Insecticide Resistance Management for *Aphis glycines* (Hemiptera: Aphididae): Understanding the Biological Mechanisms and Farmer Practices

A Dissertation

SUBMITTED TO THE FACULTY OF

UNIVERSITY OF MINNESOTA

BY

Rosa Ernestina Lozano Murcia

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Robert L. Koch (Advisor)

Amelia I. Lindsey (Co-advisor)

AUGUST 2023

Copyright Permission

Chapter 2 has previously been published (citation: Lozano, R.E., D.P. Paula, D.A. Andow, and R.L. Koch. 2022. Validation of Reference Genes Across Populations of *Aphis glycines* (Hemiptera: Aphididae) for RT-qPCR Analysis of Gene Expression Related to Pyrethroid Detoxification. J. Entomol. Sci. 57: 213–239). Permission for use here granted by the first author Rosa E. Lozano and the publisher Journal of Entomological Science.

Acknowledgements

I would like to acknowledge everyone who played a role in my academic accomplishments including, my advisors Dr. Robert Koch and Dr. Amelia Lindsey, my committee members Dr. David Andow, Dr. Aaron Lorenz, and my co-authors Dr. Débora Pires Paula, Dr. Terrance Hurley, and Dr. Janet Knodel. Each of you have provided patient advice and guidance throughout my research process. Thank you all for your unwavering support. Also, I want to thank the members of the Koch and Lindsey laboratories members and collaborators, for all the help provided. Further, I want to thank my family: my sisters Steffani Lozano and Rosita Lozano, my nieces Susana Pinilla and Verónica Pinilla, my brother-in-law Daniel Pinilla, my fiancé Zach Schenfish for all the loving support, and especially my parents Rosa Elvira Murcia Gómez and Herbert de Jesús Lozano Lozano, who supported me with love and understanding. Without you, I could never have reached where I am right now. Finally, I want to thank the funding sources supporting my work: the Minnesota Rapid Agricultural Response Program (RARF), the USDA National Institute of Food & Agriculture (NIFA) (award no. 2019-68008-29892), the MN Agricultural Experiment Station Project MIN-14-120, the Minnesota Soybean Research and Promotional Council, the North Dakota Soybean Council, the Department of Entomology Lugger-Radcliffe Summer Fellowship to REL, and a University of Minnesota Doctoral Dissertation Fellowship to REL.

Dedication

To my grandparents, Modesta Gómez Gómez, Ernestina Lozano Lozano, Berta Lozano de García, Aurelio Murcia Gómez, Luis Ernesto Lozano Rengifo, and Genaro García Andrade; and to my parents, Rosa Elvira Murcia Gómez and Herbert de Jesús Lozano Lozano.

Abstract

Soybean aphid (*Aphis glycines* Matsumura) (Hemiptera: Aphididae) is a pest that can severely impact soybean crops in the United States. Over the years, the repeated and widespread use of pyrethroid insecticides has led to the selection of resistant individuals within soybean aphid populations. The two insecticide resistance mechanisms documented in soybean aphids are metabolic detoxification and target site alteration. Farmers are key in implementing integrated pest management (IPM) and insecticide resistance management (IRM) strategies to manage soybean aphids. Resistance mechanisms are constantly evolving, insecticide resistance is becoming a barrier to soybean aphid control, and current farmer management approaches are unknown. My dissertation is focused on understanding the variability of resistance mechanisms and farmers practices to help inform IPM and IRM.

The supplementary files included are:

Chapter I:

Table SF 1. Summary of individual soybean aphid genotypes. Microsoft Excel Worksheet (.xlsx). 23.9 KB

File SF 1. SBA_Haplotype_Aligned_401_bp.fna. FASTA document 2021. 35 KB

Chapter IV:

File SF 1: Midwest 2021 Soybean Pest Management Survey. PDF document 2021. 252 KB

Table of Contents

List of Tables

List of Figures

Introduction

Soybean, *Glycine max* (L.) Merrill (Fabales: Fabaceae), is an important crop worldwide. Brazil, the United States and Argentina are the leading producers of this grain commodity (Shahbandeh 2023a). The soybean grain is composed of two major components: meal and oil. Approximately 80% is meal (protein), 97% of which is used for animal feed, and the other 20% is oil, 61% of which is used for frying and baking (NJSB 2023). Soybean is the second most planted crop in the United States. In 2022, the area planted to soybean in the United States reached 35.3 million hectares with a production of 116.5 million metric tons (USDA- NASS 2023b). Over 80% of soybeans grown in the US are cultivated in the upper Midwest (Shahbandeh 2023b). Minnesota is the third largest soybean producer with approximately 2.9 million hectares harvested in 2020 (USDA- NASS 20203c).

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is an invasive insect and the greatest pest threat to soybean in North America (Ragsdale et al. 2004). Soybean aphid is originally from Asia (Ragsdale et al. 2011) with Japan being the most likely site of origin of the infestations in the United States (Venette and Ragsdale 2004). Following its detection in 2000 near Lake Michigan (Alleman et al. 2002), soybean aphid expanded to soybean growing areas in the midwestern United States and southern Canada (Ragsdale et al. 2011). In 2001, 2003, and 2005 large soybean aphid outbreaks occurred

across multiple states (Heimpel et al. 2010). Despite the fact that infestations have decreased in some states, soybean aphid is still a persistent problem in Minnesota and parts of North Dakota, South Dakota, and Iowa (Koch et al. 2018). Accordingly, the Minnesota Invasive Terrestrial Plants and Pests Center (MITPPC) ranked soybean aphid as third in the list of terrestrial invasive species that threatens Minnesota (MITPPC, 2016). Some of the factors that enhance this species as a pest were the high dispersal capacity, presence of hosts, reproductive capacity, and impacts to yield (MITPPC, 2016).

Soybean aphid feeds by sucking out phloem sap of the soybean plants. These injuries can cause yield losses of 20-30%, and even between 50-70% when very heavy infestations are constant on soybean plants (Dai and Fan 1991, Wu et al. 2004, Tilmon et al. 2011), with billions of dollars lost annually (Song et al. 2006, Ragsdale et al. 2007). Visually yellow leaves, stunting of plants, premature defoliation, smaller seed size, and reduced root systems, are characteristics of high-density infestations of soybean aphids on soybean plants (Wu et al. 2004, Ragsdale et al. 2007). Additional collateral effects of soybean aphid on plant fitness can be attributed to its ability to transmit viruses such as soybean mosaic virus (Wang and Ghabrial 2002) and interference with photosynthesis due to the facilitation of sooty mold growth (Beckendorf et al. 2008).

Soybean aphid is a heterocious holocyclic insect species, meaning that it alternates plant hosts and goes through sexual reproduction for part of its life cycle (Wang et al. 1962). Buckthorn, *Rhamnus* spp.*,* is a soybean aphid's primary host, where the gynoparae lay eggs to overwinter (Ragsdale et al. 2004). The eggs are well adapted to low temperatures, with a supercooling point of approximately -34 Cº (Mccornack et al. 2005). These eggs hatch in the spring and alate soybean aphids migrate to soybean fields with the help of wind currents. In the summer, cultivated soybean is the main secondary host, where soybean aphid produces approximately 15 generations, undergoing asexual reproduction (parthenogenesis), producing only female aphids (Wang et al. 1962, Venette and Ragsdale 2004). In the fall soybean plants undergo senesce and a generation of winged female and male sexual morphs develop and migrate to buckthorn to reproduce and lay eggs that will overwinter and hatch in the following spring (Venette and Ragsdale 2004). In addition to soybean, horsenettle, *Solanum carolinense* L. (Solanales: Solanaceae) and red clover, *Trifolium pratenseis* (Fabales: Fabaceae) are other potential secondary hosts (Alleman et al. 2002, Clark et al. 2006).

