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Longevity and Potential Mechanisms of Fenpropathrin Resistance in Asian Citrus Psyllid, *Diaphorina citri* Kuwayama

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Abstract: The stability of resistance to fenpropathrin was assessed using five populations of *Diaphorina citri* with varying initial resistances ranging from fully susceptible (SS) to fully resistant (RR). Furthermore, we quantified the relative expression of voltage-gated sodium channel (*VGSC*) genes in crosses of field-selected and laboratory-susceptible *D. citri* lines after eight months without insecticide selection. We found that resistance to fenpropathrin remained elevated up to eight months after exposure to fenpropathrin. A real-time quantitative PCR analysis using the susceptible baseline population revealed that levels of *VGSC* gene expression were significantly higher in the RS75 cross and the RR100 fully resistant line eight months after their last fenpropathrin exposure. Our results suggest that while fenpropathrin resistance is likely unstable under field conditions when interbreeding with susceptible individuals is possible, resistance can remain stable for at least 8 months if those populations are isolated. Further, insecticide rotation and the maintenance of susceptible reservoirs of individuals should mitigate fenpropathrin resistance in *D. citri* over time. The development of a *VGSC* gene biomarker may be a useful tool for monitoring pyrethroid resistance in *D. citri* going forward.

Keywords: insecticide resistance; pyrethroid; resistance stability; voltage-gated sodium channel (VGSC); biomarker; citrus greening disease

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1. Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is a vector of *Candidatus* Liberibacter asiaticus (CLas), the putative causal agent of citrus huanglongbing (greening disease) [1–3]. In order to manage invasive *D. citri* populations and reduce the spread of greening disease in citrus and ornamental hosts, calendar-based applications of synthetic chemical insecticides are routinely employed, including applications of pyrethroids, organophosphates, and neonicotinoids [2–5]. However, the widespread and intensive use of insecticides in citrus has led to the development of insecticide resistance among *D. citri* populations [2,3,5]. Insecticide resistance was first detected in several South Florida commercial citrus-growing areas in 2009 to fenpropathrin, imidacloprid, malathion, and thiamethoxam, with the highest levels of resistance detected in LaBelle, FL (Hendry County) to imidacloprid [4]. Following these findings, area-wide management zones were implemented across the state, and coordinated insecticide class rotations were introduced. By 2013, levels of resistance in *D. citri* populations throughout Florida returned to baseline levels of susceptibility, likely due in part to those interventions [6]. However, since that

time, multiple reports have revealed widespread insecticide resistance among *D. citri* populations throughout citrus-growing regions worldwide, particularly in areas employing intensive spraying programs such as China [5], Mexico [7,8], and Pakistan [9]. However, several factors that affect resistance management programs remain unclear, including the stability and mechanisms underlying insecticide resistance in *D. citri*.

Understanding the genetic stability of insecticide resistance is useful for the development of effective strategies for prevention and mitigation [10–12]. Unstable resistance to specific molecules may prolong their effective lifespan in the field [12]. For the management of insect pests, it is important to determine the relationship between resistance development and the subsequent recovery of susceptibility over time [9,10,13]. After several generations of relaxed selection pressure, the rate of reversion will determine the stability of resistance [14]. The rate of recovery depends on the prevalence of resistant individuals in the population and on their ability to compete with susceptible individuals in terms of reproductive potential and other biotic factors [10]. We previously reported that the susceptibility of resistant *D. citri* populations to various modes of action recovers after reduced selection pressure or under the effective rotation of insecticides with different modes of action [6]. Similar instability has been reported in the diamondback moth, *Plutella xylostella* Linnaeus [10]; silverleaf whitefly, *Bemisia tabaci* Gennadius [15]; citrus red mite, *Panonychus citri* McGregor [16]; and green peach aphid, *Myzus persicae* Sulzer [17]. In contrast, DDT and cyclodiene resistance in the housefly, *Musca Domestica* Linnaeus, has persisted for more than 20 years and resistance to diazinon and dimethoate also exhibits extended persistence [18].

Pyrethroids are a class of synthetic organic insecticides derived from pyrethrins and have been used worldwide since the 1980s because of their high level of effectiveness compared to other chemicals, such as organophosphorus and carbamic ester compounds [19,20]. Pyrethroid insecticides have seen extensive worldwide use to control wide-ranging pests in the orders Coleoptera, Hemiptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera, and Thysanoptera [19,20] and are also used against phytopathogen vectors including *D. citri* [2,3,6]. However, resistance among *D. citri* populations has emerged following the extended use of pyrethroids in citrus [3,5].

