link and exploring the bacteria known to degrade insecticides within *T. castaneum* may offer novel insights into the unknown insecticide resistance mechanisms. Understanding the symbiont-associated resistance mechanism in *T. castaneum* has broader implications for developing integrative resistance management strategies.

4. Methods to monitor insecticide resistance in *T. castaneum*

Multiple reports indicate that *T. castaneum* has developed resistance to almost all insecticides [47, 51, 67, 72, 73]. The deleterious consequences of insecticide resistance in *Tribolium* have created a crisis in pest management programs. In the last decade, various research groups have identified several mechanisms involved in insecticide resistance in *T. castaneum* [39, 163, 164, 178, 208, 210]. They approached this problem at different levels and incorporated a diverse range of approaches like conventional toxicity bioassays, biochemical assays, gene expression, and functional genomic studies.

Different toxicity bioassays were used to measure resistance in the early stage in a cost-efficient manner. The conventional bioassay that is used to diagnose resistance involves collecting *T. castaneum* from the grain storage facilities and warehouses and rearing them until sufficient populations for testing. Mortality of larva, pupa, or adults is then estimated after exposure to series of concentrations of insecticide compounds. Subsequently, a Log dose probit assay is used to determine the LC_{50} or LC_{99} values of field-collected and susceptible populations. Then the resistance ratio was calculated using LC_{50} and LC_{95} values from *T. castaneum* field-collected populations compared with LC_{50} and LC_{95} values of the susceptible laboratory strain of *T. castaneum*. Different techniques such as topical application [233], residue exposure test [163], filter paper [39], diet incorporation method [234] are used to diagnose and determine the causes of pest control failure by insecticide selection pressures under field conditions.

The fully sequenced and annotated genome of *T. castaneum* has facilitated the identification of many genes involved in resistance [179]. Thus, further resistance research is centered on identifying genes involved in mediating insecticide resistance, particularly *CYP450s, GSTs*, and *CarEs*. Transcriptome profiling study identified differentially expressed miRNAs in four major life stages of *T. castaneum* and validated them by using real-time PCR experiments [235]. Several studies relied on different techniques such as Next-generation sequencing, RNA isolation, First-strand cDNA Synthesis, Real-Time PCR to evaluate the responses (upregulation or downregulation) of detoxifying genes against various insecticides in different

tissues, developmental stages [163]. However, the resistance mechanism in insects was not fully understood. In this scenario, extensive genetic analysis is critical for understanding the function of genes involved in developing insecticide resistance which can then be targeted to suppress the further evolution of resistance. Thus, the RNAi technology complemented with expression studies to investigate the correlation between mRNA and the function of genes to rule out the role of these genes in resistance [144]. This gene silencing mechanism suppressed the target gene expression in *T. castaneum*, caused rapid and widespread mortality within the pest population. Here, different steps include target genes selection, isolation of *T. castaneum* at the proper stage for injection, establishing dsRNA production methodology, knockdown of target genes by injecting dsRNA directly into egg and larva and relative expression of knocked down gene-specific transcripts were employed to accomplish gene silencing [236]. To evaluate the specificity of dsRNA effects, quantitative PCR experiments were also carried out to check the expression of housekeeping genes such as actin or tubulin as a control [236]. RNAi-based gene silencing has the potential to down-regulate the expression of resistance-relevant genes and accelerate the discovery of gene function in *T. castaneum*. Thus, knock-down of upregulated resistance-relevant genes in *T. castaneum* is incredibly valuable in elucidating gene functions and provides information on the process that makes *T. castaneum* resistant or susceptible.

Resistance research on this beetle further improved by the most advanced approach Clustered regularly interspaced short palindromic repeats (CRISPR) system [237]. Recently, CRISPR is the best available method on vogue in order to explore the functional genes relevant to resistance in *T. castaneum* [238]. Considering the relevance of resistance inducing genes in *T. castaneum,* genome editing technology plays an important role in determining the functional genes involved in insecticide resistance. An enhanced conceptual understanding of Genome editing in *T. castaneum* will facilitate the application of CRISPR for dis-section of gene function and fast-track the application of CRISPR to control these destructive pests [238].

Further, on the basis of gene expression, knockdown and genome editing studies, we could generate information on insecticide resistance levels of pest populations in our country. This information would offer a unique opportunity for overcoming or delaying resistance in *T. castaneum*. With the introduction of these advanced genetic technologies, the food security of our country could outstrip the population growth and ensure the supply of high-quality, safe, and economically stored grain products.