Crop production faces many challenges that require the adoption of Integrated Pest Management (IPM) strategies to suppress pests before they cause economic damage. Peterson et al. (2018) updated IPM's definition as "a

comprehensive approach to managing host stress that is economically and ecologically sustainable." IPM should be use for sustainable protection of soybean from soybean aphid, including several tactics such as cultural control, host plant resistance, biological control, and chemical control (Hodgson et al. 2012). From these tactics, chemical control with foliar insecticides has prevailed as the main management tactic in the United States (Hodgson et al. 2012, Koch et al. 2018). To date there are seven groups of foliar insecticides registered for soybean aphid management on soybean: carbamates (IRAC group 1A), organophosphates (IRAC group 1B), pyrethroids and pyrethrins (IRAC group 3A), neonicotinoids (IRAC group 4A), sulfoxamines (IRAC group 4C), butenolides (IRAC group 4B), and pyropenes (IRAC group 9D) (Koch et al. 2022). However, pyrethroids and organophosphates are the primary insecticides used for soybean aphid management (Hodgson et al. 2012). To determine the correct time to intervene with a management action for soybean aphid an economic threshold (ET) has been set for soybean aphid at 250 aphids per plant, with more than 80% of the plants infested, and aphid populations increasing overtime (Ragsdale et al. 2007).

Pyrethroids were introduced more than five decades ago for various purposes including pest control in agricultural settings and to control insect-borne vectors to support human health (Soderlund 2010). Initially synthetic pyrethroids were designed from natural pyrethrins found in chrysanthemum flowers and

characteristics such as photostability and insecticidal effectiveness were improved (Elliott and Janes 1978, Soderlund 2010). Pyrethroids are classified by the Insecticide Resistance Action Committee (IRAC) as sodium channel modulators (IRAC 2020a). The site of action for pyrethroids is the voltage-sensitive sodium channel (Soderlund and Bloomquist 1989, Soderlund 2005). The neuron has an unequal distribution of positive and negative charges, referred to as polarity (Kandel 1991, Kim and Zhen 2009). The sodium channels open and close in succession along the neuron allowing a pulse of positive charged sodium ions into the neuron, leading to the creation of the nerve impulse. However, pyrethroid insecticides bind to and keep the sodium channel open, causing a continuous influx of sodium ions into the neuron cell, leading to the disruption of the normal balance of the electrical charges, resulting in the paralysis and death of the insect (Soderlund 2012).

Tabashnik et al. (2014) defines insecticide resistance as a “genetically based decrease in susceptibility to a pesticide in a population caused by exposure to the pesticide in the field”. Since the first case of insecticide resistance was reported in 1914 for *Aspidiotus perniciosus* Comstock (Hemiptera: Diaspididae) (Melander 1914), the number of cases of insecticide resistance in arthropods has increased to 11,403 cases and have been documented in 583 species for 342 compounds (Carlson et al. 2014). Despite the development of IPM monitoring practices such as scouting and an economic

threshold, resistance to pyrethroid insecticides was documented in soybean aphid in Minnesota in 2015 (Hanson et al. 2017). Failures of foliar pyrethroid applications for soybean aphid management have been documented in the field and confirmed with laboratory bioassays over multiple years with cases in Minnesota, Iowa, North Dakota, South Dakota, and Manitoba (Hanson et al. 2017, Koch et al. 2018, 2022, Menger et al. 2020). The development of soybean aphid's resistance to insecticides is associated with frequent infestations, limited number of available insecticide groups, and overuse of the available insecticides (Koch et al. 2022). To reduce the risk for further insecticide resistance, it is recommended to implement non-chemical control tactics, follow the insecticide labels, rely on economic thresholds for spraying decisions, and rotate active ingredients to avoid unnecessary applications and reduce the chances of management failure (Koch et al. 2018, 2022).

Insecticide resistance can be due to either behavioral resistance or physiological resistance. Physiological resistance includes reduced penetrance of the cuticle, target site insensitivity, and increased metabolic detoxification (Simon 2011). In this document I will focus on target site insensitivity and metabolic detoxification. The first mechanism of resistance involves the loss of sensitivity on the target site of a xenobiotic. Target-site resistance is the primary mechanism involved in pyrethroid resistance in several insect species (Khambay and Jewess 2005). The first pyrethroid resistance case was found and described

in the 1950's in *Musca domestica* Linnaeus (Diptera: Muscidae) and named *kdr* mutation, after the phenotype "knock down resistant" (Busvine 1951, Milani 1954). Additionally, a second mutation that attributes greater resistance to pyrethroids when in association with *kdr* or by itself, was later identified and called super-*kdr* (*s-kdr*) (Sawicki 1978, Soderlund and Knipple 1999, Davies and Williamson 2009). The *kdr* mutations in insects are located in the voltage-gated sodium channel (VGSC), with the *para*-type sodium channel being the target of pyrethroid insecticides (Khambay and Jewess 2005, Xu et al. 2012). The structural changes in the target proteins of the resistant organisms prevent pyrethroids from binding to the sodium channel, thus maintaining the normal function of the neuron (Khambay and Jewess 2005). The insect VGSC includes three subunits: α-subunit, auxiliary β subunit, and auxiliary tipE subunit (Soderlund 2005). Pyrethroid binding is inherent to the α-subunit proteins (Soderlund 2012). The sodium channel α-subunit is comprised for four repeat domains (I–IV) and six hydrophobic membrane segments arranged in each segment (S1–S6), where S1-S4 are voltage-sensing module segments and S5-S6 are termed as the P-region (Dong et al. 2014). Most of the pyrethroid-resistance conferring target-site mutations reported in arthropod species are located in the regions encoding for the S5-S6 segments of the VGSC α-subunit and more precisely on the domain II, suggesting it is a significant section for pyrethroid resistance (Soderlund 2005). A point mutation resulting in a change

from methionine to threonine (M918T) is a recurring s-*kdr* mutation in insects, usually located in the in the region encoding for the domain II or III of the VGSC between S5-S6 (Williamson et al. 1996). Additionally, a non-synonymous change in the in the nucleotide sequence resulting in a change from leucine to phenylamine (L1014F) is the most common *kdr* mutation associated with resistance to pyrethroid insecticides (Haddi et al. 2012, Xu et al. 2012). For example, target site insensitivity in resistant strains of the maize weevil, *Sitophilus spp.* was found at the amino acid position L1014F (*kdr*) or T229I (s-*kdr*) (Haddi et al. 2018). In *B. tabaci,* the substitution of three nucleotides resulted in the following changes: leucine for isoleucine (L925I), threonine for valine (T929V), and methionine for valine (M818V) conferred target site insensitivity to pyrethroid insecticides (Basit 2019). For soybean aphids, four-point mutations resulted in non-synonyms amino acid changes (i.e., L1014F, L925M, M918L, and M918I) in the region encoding for the domain II between S4-S6 segments were associated with the loss of pyrethroid susceptibility (Paula et al. 2021, Valmorbida et al. 2022b).