Voltage-gated sodium channel (*VGSC*) genes are associated with a group of integral transmembrane proteins that are responsible for the initiation and propagation of action potentials in almost all excitable cells [21]. Due to their crucial role in regulating cell excitability, the *VGSC* genes are the primary targets of several classes of chemical insecticides [21,22]. A *VGSC* is a large transmembrane protein consisting of four internally repeated homologous domains (I to IV), each with six membrane-spanning segments (S1–S6) [22,23]. A substitution was identified within the domain IIS4-IIS5 in the super-*kdr* strain of *M. domestica* [24]. These substitutions have subsequently been reported in a wide range of insect species. In addition to these substitutions, a number of substitutions outside of domain II have also been reported [25]. Pyrethroids are among the earliest synthetic compounds identified to target the sodium channel and they prolong sodium channel opening, resulting in repetitive nerve firing and membrane depolarization [26]. Liu et al. (2017) demonstrated that target site insensitivity is a potential basis for insecticide resistance to pyrethroids in *D. citri* [23].

In this study, populations of *D. citri* with varying degrees of fenpropathrin resistance, ranging from fully susceptible (SS) to fully resistant (RR), were established under laboratory conditions. Subsequently, the stability of fenpropathrin resistance was investigated over an eight-month period without selection pressure. Furthermore, the potential relationship between fenpropathrin resistance in *D. citri* and *VGSC* gene expression was investigated to gain insight into the potential mechanism(s) of pyrethroid resistance in *D. citri*.

2. Materials and Methods

2.1. Insects, Plants, and Chemicals

A susceptible laboratory population of *D. citri* was reared in a greenhouse at the Citrus Research and Education Center, University of Florida, Lake Alfred, FL. This strain was collected in 2000 and has been reared without exposure to pesticides. The culture was maintained on sweet orange (*Citrus sinensis* (L.) Osbeck) in a greenhouse at 27 ± 1 °C, with a 60–65% relative humidity and a 14:10 h (light/dark) photoperiod. To develop a fenpropathrin-resistant population, adult *D. citri* were collected from a commercial citrus grove in Wauchula, FL, USA (N: 27°36′21″ W: 81°49′1″) on 27 March 2018 and were further selected for fenpropathrin (Danitol 2.4EC) resistance over ten generations using a previously described method [27]. The citrus plants were purchased from a local nursery (Southern Citrus, Dundee, FL, USA). The chemical fenpropathrin (Danitol 2.4 EC) was obtained from Valent Inc. (Walnut creek, CA, USA).

2.2. Stability of Fenpropathrin Resistance

Fenpropathrin toxicity was evaluated bimonthly in five populations of *D. citri* that were derived from various initial crosses and ranged from initially highly susceptible SS100 (RR00 + SS100) to highly resistant RR100 (RR100 + SS00), with three susceptible × resistant crosses: (1) RS75, (2) RS50, and (3) RS25. This resulted in initial percentages of resistance of 100, 75, 50, 25, and 0% as described previously in the protocol used by [13]. Each population was initiated using 100 randomly collected *D. citri* individuals from the selected highly resistant and susceptible populations. Each population was held on four *C. sinensis* plants (described above) in mesh cages (90 cm × 60 cm × 60 cm). The cages were maintained under the greenhouse conditions described above. Under these conditions, the developmental period from egg to adult was approximately 15 days and the mean generation time was approximately 25 days. The initial populations were established in cages on 19 March 2020.

Toxicity bioassays were performed every two months for an eight-month period. In total, 140 to 300 individual were tested on each sampling date for each insecticide over a range of concentrations using the leaf dip bioassay [9,28]. Fenpropathrin (Danitol 2.4EC) was diluted in distilled water in eight concentrations. Leaf discs (35 mm diameter) were cut from citrus leaves collected from 'Valencia' orange trees that were not previously treated with insecticide. The leaf discs were dipped in test solutions for 30 s and allowed to dry in a fume hood for 30 min. Leaf discs dipped in water alone served as controls. Each concentration of fenpropathrin was replicated three to four times. Petri dishes (Thermo Fisher Scientific, Waltham, MA, USA), 35 mm in size, were coated with 0.3 mL of 1.5% agar solution to prevent the desiccation of leaf discs during the assays [28]. Mortality counts were taken 48 h after transfer; insects found on their side or back that were unable to move when a Petri dish was tapped gently were considered dead and included in mortality counts [28]. Each assay was replicated three to four times and assays were conducted under the environmental conditions described above for insect rearing.