5. Conclusion

Insecticide resistance poses a major threat to global pest control efforts and elucidating the underlying mechanisms is critical for effective pest management. Most of the important pests of stored products have evolved resistance to commonly used insecticides the world over. *T. castaneum* is a pest of stored products that causes significant damage to cereal products, flour, grain, and rice bran. This omnivorous beetle has become so resistant to a range of insecticides that it can tolerate exposure to any insecticide. Many key factors such as intensive application of insecticides, control operations, mode of inheritance of resistance genes, change in fitness of individuals, and genetic background of *T. castaneum* influence the resistance.

This review attempts to address critical questions around how insecticide resistance emerges in *T. castaneum*, such as how many resistance mechanisms exist in a species genome? How do the different point mutations in ion channel receptors

cause resistance? How does this mutation transfer globally? How many single or multiple mutations give rise to resistance? How many detoxifying genes are involved in resistance development? How do these genes provide resistance to all insecticides? How does their expression control resistance? What new mechanisms are still unexplored and what might be their role?

The availability of whole-genome sequence and applications of RNAi have made significant progress in understanding resistance mechanisms in *T. castaneum*. If *T. castaneum* comes into contact with an insecticide, the cuticle may be modified or thickened, eventually slowing down the penetration of insecticide molecules beyond the cuticular layer. If the insecticides enter the insect's body, *T. castaneum* can increase the expression of several genes from metabolic enzyme families (e.g., esterases, mixed-function oxidases, glutathione S-transferases) to detoxify the insecticidal effect. Eventually, if the insecticides enter the nervous system to act on the target sites, mutations are introduced into the active site of genes (e.g., *kdr* mutations, super *kdr* mutations), which can decrease the sensitivity of the target site to the insecticide. These are the three genetic modifications that reduce the lethal effects of an insecticide, thus developing pest resistance to organophosphates, pyrethroids, and neonicotinoids. Although metabolic detoxification and reduced target site insensitivity have been extensively studied, reduced cuticular penetrance mechanisms exist outside these paradigms. However, there are reasons beyond these factors that could shape resistance in insects, including microorganisms that enhance the degradation of insecticides. Many fundamental questions about the microbial shifts in response to insecticides and the functions of particular microorganisms in mediating resistance *in T. castaneum* remain unresolved.

The comprehensive genomic and transcriptomic analysis have improved the identification of the key genes encoding detoxifying enzymes such as CY*P450s*, *GSTs* and *CarEs*. The understanding of the detoxification mechanism responsible for insecticide resistance allows us to detect resistance at an early stage, remove the particular insecticide before the resistance alleles become fixed in the populations, and design an effective molecule of insecticide. Thus, understanding which insecticide is degraded by what genes is crucial to tackling the resistance problem. However, the contribution of most of the genes still needs to be confirmed by extensive genetic analysis such as RNAi and gene functional characterization. Knockdown of upregulated detoxifying genes in *T. castaneum* is immensely valuable in elucidating gene function and provides information on the factors that make *T. castaneum* resistant or susceptible. This double-stranded RNAi-mediated experiment paves the way for understanding the mechanisms causing resistance in *T. castaneum*.

T. castaneum resistance research has progressed effectively, from initial single-mutation study to multiple mutations in *Rdl* gene of GABA receptor, from examining the mutation in *Ace* gene to functional characterization of *Ace* gene of Acetylcholineesterase receptor, from sequencing the amino acid substitution of the *para* sodium channel to gene knockdown characterization, from single-gene sequencing to whole-genome analysis, from exploring transcriptional gene expression to functional expression analysis and from synergistic measures of metabolic detoxification to specific gene expression quantitation. The outcomes of these efforts have provided a clearer picture of molecular targets of different insecticides, complex resistance mechanisms, detoxifying genes, gene expressions, modification of target receptors, and have generated fresh insights for the development of targeted novel insecticides. Thus, this detailed review on complex resistance mechanisms and the genes involved in resistance will enhance the knowledge pool of all possible insecticide targets in *T. castaneum* and render greater selectivity in insecticide design, thereby improving the efficacy of insecticides. The consolidated

insights from the literature review will provide much-needed insights as to what makes *T. castaneum* resistant to insecticides. The comprehensive overview will help successive research to initiate focussed monitoring of resistance. It contributes towards a deeper understanding of insecticide resistance and improved management of this destructive pest that threatens food storage, food safety, health, and economic security. Scientific efforts that make use of this pool of knowledge can lead to more sustainable agricultural practices.

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