Insects can present multiple resistance (resistance to two or more insecticidal compounds through multiple mechanisms) or cross resistance (resistance to two or more insecticidal compounds through a single mechanism) to xenobiotics (Denholm and Devine 2013). For example, in laboratory strains of *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae) there is multiple resistance

via both target site insensitivity and metabolic detoxification, both conferring permethrin resistance (Sonoda 2010). Therefore, to increase the understanding of pyrethroid resistance in soybean aphid and to make informed resistance management strategies it is essential to evaluate the diversity and distribution of target-site resistance in the field.

Metabolic detoxification is the most common mechanism of insecticide resistance (IRAC 2020b), resulting in the breakdown and elimination of toxic compounds. Resistant insects can have an enhanced metabolism typically involving the overexpression or upregulation of specific detoxifying enzymes that can metabolize xenobiotics (insecticides) before they reach the target site and upset the normal nerve function (Denholm and Devine 2013). Resistant insects predominantly use three gene families to detoxify xenobiotics: cytochrome P450-mediated monooxygenases (CYPs), glutathione-S-transferases (GSTs), and esterases (CE, E4 and FE4) (Soderlund and Bloomquist 1990, Panini et al. 2016). The CYP family is linked to resistance to a wide range of insecticides, including pyrethroids (David et al. 2013). The detoxification of xenobiotics occurs in two phases. In these phases, lipophilic insecticides such as pyrethroids can be converted by the detoxification enzymes into hydrophilic compounds that later will be eliminated by the resistant insect via excretion (Saha 2016). In phase I, the CYPs are responsible for the oxidation and reduction of the insecticidal molecule with the loss or addition of electrons (Feyereisen 2005), and the

esterases are responsible for the hydrolysis (Oakeshott et al. 2005) of the insecticidal compound, changing its chemical properties. In phase II, the resulting products of phase I undergo conjugation mediated by the GSTs to obtain lipophobic compounds that can be subsequently eliminated (Ranson and Hemingway 2005).

There are several case studies in arthropod species where metabolic resistance plays a key role in insecticide detoxification. Specifically, for pyrethroid resistance, in *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) the increased expression of P450 proteins was associated with acrinathrin resistance (Scharf et al. 2001). Additionally, esterases conferred permethrin resistance in *Bemisia tabaci* Gennadius (Hemiptera: Alerodidae) (Byrne et al. 2000) and GST overexpression was related with *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) resistance to deltametrin (Silva et al. 2015). In soybean aphid populations, pyrethroid resistance has been confirmed in China (Xi et al. 2015) and the U.S (Paula et al. 2020) with CYP overexpression as the predominant mechanism. However, in a synergistic study evidence of metabolic resistance was not found (Valmorbida et al. 2022b).

qRT-PCR or quantitative reverse transcription polymerase chain reaction is a commonly used technique in molecular biology to measure gene expression levels. By quantifying the mRNA levels of these genes, qRT-PCR can provide valuable information about the activity of metabolic detoxification pathways in

cells or tissues (VanGuilder et al. 2008). In order to normalize gene expression of detoxification enzymes, the identification of reference genes (often referred as housekeeping genes (HKGs)) that exhibit stable expression across different populations and conditions is essential (Butte et al. 2001, Koramutla et al. 2016). This is important because variation in gene expression due to population- or environment-specific factors can confound the interpretation of experimental results (Huggett et al. 2005, Kozera and Rapacz 2013). Therefore, looking rigorously for properties such as stability is important to ensure consistent expression across samples (Bustin and Mueller 2005) is vital for ensuring data comparability, consistency, reproducibility, and accurate interpretation in gene expression studies (Sun et al. 2010, Lu et al. 2013, Smitha et al. 2019). The most frequently used reference genes for expression analysis in insect species are Actin, *RPL*, Tubulin, *GAPDH, RPS, 18S, EF1A, TATA, HSP*, and *SDHA* (Lü et al. 2018). Additionally, the recurrent experimental conditions tested in insect populations are developmental stage, tissue, temperature, insecticide exposure, diet, population, virus infection, sex, photoperiod, and starvation (Lü et al. 2018). However, the expression levels of some reference genes can fluctuate under experimental conditions other than the ones that were originally tested (Gutierrez et al. 2008, Leal et al. 2015). Further, Freitas et al. (2019) examined the stability of reference genes across different stingless bee species (Hymenoptera: Apidae: Meliponini) subjected to different experimental conditions. They found that widely

used reference genes, such as *EF1A,* and *GAPDH* displayed significant variation in expression levels across populations. These findings emphasize the importance of validating the appropriate reference genes for the specific experimental variables and subject (Zhou et al. 2019a) such is the case of soybean aphid, an important agricultural pest. Additionally, understanding the potential of metabolic detoxification as a mechanism for pyrethroid resistance in soybean aphid field-collected populations is crucial.

Farmers play a crucial role in managing insecticide resistance by implementing appropriate management practices and making informed decisions(Leach et al. 2019, Hurley et al. 2023). This involves adopting IPM and Insecticide Resistance Management (IRM) strategies. Monitoring of soybean aphid populations, rotation of insecticides, adherence to label instructions, and the use of pest-resistant soybean varieties are some key chemical and non-chemical management practices that can mitigate the selection for resistant populations (Roush and McKenzie 1987, McCarville and O'Neal 2013, Koch et al. 2018). Agricultural advisors play a crucial role by ensuring that farmers are equipped with the necessary knowledge and tools to make informed decisions regarding pest management, technology adoption, and the use of insecticides while improving productivity, efficiency, and sustainability in their farming operations (Haigh et al. 2015). It is worth noting that specific management practices can vary between regions, and are influenced by factors such as

climate, pest pressure, and local regulations. Surveys have shown that more than half of the farmers in the Upper Midwest consider scouting as an important practice for insecticide application decision making. Additionally, farmers consider a threshold of 250 aphids per plant to be cost-effective (Olson et al. 2008). Also, the most used insecticides to manage soybean aphid are organophosphates and pyrethroids (Olson et al. 2008). However, farmers may not understand subtle differences in IPM terminology, such as “economic thresholds” and “economic injury levels”. Because of this, the implementation of this arduous practice is not often adopted (Hoidal and Koch 2021). Farmers' soybean aphid management practices and their perceptions on insecticide resistance are not well understood since the onset of insecticide resistance in this pest. Continuous research and monitoring help inform farmers about the most effective management practices for soybean aphids.

Monitoring resistance is not a widely used practice (Van Leeuwen et al. 2020). However, the timely identification and diagnosis of the resistance mechanisms can inform and facilitate management decisions, making good use of the insecticide toolbox, preventing the continuation of the selection pressure and enabling the possible recovery of the susceptible population (David et al. 2013). Resistance mechanisms are constantly evolving (Donnelly et al. 2016), insecticide resistance is becoming a barrier to soybean aphid control, and current farmer management is unknown. Therefore, this work aims to further understand

the mechanisms involved with the enzymatic regulation and loss of sensitivity in this important agricultural pest, and to understand farmer management practices and perceptions. The overall objectives were to 1) determine the variation and frequency of mutations from field-collected populations of soybean aphid, 2) validate reference genes for the analysis of gene expression of detoxification genes related to pyrethroid resistance in soybean aphid populations, 3) identify and quantify expression of detoxification enzymes in field-collected populations of soybean aphid, and 4) understand farmers' practices and decision-making processes. The results will play a crucial role for IRM to preserve the limited chemical toolbox for management of soybean aphid. Overall, insecticide resistance is a major problem for the sustainability of agriculture in the United States and the soybean industry, therefore, understanding the variability of resistance mechanisms and the farmers management practices will inform IPM and IRM.