2.3. Nucleic Acid Extraction and cDNA Synthesis

RNA was extracted from adult *D. citri* using the RNeasy mini prep kit (QIAGEN, Hilden, Germany). Samples were collected from insecticide-resistant (RR100), susceptible (SS100), and crossed (RS75, RS50, and RS25) populations. For each population, six tubes containing ten *D. citri* were used for RNA extractions. The quality of RNA from each sample was assessed on a Nano Drop 2000 Spectrophotometer (Thermo Fisher Scientific) using A260/A280 ratios at approximately 2.0. Thereafter, 500 ng of total RNA was used for cDNA synthesis (Verso cDNA synthesis kit, Thermo Fisher Scientific).

2.4. Real-Time Quantitative PCR for Expression

The cDNA used for this study was synthesized from total RNA, as described above. Gene-specific primers were used to amplify the *VGSC* gene (Table 1). The cDNA encoding actin was amplified using gene-specific primers to normalize the threshold cycle (Ct)

value for *VGSC* amplification. The primers specific to the *VGSC* gene and actin were confirmed to have similar amplification efficiencies using PowerUp SYBR Green Master in a thermal cycler (ABI Prism 7500; Applied Biosystems), according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Three biological replicates were performed for each gene. The relative expression of each gene among the populations was compared using the $2^{-\Delta\Delta}$ CT method [29]. *DcActin* was used as a calibrator and then normalization was performed on the *D. citri* laboratory colony.

Table 1. Quantitative real-time PCR primer details for the *Diaphorina citri VGSC* gene.

Gene	Forward/Reverse	Sequence (Forward/Reverse)	Reference
VGSC	F	AGCGGAAAATTACACGTGGG	[23]
	R	CGGATACCTTTGGCCCCTTT	
Actin	F	CCCTGGACTTTGAACAGGAA	[27]
	R	CTCGTGGATACCGCAAGATT	

2.5. Statistical Analysis

To estimate the parameters of the dose–mortality regression line for each bioassay, a probit analysis was conducted with SAS [30]. Resistance ratios (RRs) at the LC_{50} level and LC_{90} levels (RR_{50} and RR_{90}), and their associated 95% confidence intervals (CIs), were calculated as outlined in Robertson and Preisler [31]. The 95% CIs of the RRs were calculated by dividing the relative 95% confidence intervals associated with LC_{50} or LC_{90} values of the selected population by the 95% confidence intervals associated with LC_{50} or LC_{90} values of the laboratory susceptible population, according to Robertson and Preisler [31]. The normality and homogeneity of variance of the gene expression data were investigated using the Shapiro–Wilk test followed by Levene's test. The data met assumptions of normality. For each population sampled (RR, SS, and RS cross populations), the gene expression data were analyzed using one-way analysis of variance (ANOVA). The mean gene expressions between the RS cross, RR, and SS populations were compared using Tukey studentized range [HSD] tests at $p < 0.05$ [30].

3. Results

3.1. Stability of Fenpropathrin Resistance among D. citri Populations

There were no changes in dose–mortality relationships for *D. citri* from the susceptible strain (SS100: 00RR + 100SS) over eight months of testing [equivalent to approximately 9 generations of *D. citri* [32], during which there was no further selection pressure (Table 2). In the highly resistant strain (RR100: 100RR + 00SS), resistance to fenpropathrin gradually decreased over time but was still present at RR50 = 10.83 when assessed after eight months without further exposure to fenpropathrin (Table 2). The fenpropathrin-resistant crosses (RS75: 75RR + 25SS; RS50: 50RR + 50SS, and RS25: 25RR + 75SS) exhibited a greater decline in resistance over time than the RR100 strain (Table 2). However, the rate of decline was slower in the cross with the lowest initial percentage of insecticide-resistant *D. citri* individuals (RS25) than in populations with higher initial percentages of resistant individuals (RS75 and 50RS) (Tables 2 and 3).

Table 2. Mortality of *Diaphorina citri* populations with initial gene frequencies of RR100, RS75, RS50, RS25, and SS00 in response to fenpropathrin during bimonthly tests over time.