Chapter I: Population Genetics of Pyrethroid Resistance in Soybean Aphid (*Aphis glycines* Matsumura)

Rosa E. Lozano, Amelia R. I. Lindsey, Débora P. Paula, David A. Andow, and Robert L. Koch

Abstract

The soybean aphid is a significant pest of soybean in the Midwest of the United States. In the last decade, reports of resistance to the major insecticide group used for soybean aphid control, pyrethroids, have become increasingly common. This is the first study to assess diversity in the voltage-gated sodium channel (VGSC) gene, the target site for pyrethroids, of 32 field-collected soybean aphid populations throughout the state of Minnesota, US. Using Amplicon sequencing (Amplicon-seq) of a region of the VGSC gene associated to pyrethroid resistance of individual aphids collected across Minnesota, we identified 33 variants including four variants previously characterized for their role in conferring pyrethroid resistance (M918l, M918L, L925M, and L1014F), and 43 unique haplotypes indicating high diversity among the soybean aphid populations. Overall, 55.40% of all the haplotypes and 74.0% of the soybean aphids had at least one resistance-associated variant.

Broadly, the presence of resistance variants can be significant contributors to pyrethroid resistance in soybean aphids and poses a considerable challenge for pest control programs that rely on pyrethroids as a primary method of control.

Understanding the genetic basis of pyrethroid resistance and monitoring its prevalence in soybean aphids can help to develop new control strategies for managing this pest in agricultural settings.

Introduction

Soybean production in North Central United States is threatened by the soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) (Ragsdale et al. 2004, 2011), an invasive insect pest originally from Asia (Venette and Ragsdale 2004). Soybean aphid feeds on phloem sap of the soybean plants and can quickly reach high densities causing up to 50% yield losses (Tilmon et al. 2011), with billions of dollars lost annually (Ragsdale et al. 2007). Even though soybean aphid infestations have decreased in some states, it remains a persistent problem in Minnesota and parts of North Dakota, South Dakota, and Iowa (Koch et al. 2018).

Chemical control with insecticides, such as organophosphates and pyrethroids (Koch et al. 2019), has been the main management tactic for this pest in the US (Hodgson et al. 2012, Koch et al. 2018). Following the first detection of soybean aphid in 2000 (Alleman et al. 2002), insecticide use increased considerably in soybean to protect the crop from this pest (Ragsdale et al. 2011). Consequently, soybean aphid's resistance to pyrethroid insecticides was first documented in 2015 in Minnesota (Hanson et al. 2017). Pyrethroid resistance has persisted in soybean aphid populations across the region, with resistance to bifenthrin and lambda-cyhalothrin documented with bioassays on

populations from Minnesota, South Dakota, North Dakota, Iowa, and Manitoba (Hanson et al. 2017, Menger et al. 2020). Examination of data from insecticide efficacy trials performed under field conditions showed the onset of practical resistance (i.e., decreased efficacy of field applications) to pyrethroids occurred after 2014 in Minnesota (Menger et al. 2022a).

Pyrethroids are commonly used neurotoxic synthetic organic pesticides that are similar to the natural pyrethrins produced by some chrysanthemum flowers. These compounds prevent the closure of voltage-gated sodium channels (VGSC) in the axonal membrane (Soderlund and Bloomquist 1989, Soderlund 2005, IRAC 2020). Target-site resistance is the primary mechanism involved in pyrethroid resistance across many insect species (Khambay and Jewess 2005). Specifically, pyrethroids bind to the α-subunit of the VGSC (Soderlund 2012), which contains four repeated domains (I–IV) and six hydrophobic transmembrane segments (S1–S6), where S1-S4 are the voltage-sensing module and S5-S6 are termed the P-region (Dong et al. 2014, Appendix B). Most of the amino acid variants that confer pyrethroid resistance are in the S5-S6 segments of the VGSC α-subunit, on domain II (Soderlund 2005). Examples of this include *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) with M918T (Eleftherianos et al. 2008a), *Psylliodes chrysocephala* Linnaeus (Coleoptera: Chrysomelidae) with L1014F (Zimmer et al. 2014), and *Aedes albopictus* Skuse (Diptera: Culicidae) with V1016G (Zhou et al. 2019b). For soybean aphid in particular, variants such as M918L (i.e., super knockdown resistance "*skdr*"),

M918I, L925M, and L1014F (i.e., knockdown resistance "*kdr*") have been identified in aphid colonies with confirmed pyrethroid resistance (Paula et al. 2021, Valmorbida et al. 2022b). These findings, however, were obtained from a limited number of soybean populations, thus much remains unknown about the broader diversity and frequency of target-site resistance to pyrethroids in soybean aphid.

Importantly, assessing the in-field diversity and distribution of target-site resistance is necessary for developing informed resistance management strategies. Therefore, to further understand the role of pyrethroid resistance in this important agricultural pest, we used high throughput sequencing to determine the genetic diversity of the VGSC in soybean aphids along with the determination of the spatial and temporal variability across Minnesota, US. The results of this study can subsidize the development of Integrated Pest Management (IPM) and Integrated Resistance Management (IRM) strategies to effectively minimize the development and spread of resistance in soybean aphid populations.

Materials and Methods

Soybean Aphid Collections

To assess variability of the VGSC in soybean aphid across Minnesota, we sampled a total of 32 field populations across a three-year period (12 populations in 2019, 10 populations in 2020, and 10 populations in 2021) (Fig 1.1). Some but

not all locations were repeated across multiple years. In 2019 and 2020, samples were collected from five sites in each field with at least 20 meters between sites. At each site, soybean aphid-infested plant material was taken from within a 1m2 area. There were two types of collections taken from each site. For the first type of collection, soybean aphids were kept alive on soybean branches carefully inserted in florist foam to maintain moisture and placed into coolers with ice packs for transport back to the laboratory where they were bioassayed within 24 hours of collection. For the second type of collection, soybean aphids were flash-frozen in liquid nitrogen in the field and transported to the laboratory, where they were transferred to 1.5-ml microtubes with RNAlater (Thermo Fisher Scientific) and kept at -80ºC.

In 2021, drought conditions resulted in soybean aphid infestations being considerably lower than in previous years across Minnesota. Therefore, the sampling methodology was modified, and soybean aphid-infested material was collected from fifteen sites in each field. Separate laboratory colonies for each field population were initiated with ~40 apterous female soybean aphid founders. The laboratory colonies were maintained in environmental growth chambers in 60-cm2 cages containing soybean plants (SD01-76R cultivar) kept at approximately 70% relative humidity, 25°C, and 16:8 (L:D) h photoperiod. After one week where aphids clonally reproduced, two types of collections were taken from each population. Following the methods described above, the first collection

was maintained alive for bioassays and the second collection was flash-frozen in liquid nitrogen and preserved in RNAlater in a -80ºC ultra-freezer for sequencing.

Pyrethroid Bioassays

Following the methods of Menger et al. (2020), the soybean aphids from each population, including an insecticide-susceptible laboratory population (i.e., Biotype I soybean aphid colony) as positive control, were exposed to the pyrethroid insecticides bifenthrin and lambda cyhalothrin in a diagnostic-concentration (LC99) bioassay to assess levels of resistance. An exception was Pomroy in 2021 which was only exposed to lambda cyhalothrin due to sample shortage. Technical grade bifenthrin (99.1% purity, FMC Corporation, Philadelphia, PA) and lambda cyhalothrin (98.7 % purity, Sigma–Aldrich, St. Louis, MO) were diluted separately in acetone (99.1% purity) through a series of serial dilutions to a final concentration of 0.0215 µg A.I. bifenthrin or 0.252 µg A.I. lambda cyhalothrin. Then, 0.5 ml of the insecticide solution (or solvent only as control-group) was used to coat the internal surfaces of 20-ml glass scintillation vials (Fisher Scientific, Hampton, NH). Vials were dried uncapped on a hotdog roller (Model # RDB24SS, Funtime Popcorn Company, Ontario, CA) without heat until complete evaporation of the acetone. After drying, treated vials were capped and stored in the dark at room temperature for no more than 2 weeks to ensure vial efficacy (Menger et al. 2020). Each treatment was replicated four times, with each replicate vial containing 10 apterous female soybean aphids from the same population. Soybean aphids that survived after 4 h of exposure were scored as

resistant. Soybean aphids that were moribund or dead after 4 h were scored as dead. The survivorship of soybean aphids was analyzed using a generalized linear model based on a binomial response (dead or alive) (package: stats, code: glm, family = binomial) with population as predictor. The resistance of soybean aphid to bifenthrin and lambda cyhalothrin was compared among populations with a likelihood-ratio chi-square test (package: car, code: Anova) and means were compared with Tukey's test (package: lsmeans, function: lsmeans). Mortality was determined by using the Henderson-Tilton formula for each field population (Henderson and Tilton 1955).

RNA Extractions and cDNA Synthesis

Fifteen apterous female soybean aphids per field (for 2019 and 2021, three from each of the five sites in each field; for 2021, fifteen collected at random from each colony, except for Pomroy where we only collected 10 individuals for the reasons explained above) that were preserved for sequencing were placed in 2.0-ml screw-cap tubes containing two 5.0-mm borosilicate beads and 200 µl of RNeasy Lysis Buffer obtained from the RNeasy Mini Kit (Qiagen). Sample homogenization was carried out using a FastPrep-24 Instrument (MP Biomedicals) for 20 s at 4 m/s. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and DNA was removed by on-column DNase digestion following the manufacturer's protocol. RNA was quantified with a Qubit 3.0. RNA HS Assay Kit and Qubit 3 Fluorometer (Thermo Fisher Scientific). cDNA was synthesized using the SuperScript IV First-Strand Synthesis System for RT-PCR kit (Thermo

Fisher Scientific) using 20 ng total RNA as input and was reverse transcribed with the provided oligo-d(T) and random hexamer mix.

Primer Design, Library Construction, and Sequencing

To determine the genetic diversity in the resistance-associated region of the VGSC, we designed PCR primers to amplify a previously identified region of the *A. glycines* VGSC gene in the domain II, where resistance variants were identified in laboratory colonies of soybean aphid (Paula et al. 2021) and in a variety of other insects (Soderlund 2005, Marshall et al. 2012, MacKenzie et al. 2018). The *A. glycines* voltage-gated sodium channel para-like heterodimer 1 alternatively spliced variant X2 (AgNavH1 X2, GeneBank: MT379843.1) was used to design PCR primers that generated a 401 bp amplicon. Specifically, we designed a forward exon-exon junction primer bridging exons 17 and 18, and a reverse primer in exon 20, using Primer3 web version 4.1.0 (Untergasser et al. 2012) (forward primer sequence: AglyVGSC-17-18-F: 5-’GCGTTCATTTCGTTTGCTTCG-3’, reverse primer sequence: AglyVGSC-20-R: 5’-TCAGCAACAACGCCAAGAAA-3’) (Fig 1.2). We first ran a primary PCR with the AglyVGSC primer-pair followed by a secondary amplification in which primers contained Nextera compatible adaptors (lower case nucleotides) to generate a 509 bp product (forward primer sequence: Nextera-AglyVGSC-17-18-F: 5-’ tcgtcggcagcgtcagatgtgtataagagacagGCGTTCATTTCGTTTGCTTCG-3’, reverse primer sequence: Nextera-AglyVGSC-20-R: 5’-gtctcgtgggctcggagatgtgtataagagacagTCAGCAACAACGCCAAGAAA-3’). All

PCRs were performed using a Mastercycler Nexus (Eppendorf) with Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs) in a 20 µl reaction, with 500 nM of each primer and 1 µl of cDNA template. The cycling protocol started with an initial denaturation at 98°C for 2 minutes, then 40 cycles of a three-step amplification including denaturation at 98°C for 10 seconds and annealing at 65°C for 10 seconds and extension 72°C for 10 seconds, followed by final extension at 72°C for 2 minutes and a final hold at 4°C. Amplification was checked on a 1% agarose gel and visualized by GelRed staining (Biotium).

Amplicons containing Nextera-adaptors were cleaned and size-selected (to remove primer dimers) using AMPure XP SPRI beads following manufacturer's protocols. Purified amplicons were quantified by Picogreen, normalized to 2 ng/µl with the SequalPrep Normalization Kit, and barcoded as previously described (Gohl et al. 2016). Each library was normalized to 2 nM and pooled, to be sequenced in one MiSeq sequencing lane (2x300 bp v3 kit) at a concentration of 10 pM + 5% PhiX.

Bioinformatics and Statistical Analysis

Fastq data was demultiplexed using bcl2fastq version 2.20.0. Analyses were carried out with shell commands in Linux-based programs (Ubuntu 20.04.3 LTS, GNU/Linux 4.4.0-19041-Microsoft x86_64). Two filtering steps were performed to ensure accurate variant detection. First, library adapters were trimmed and low-quality reads (Phred quality score <15) with at least 100 bp length after trimming (MINLEN:100) were discarded using Trimmomatic version

0.39 (Bolger et al. 2014). Additional quality checks were performed with Fastqc version 0.11.9 (Andrews 2010). The trimmed reads were aligned to the reference soybean aphid VGSC coding sequence (GenBank accession number KAE9541230.1) with BWA MEM version 0.7.17 with baseline parameters (Li and Durbin 2009). Alignment files were converted and sorted with SAMtools version 1.10 using baseline parameters (Li and Durbin 2009). Variant calling was performed with the GATK HaplotypeCaller tool version 4.1.2 using mode -ERC GVCF (Poplin et al. 2018). During the second more stringent filtering step, variants were filtered using the baseline parameters provided by the Broad (Khalfan 2020) to achieve a high degree of sensitivity and reduce false positives (SNPs were filtered using: QD < 2.0, FS > 60.0, MQ < 40.0, SOR > 4.0, MQRankSum < -12.5, and ReadPosRankSum < -8.0; indels were filtered using: QD < 2.0, FS > 200.0, and SOR > 10.0). Finally, Beagle version 5.4 with baseline parameters (Browning et al. 2021) was used for phasing genotypes and to infer haplotypes. Haplotype visualization was performed using GIS software ArcGIS Dashboard and ArcGIS Pro (Ormsby et al, 2009), and R studio (RStudio Team, 2015).

The Fixation Index (FST) analysis of population differentiation based on population structure (Holsinger and Weir 2009) was calculated with vcftools version 0.1.17 using baseline parameters (Danecek et al. 2011) and R studio to visualize the results (RStudio Team, 2015). The calculations were done on a per-site basis. Hardy Weinberg Equilibrium (HWE) was checked using the package

PLINK version 2.0 (Chang et al. 2015) to estimate if the genotype frequencies in the populations remained constant. Significance was defined at α= 0.05. Finally, the correlation between mortality (as inferred by the bifenthrin and lambda cyhalothrin glass-vial bioassays) and the proportion of soybean aphids that were (1) susceptible (S*/S*), (2) heterozygous for a resistance-associated haplotype (S*/R*), (3) homozygous for a single resistance-associated haplotype (Ra/Ra), (4) heterozygous for different resistance-associated haplotypes (Ra/Rb), or (5) with any two resistance-associated haplotypes, regardless of zygosity (R*/R*) was analyzed using R using the function cortes() and the method = 'spearman'.