Line	Month	Slope ± SE	LC_{50} (ng/µL) (95 % Confidence Interval)	LC_{90} (ng/µL) (95 % Confidence Interval)
100 RR + 00 SS	0	0.78 ± 0.17	18.57 (3.31–161.99)	912.09 (107.592–3966)

Table 2. *Cont.*

Line	Month	Slope ± SE	LC_{50} (ng/µL) (95 % Confidence Interval)	LC_{90} (ng/µL) (95 % Confidence Interval)
100 RR + 00 SS	2	0.69 ± 0.15	10.70 (1.52–134.51)	781.53 (76.23–547546)
	4	0.82 ± 0.12	11.90 (5.01–30.69)	436.53 (131.87–3111)
	6	0.82 ± 0.13	14.75 (5.324–3.67)	532.98 (142.90–5757.00)
	8	0.69 ± 0.12	5.02 (1.67–15.54)	249.68 (61.72–3256.98)
75 RR + 25 SS	2	0.88 ± 0.31	4.84 (2.21–11.13)	570.42 (174.23–3169)
	4	0.81 ± 0.14	6.54 (1.50–35.52)	329.94 (54.15–18627)
	6	0.61 ± 0.09	7.50 (3.06–19.77)	389.60 (59.00–1082)
	8	0.89 ± 0.09	3.31 (1.42–7.54)	91.38 (33.59–418)
50 RR + 50 SS	2	–	–	–
	4	0.60 ± 0.20	5.57 (1.18–35.54)	303.57 (46.09–22548)
	6	0.51 ± 0.07	2.51 (0.81–8.43)	794.56 (144.00–13207)
	8	0.88 ± 0.13	1.99 (0.80–5.12)	102.19 (30.70–671.50)
25 RR + 75 SS	2	0.42 ± 0.06	0.91 (0.30–2.63)	314.73 (70.41–3281)
	4	0.56 ± 0.09	0.85 (0.15–5.34)	164.11 (19.18–16330)
	6	0.67 ± 0.09	0.81 (0.33–2.00)	33.76 (13.13–235.75)
	8	0.89 ± 0.13	0.77 (0.42–1.42)	64.23 (27.35–196.74)
00 RR + 100 SS	0	0.89 ± 0.12	0.35 (0.16–0.78)	9.73 (3.803–8.46)
	2	1.11 ± 0.14	0.40 (0.22–0.77)	5.71 (2.75–16.59)
	4	0.76 ± 0.16	0.40 (0.06–3.62)	19.36 (2.71–475.16)
	6	0.70 ± 0.10	0.74 (0.29–1.90)	48.70 (14.41–317.51)
	8	0.79 ± 0.11	0.43 (0.27–0.67)	16.67 (9.84–31.78)

Table 3. Resistance ratios (95% confidence intervals) of *Diaphorina citri* populations with initial gene frequencies of RR100, RS75, RS50, RS25, and SS00 at two lethal concentration levels (LC_{50} and LC_{90}) obtained using fenpropathrin during bimonthly tests over eight months.

Line	Month	RR_{50}	RR_{90}
100 RR + 00 SS	0	60.14 (16.99–212.83)	951.00 (82.00–10941.00)
	2	34.00 (11.73–100.09)	80.32 (13.00–472.00)
	4	32.85 (13.63–79.14)	31.07 (7.3–7131.01)
	6	19.96 (5.64–70.63)	10.95 (1.37–87.25)
	8	10.83 (2.91–40.34)	20.14 (3.16–128.36)

Table 3. *Cont.*

Line	Month	RR_{50}	RR_{90}
75 RR + 25 SS	2	13.92 (4.56–42.48)	58.60 (9.61–357.47)
	4	18.06 (7.38–44.17)	23.48 (5.37–102.64)
	6	10.15 (2.78–37.09)	35.20 (0.93–69.70)
	8	7.61 (2.20–26.32)	5.48 (1.04–28.81)
50 RR + 50 SS	2	–	–
	4	15.85 (6.46–38.95)	22.57 (5.14–99.17)
	6	3.39 (0.78–14.75)	16.32 (1.20–221.31)
	8	2.69 (0.88–8.27)	2.10 (0.34–12.84)
25 RR + 75 SS	2	3.89 (0.84–18.09)	30.45 (3.69–251.60)
	4	2.35 (0.77–7.20)	11.68 (1.75–77.59)
	6	1.15 (0.2–83.94)	1.32 (0.16–11.15)
	8	1.97 (0.51–7.57)	1.41 (0.22–9.16)
00 RR + 100 SS	0	1 (0.34–2.98)	1 (0.19–5.47)
	2	1 (0.35–2.83)	1 (0.18–5.47)
	4	1 (0.40–2.47)	1 (0.23–4.26)
	6	1 (0.27–3.75)	1 (0.11–9.23)
	8	1 (0.22–4.55)	1 (0.14–7.03)