Results

Resistance to pyrethroids varied across Minnesota

Within each year, mortality of soybean aphids exposed to bifenthrin differed significantly among populations (2019: X2= 166.83, df= 12, P < 0.001; 2020 X2= 158.5, df= 10, P < 0.001, and 2021: X2= 60.259, df= 9, P < 0.001). In all years, mortality of aphids from the insecticide-susceptible laboratory population (“Biotype 1”) treated with bifenthrin and lambda cyhalothrin was 100%. Mortality of the field-collected populations treated with bifenthrin ranged from 0% to 100% in 2019 and 2020, and from 30% to 100% in 2021 (Appendix C). The populations with the lowest mortality were Rosemount 1 and Rosemount 2 in 2019 (Appendix Ca), Grand Meadow, Little Chicago 1, and Little Chicago 2 in 2020 (Appendix Cb), and North Branch in 2021 (Appendix Cc). In the case of

lambda cyhalothrin the mortality of soybean aphids exposed was significantly different between populations only for the 2019 and 2020 (2019: X2= 900.8, df= 12, P < 0.001; 2020 X2= 118.15, df= 10, P < 0.001; and 2021: X2= 1.3106, df= 10, P = 0.999). However, the mortality due to lambda cyhalothrin was very high, with the lowest mortality each year of 65% at Rosemount 1 and 2 in 2019, 40% at Grand Meadow in 2020, and 95% at Gilmanton in 2021 (Appendix D).

VGSC diversity

Identification of pyrethroid resistance variants among populations

We sequenced a 401 bp region of the VGSC domain II from 475 individual soybean aphids using Amplicon-seq. These aphids comprised 15 individuals from each of 31 field-collected populations, and 10 individuals from Pomroy in 2021 (for the reasons described above). We generated an average of 11,877 paired-end reads per sample after. Samples had an average of 50487x sequence coverage. After aligning reads to the reference soybean aphid VGSC coding sequence (GenBank accession number KAE9541230.1), we identified a total of 33 variants (Table 1.1, File SF 1), which comprised 29 SNPs and 4 indels. All 475 aphids had the in-frame deletion of three nucleotides of the codon of the amino acid 1016. Pyrethroid resistance-associated variants included the following non-synonymous changes: two polymorphisms in codon 918 (M918l or M918L), L925M, and L1014F, which were present in 14.7%, 12.0%, and 30.4% of aphids, respectively.

Multiple pyrethroid resistance variants identified across Minnesotan soybean aphids

We identified 43 haplotypes across all the 32 soybean aphid populations (Table 1.2, Table SF 1, see the nucleotide alignment in the supplementary information files). The four most abundant haplotypes accounted for 93.0% of the total across the three years (Fig 1.3). Haplotype 1, which accounted for 41.6% of the total, contained only the codon deletion unknown to be associated with resistance (V1016). In contrast, haplotypes 2, 3, and 4 each had one pyrethroid resistance-associated variant, and accounted for 27.8%, 12.9%, and 10.9%, of the total of the haplotypes, respectively. Overall, 55.4% of all the haplotypes and 74.0% of the soybean aphids had at least one pyrethroid resistance-associated variant. Of the 55.4% of haplotypes containing a pyrethroid resistance-associated variant, the most incident haplotypes (i.e., Haplotypes 2, 3 and 4) contained a single resistance-associated variant, which accounted for 53.7% of the individuals (Appendix F). Only5 haplotypes (1.5%) were identified with two or three variants related to pyrethroid resistance. They were present in low frequencies, ranging from 0.1 to 0.7% (0.3 to 1.5% of aphids respectively; Appendix F).

VGSC-II heterozygosity is common

At the VGSC amplicon region, 64.4% of the soybean aphids of all populations were heterozygous (Fig 1.4a). The same pattern was observed for individual populations collected across the three years, where 26 of the 32

populations contained >50% of aphids that were heterozygous (Fig 1.4b). Additionally, most of the variants were in HWE ($P < 0.05$), except for variants at amino acid positions 964, 965, 969, 997, and 1004, which showed a deficit of heterozygous individuals, and variant L1014F with an excess of heterozygous individuals (Table 1.3, Appendix A).

High VGSC diversity within populations across Minnesota

There was evidence of a high VGSC-II haplotype diversity in most populations across Minnesota (Fig 1.5). Haplotypes with only one variant known to confer pyrethroid resistance (Haplotypes 2, 3, and 4) and the haplotype with a deletion at amino acid position 1016 (Haplotype 1), were the most abundant and were widespread across Minnesota in almost all populations (Fig 1.5). These haplotypes included the variants L1014F (Haplotype 2), M918I (Haplotype 3), and L925M (Haplotype 4). Within each year from pyrethroid resistance-associated variants, Haplotype 2 and Haplotype 3 were predominant (Fig 1.5, Fig 1.6a, Appendix E). However, the variation in the soybean aphid haplotype proportions did not change significantly among the three years (Fig 1.6a). Additionally, the fixation index (FST) across the years of 2019, 2020, and 2021 was below 0.15, suggesting that there was no significant population differentiation across the VGSC-II region (Appendix G).

Survival to pyrethroids is associated with resistance variants

The proportion of individuals that were susceptible (S*/S*), heterozygous for a pyrethroid resistance-associated haplotype (S*/R*), or with any two

pyrethroid resistance-associated haplotypes (Ra/Ra or Ra/Rb) varied across soybean aphid populations, with the highest for the soybean aphids that were heterozygous for a pyrethroid resistance-associated haplotype (S*/R*) (average 35.8%) (Fig 1.7). Individuals with haplotypes containing pyrethroid resistance-associated variants were present in populations at average proportions of 26.7% for heterozygous (Ra/Rb) and 11.1% for homozygous (Ra/Ra) (Fig 1.7). For individuals exposed to bifenthrin, there was not a significant correlation between mortality and proportion of individuals that were susceptible (S*/S*) to pyrethroid or heterozygous for a pyrethroid resistance-associated haplotype (S*/R*) ($P>0.05$) (Fig 1.8a, b). However, there was a significant negative correlation between mortality and the proportion of individuals that were homozygous for a single pyrethroid resistance-associated haplotype (Ra/Ra) (rho =-0.4338, P= 0.0148) or heterozygous for different pyrethroid resistance-associated haplotypes (Ra/Rb) (rho= -0.3687, P= 0.0412) (Fig 1.8c, d). Furthermore, individuals with any two pyrethroid resistance-associated haplotypes, regardless of zygosity (R*/R*), had a stronger negative correlation (rho= -0.4771, P= 0.0067) (Fig 1.8e). For individuals exposed to lambda cyhalothrin, there was not a significant correlation between mortality and proportion of individuals that were pyrethroid susceptible (S*/S*), heterozygous for a pyrethroid resistance-associated haplotype (S*/R*), or homozygous for a single pyrethroid resistance-associated haplotype (Ra/Ra) ($P>0.05$) (Fig 1.9a-c). However, there was a significant negative correlation between mortality and the proportion of individuals that were

heterozygous for different pyrethroid resistance-associated haplotypes (Ra/Rb) (rho =-0.6222, P= 0.0001) and (Fig 1.9d). Furthermore, individuals with any two pyrethroid resistance-associated haplotypes, regardless of zygosity (R*/R*), showed a stronger negative correlation (rho= -0.5031, P= 0.0034) (Fig 1.9e).

Discussion

The continued use of pyrethroid insecticides (e.g., bifenthrin and lambda cyhalothrin) has led to the evolution of resistance in soybean aphid populations (Hanson et al. 2017, Menger et al. 2020, Menger et al. 2022a). Target-site insensitivity has been found to be an important mechanism for resistance in this pest (Paula et al. 2021, Valmorbida et al. 2022b). This is the first assessment of the genetic diversity of the VGSC-II among field populations of soybean aphid throughout Minnesota-US, a state that has historically struggled with this pest (Hanson et al. 2017).

From the 33 VGSC-II variants we identified in Minnesota-US soybean aphids, four of them (M918L, M918I, L925M, and L1014F) have been associated with pyrethroid resistance in several important pest species around the world, including the soybean aphid and other aphid species (Foster et al. 2014, MacKenzie et al. 2018, Paula et al. 2021). In the majority of cases, only one of these resistance variants was present in a given haplotype, though haplotypes with multiple resistance associated variants were found in very low proportions (Appendix F). In addition to known pyrethroid resistance variants present in the

samples analyzed, a single amino acid deletion (V1016) was found ubiquitously (Table 1.1). We are not aware of this deletion having been associated with pyrethroid resistance in aphids, and it appears to be fixed in the populations examined. Furthermore, to our knowledge there is no record in the literature of this deletion occurring in other arthropod species. However, polymorphisms at amino acid position 1016 (V1016G, V1016I) are correlated with pyrethroid resistance in *Aedes aegypti* (Linnaeus) (Deming et al. 2016, Saingamsook et al. 2017).

Historically, the *kdr* (knock-down resistance) and *skdr* (super-kdr) variants have been reported as recessive in many important insect pests (Davies et al. 2007). In contrast, aphids that are heterozygous for M918l (*skdr*), M918L, L925M, or L1014F (*kdr*) have a pyrethroid resistance phenotype (Fontaine et al. 2011, Paula et al. 2021, Valmorbida et al. 2022b). This corroborates a recent study where it was found that resistance to pyrethroids is haplosufficient (MacKenzie et al. 2018). In addition to the impacts of zygosity and dominance on the resistance phenotype, there may also be negative impacts on other traits such as reproductive success, or survival under environmental stress (Kliot and Ghanim 2012, Platt et al. 2015). These traits can confer advantages in one context, such as when exposed to insecticides, but result in disadvantages in other contexts, such as when not exposed to insecticides, leading to a trade-off between adaptation and fitness (Foster et al. 2011). For example, heterozygosity for pyrethroid resistance at the VGSC may confer some degree of resistance, but

have lower fitness costs (e.g., effects on survivorship or reproductive success) than homozygous individuals that may have a higher degree of resistance, but also a higher fitness cost (Fenton et al. 2010, Foster et al. 2011, Brito et al. 2013, Homem et al. 2020). However, such fitness costs have not been documented for soybean aphid (Menger et al. 2022b, Valmorbida et al. 2022a). While we found that homozygous individuals with variants associated to the pyrethroid resistance were notably more frequent than heterozygous in some populations (e.g., in Amador and LittleChicago2 (Fig 1.4)), heterozygous aphids were overall more common. Furthermore, the variant L1014F with evidence for Hardy-Weinberg disequilibrium presented excess of heterozygosity, a pattern also seen in *Anopheles gambiae* Giles (Diptera: Culicidae) (Pinto et al. 2006, Fassinou et al. 2019). In aphids, this disequilibrium could be partially due to clonal propagation (making it difficult to get two copies of the same allele) (Rasmussen and Kollmann 2008), and interactions with fitness consequences for having this particular VGSC-II variant (Stern 1943, Mayo 2008). Finally, there is a potential for synergistic or additive effects when multiple resistance variants are present (Hardstone and Scott 2010). This has been seen in populations of *M. persicae* where individuals that were heterozygous for both *kdr* variants L1014F and M918T had higher pyrethroid resistance levels than individuals with only one of the variants (Eleftherianos et al. 2008b).

Given the diversity of patterns across insects with regards to the haplosufficiency of target-site mediated pyrethroid resistance (MacKenzie et al.

2018), we used our complementary genetic and mortality data to determine if there were any population level correlations between susceptibility and genotype. For bifenthrin, mortality was negatively correlated with the proportion of soybean aphids with any two resistance-associated haplotypes, regardless of zygosity (R*/R*) (Fig 1.8c, d, e). In the case of lambda cyhalothrin, mortality was negatively correlated with the proportion of soybean aphids that were heterozygous for different resistance-associated haplotypes (Ra/Rb) (Fig 1.8d). Similar instances have been reported in other insect species such as *Anopheles arabiensis* (Hemming-Schroeder et al. 2018). Unfortunately, the soybean aphids used for the phenotypic and genotypic analysis were not the same individuals, therefore, this analysis is not a direct correlation between phenotype and genotype and could lead to underestimating or overestimating some of the relationships.

The absence of spatial structuring across the geographical area might be attributed to long-distance movements of the soybean aphids (Schmidt et al. 2012) and the likelihood of having different aphid genotypes colonizing an area each year. Additionally, sexual reproduction and recombination in the fall on buckthorn (the primary host), or the absence of insecticide applications because of the low aphid infestations in the years sampled (Bass et al. 2014, Charaabi et al. 2016) could have contributed to the lack of spatial structuring.

The pyrethroid resistance variants in the soybean aphid populations studied have significant implications for its control as they can be used as

markers to assess the genetic basis of resistance. Two main contributions of our work to the pest control and resistance programs are that the genetic basis of resistance may be widely spread in a large geographical area, as it was identified for almost all Minnesotan soybean aphid field populations, and may consistently persist over the years, as they were identified in all the three-years period of evaluation of the field-collected populations. This information can serve as a baseline for future monitoring efforts of the evolution of the resistance (Yainna et al. 2021), for example demonstrating the necessity of refuge areas around the soybean cultivation. Furthermore, all farmers across this geography should consider implementation of IPM and IRM. Future work could focus on the functional testing of the resistance variants, the continuation of the monitoring efforts, and the assay of a broader geographical area, such as multiple states.

Acknowledgments

Thank you to Christy Henzler and Thomas Kono for their bioinformatics assistance, and Arthur Vieira for the statistical advice. Also, thank you to Lisa Behnken, Bruce Potter and Jonathan Dregni for helping to locate fields with soybean aphid infestations, and James Menger for assistance with bioassays and keeping the soybean aphid colonies. This work was supported by the Minnesota Rapid Agricultural Response Program (RARF), the USDA National Institute of Food & Agriculture (NIFA) (award no. 2019-68008-29892), the

Department of Entomology Lugger-Radcliffe Summer Fellowship, and a University of Minnesota Doctoral Dissertation Fellowship.

Tables

Table 1.1. VGSC-II variants identified in the *Aphis glycines* 32 field populations from Minnesota-US sampled in from 2019 to 2021. The variants associated with pyrethroid resistance in other insect pests are indicated in bold.