3.2. Expression of VGSC in Insecticide-Resistant, Susceptible and Crossed Populations

VGSC gene expression was examined using qPCR with cDNA that was individually synthesized from adults, obtained from the highly susceptible (SS100), resistant (RR100), and crossed (RS75, RS50, and RS25) populations. The relative expression of the *VGSC* gene was estimated using qPCR. Transcript levels were detected in all five populations investigated. There was a significant effect of resistance level on *VGSC* gene expression (df = 4, 10; F = 30.01; $p < 0.001$) (Figure 1). *VGSC* gene expression was significantly higher in the two populations with the highest initial resistance to fenpropathrin (RS75 and RR 100) than in the other populations examined (Figure 1).

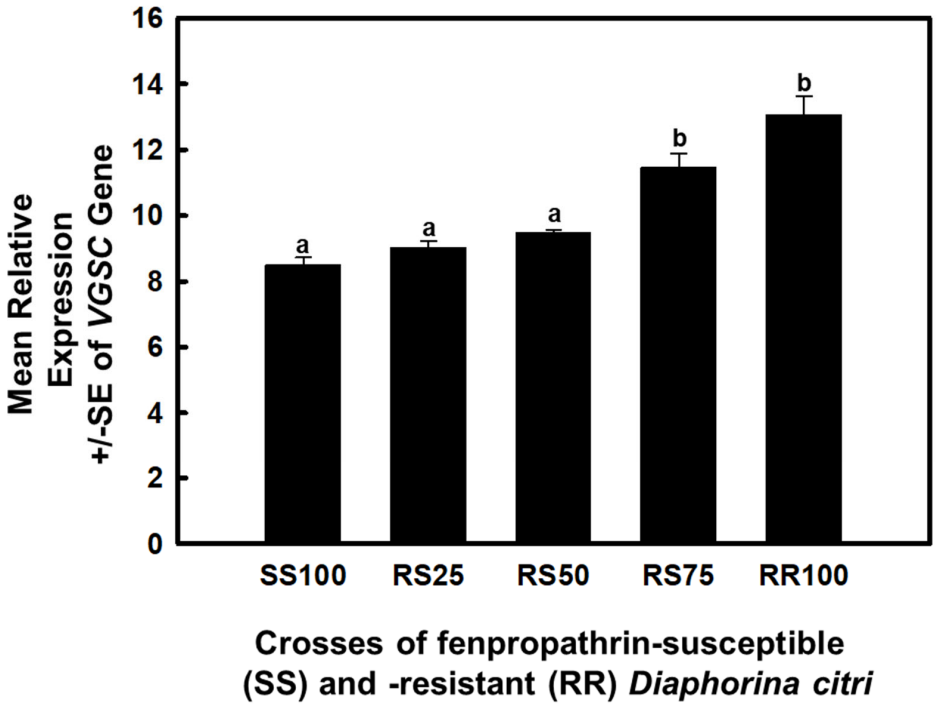

Figure 1. Quantification of *VGSC* gene expression in populations of *Diaphorina citri* exhibiting differing levels of resistance to fenpropathrin (±SE) (means with the same letter are not statistically different according to Tukey test α = 0.05).

4. Discussion

Pyrethroids have been one of the main classes of insecticides used for the suppression of *D. citri* populations and as a result, resistance to this chemical has evolved among managed populations worldwide [3,5]. Our results indicate that from a starting point of very high fenpropathrin resistance (RR = 60.14-fold), resistance in *D. citri* remained relatively stable in the absence of selection pressure over approximately nine generations. However, the introgression of susceptible alleles through crossbreeding reduced initial resistance levels and RR × SS crosses tended to reverse to a susceptible state at a slightly faster rate, resulting in populations expressing only 2.0–7.6-fold resistance after nine generations. A reversal to the susceptible state appeared to be driven more by the introgression of susceptible alleles through crossbreeding between RR and SS populations than by a gradual reversal caused by possible fitness consequences associated with resistance (Table 3).

The relative stability of fenpropathrin resistance in *D. citri* populations isolated from crossbreeding would suggest negligible fitness disadvantages associated with a highly resistant population with a low frequency of susceptible alleles [27]. Similar results were observed with a highly resistant strain (RR = 669-fold) of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) after 13 generations of laboratory selection to spinosad; only a 1.4-fold reversion to susceptibility occurred after five generations of relaxed selection pressure [33]. Similarly, Robb (1989) observed that a strain of *F. occidentalis* retained resistance to dimethoate up to seven years after exposure [33]. Kontsedalov et al. (1998) found the occurrence of sustained resistance to cipermethrin in a laboratory-reared strain of *F. occidentalis* that lasted seven years in the absence of selection pressure [34]. Collectively, our results suggest that fenpropathrin resistance in *D. citri* is persistent and slow to reverse in the absence of SS gene introgression, indicating that appropriate rotations of MoAs and/or the maintenance of susceptible allele reservoirs may be of particular importance to managing resistance for this chemistry [35]. These results also warrant testing the hypothesis that fitness costs associated with fenpropathrin resistance in *D. citri* may be negligible, given that high fitness costs were observed with the evolution of resistance to neonicotinoids in this species [35].

Within resistant populations, the main factors influencing the rate of reversion are relative fitness differences between SS and RR phenotypes, initial gene frequencies, and dominance relationships of the resistant and susceptible alleles underlying the phenotypes [36]. The persistence of fenpropathrin resistance in an isolated, resistant strain without selection suggests a low fitness cost. However, comparisons of net reproductive rates and finite rates of increase between SS and RR populations will be required to test this hypothesis. A thorough understanding of resistance stability can contribute to the development of successful resistance management strategies [10–12]. The reversal of fenpropathrin resistance has been previously reported in *Spodoptera litura* Fabricius [37] and *Phenacoccus solenopsis* Tinsley [11]. Although our data suggest that fenpropathrin resistance remains relatively stable in isolation, breeding between fenpropathrin-resistant and -susceptible *D. citri* under natural field conditions should contribute to reversion. This scenario is congruent with field results indicating that the susceptibility of *D. citri* to fenpropathrin can be maintained when this chemical is rotated in sequence among four other modes of action that do not exhibit cross-resistance with pyrethroid products [27]. Collectively, our results suggest that pyrethroid resistance in *D. citri* is relatively more stable than that observed previously with neonicotinoids [5] and, therefore, indicates that managing the resistance to pyrethroids in this species may require the application of an appropriate counteracting selection pressure such as alternative modes of action without cross-resistance to reverse the problem when it is identified.

Resistance to pyrethroids is typically caused by target site insensitivity, with enhanced metabolic detoxification contributing in part [23,38]. The molecular basis of pyrethroid resistance has been investigated in detail in several insect species, for example, *Drosophila melanogaster* Meigen [39] and *P. xylostella* [10]. An initial characterization of the sodium channel in *D. citri* determined that the likelihood for development of knockdown resistance

(*kdr*) through single-nucleotide polymorphisms is lower than in other hemipterans such as aphids [23]. In the current investigation, the expression of *VGSC* genes in *D. citri* increased with increasing levels of resistance to fenpropathrin. Similarly, there is a strong correlation between *VGSC* gene expression and the level of insecticide resistance in house flies *M. domestica* L. and German cockroaches *Blattella germanica* L. [40]. However, the alternative splicing of the *para*-sodium channel gene appears to play a role in modulating the sensitivity to pyrethroids in other cases, such as the diamondback moth, *P. xylostella* [41]. These results suggest that the post-transcriptional modification of sodium channel gene expression may be involved in the development of insecticide resistance to fenpropathrin in *D. citri*. It may be possible to use the *VGSC* gene as a biomarker for monitoring pyrethroid resistance in *D. citri*, although this will require the identification of specific point mutations conferring resistance.

5. Conclusions

Collectively, our results suggest that fenpropathrin resistance can remain relatively stable in the absence of cross-breeding between resistant and susceptible populations. However, it is rendered unstable under more natural field conditions, where it reverses to the susceptible state in proportion to the frequency of susceptible allele introgression into the resistant population (Table 3). Our findings also show that the post-transcriptional modification of sodium channel gene expression may be involved in the development of insecticide resistance to fenpropathrin in this species (Figure 1). Although target site insensitivity may play an important role in the expression of the resistance phenotype in selected strains of *D. citri*, it remains possible that metabolic detoxification also plays a role [27]. Based on previous observations made by Chen et al. [27] with resistant field populations of *D. citri* and those obtained here with laboratory crosses between RR and SS strains, it appears that multiple mechanisms or genes are most likely responsible for conferring overall resistance to this chemistry [27]. Whether these resistance genes share similar transcriptional regulation in response to insecticide selection pressure remains to be determined.

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