Variant number	Nucleotide position in the amplicon	Amino acid position in *A. glycines**	*Musca domestica* amino acid position**	Nucleotide in reference	Alternate	Reference Amino Acid > Variant amino acid	Variant Proportion	Resistance Documented?
1	35	890	905	G	C	Alanine > Proline	0.001	No
2	39	891	906	A	G	Lysine > Arginine	0.001	No
3	46	893	908	G	A	Tryptophan > Nonsense	0.002	No
4	60	898	913	T	C	Leucine > Proline	0.002	No
5	**74**	**903**	**918**	**A**	**T**	**Methionine > Leucine**	0.005	Yes (Eleftherianos et al. 2008, Marshall et al. 2012, Paula et al. 2021, Valmorbida et al. 2022b)

6	**76**	**903**	**918**	**G**	**A**	**Methionine > Isoleucine**	0.142	Yes (Sonoda et al. 2012, Paula et al. 2021, Soh and Veera Singham 2021, Valmorbida et al. 2022b)
7	88	907	922	T	C	Isoleucine > Isoleucine	0.001	No
8	**95**	**910**	**925**	**T**	**A**	**Leucine > Methionine**	0.119	Yes (Millán-Leiva et al. 2021, Paula et al. 2021, De Beer et al. 2022, Valmorbida et al. 2022b)
9	97	910	925	G	T	Leucine > Phenylalanine	0.001	No
10	123	919	934	T	C	Isoleucine > Threonine	0.002	No
11	139	924	939	C	A	Phenylalanine > Leucine	0.001	No

12	148	927	942	G	T	Methionine > Isoleucine	0.001	No
13	149	928	943	G	T	Glycine > Cysteine	0.001	No
14	155	930	945	C	T	Glutamine > Nonsense	0.003	No
15	179	937	953	G	-	Threonine > Frame shift	0.001	No
16	180	938	953	A	G	Glutamic Acid > Glycine	0.001	No
17	197	943	958	A	-	Phenylalanine > Frame shift	0.001	No
18	213	949	964	C	G	Proline > Arginine	0.002	No
19	215	950	965	G	-	Arginine > Frame shift	0.001	No
20	228	954	969	C	A	Threonine > Asparagine	0.003	No
21	230	955	970	G	A	Aspartic Acid > Asparagine	0.001	No
22	272	969	984	G	T	Glycine > Cysteine	0.001	No
23	272	969	984	G	A	Glycine > Serine	0.001	No

24	273	969	984	G	T	Glycine > Valine	0.001	No
25	294	976	991	G	T	Tryptophan Leucine	0.002	No
26	311	982	997	G	T	Glycine > Nonsense	0.004	No
27	314	983	998	G	A	Glutamic Acid > Lysine	0.001	No
28	319	984	999	A	G	Proline > Proline	0.001	No
29	334	989	1004	C	A	Phenylalanine > Leucine	0.004	No
30	357	997	1012	G	T	Glycine > Valine	0.001	No
31	**362**	**999**	**1014**	**C**	**T**	**Leucine > Phenylalanine**	0.304	Yes (Martinez-Torres et al. 1999, Fontaine et al. 2011, Safiyanu et al. 2019, Paula et al. 2021, Valmorbida et al. 2022b)

32	368	1000	1016	GTA	-	Valine > Deletion	1	No
33	381	1005	1020	T	G	Leucine > Arginine	0.005	No

*Amino acid position of translated *A. glycines* reference (AgNavH1 X2, GeneBank: MT379843.1)
***Musca domestica* amino acid position used fo *kdr* nomenclature (GenBank: U38813)

Table 1.2. VGSC-II haplotypes identified in 32 field populations of *Aphis glycines* from Minnesota-US sampled in 2019 to 2021. Haplotypes and variants related to pyrethroid resistance are indicated in bold.

Haplotypes	Variants in Haplotype[1]	Variant(s) related to pyrethroid resistance	Incidence (%)
Haplotype 1	32		41.7895
Haplotype 2	**31**,32	L1014F	27.8947
Haplotype 3	**6**,32	M918I	12.9474
Haplotype 4	**8**,32	L925M	10.9474
Haplotype 5	**6**,**31**,32	M918I, L1014F	0.736842
Haplotype 6	26,32		0.421053
Haplotype 7	**5**,**31**,32	M918L, L1014F	0.315789
Haplotype 8	**6**,**8**,**31**,32	M918I, M918I, L1014F	0.315789
Haplotype 9	20,29,32		0.315789
Haplotype 10	32,33		0.315789
Haplotype 11	**5**,32	M918L	0.210526
Haplotype 12	**6**,**8**,32	M918I, L925M	0.210526
Haplotype 13	13,**31**,32	L1014F	0.210526
Haplotype 14	25,32		0.210526
Haplotype 15	**31**,32,33	L1014F	0.210526
Haplotype 16	1,2,4,32		0.105263
Haplotype 17	3,**8**,32	L925M	0.105263
Haplotype 18	3,32		0.105263
Haplotype 19	4,**6**,**31**,32	M918I, L1014F	0.105263
Haplotype 20	4,32		0.105263
Haplotype 21	**6**,10,32	M918I	0.105263
Haplotype 22	**6**,16,32	M918I	0.105263
Haplotype 23	**6**,17,32	M918I	0.105263
Haplotype 24	**6**,27,32	M918I	0.105263
Haplotype 25	7,32		0.105263
Haplotype 26	**8**,11,32	L925M	0.105263
Haplotype 27	**8**,29,32	L925M	0.105263
Haplotype 28	**8**,**31**,32	L925M, L1014F	0.105263
Haplotype 29	9,32		0.105263
Haplotype 30	10,**31**,32	L1014F	0.105263

Haplotype 31	12,32		0.105263
Haplotype 32	13,32		0.105263
Haplotype 33	13,32		0.105263
Haplotype 34	15,32		0.105263
Haplotype 35	18,**31**,32	L1014F	0.105263
Haplotype 36	18,32		0.105263
Haplotype 37	19,**31**,32	L1014F	0.105263
Haplotype 38	21,**31**,32	L1014F	0.105263
Haplotype 39	23,32		0.105263
Haplotype 40	23,32		0.105263
Haplotype 41	24,32		0.105263
Haplotype 42	28,32		0.105263
Haplotype 43	30,**31**,32	L1014F	0.105263

[1]As per Table 1.1

Table 1.3. Hardy-Weinberg Equilibrium (HWE) genotype frequencies and test statistics for VGSC-II variants at amino acid positions 918, 925, 1014, and 1016 associated to pyrethroid resistance and identified in *Aphis glycines* from 32 field populations sampled in Minnesota-US from 2019 to 2021. Heterozygous, homozygous reference allele genotypes (Homozygous 1), homozygous alternate allele genotypes (Homozygous 2), and Chi-square test (ChiSq).

Musca domestica Amino Acid Position*	Observed frequency (Homozygous 1/ Heterozygous / Homozygous 2)	Expected frequency (Homozygous 1/ Heterozygous / Homozygous 2)	ChiSq	*p*-value	*p*-value Heterozygous Deficit	*p*- value Heterozygous Excess
918	463/5/0	463.01/4.97/0.01	1.35E-02	1.00E+00	1.00E+00	9.89E-01
918	342/113/11	340.78/115.45/9.78	2.09E-01	5.79E-01	3.73E-01	7.54E-01
925	362/93/10	358.87/99.27/6.87	1.85E+00	1.89E-01	1.23E-01	9.40E-01
925	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
1014	214/223/33	225.43/200.15/44.43	6.13E+00	1.69E-02	9.95E-01	8.75E-03
1016	0/0/467	0.00/0.00/467.00	-nan	1.00E+00	1.00E+00	1.00E+00

**Musca domestica* amino acid position used for *kdr* nomenclature (GenBank:U38813).
-nan = SNP is monomorphic (mono-allelic).

Figures

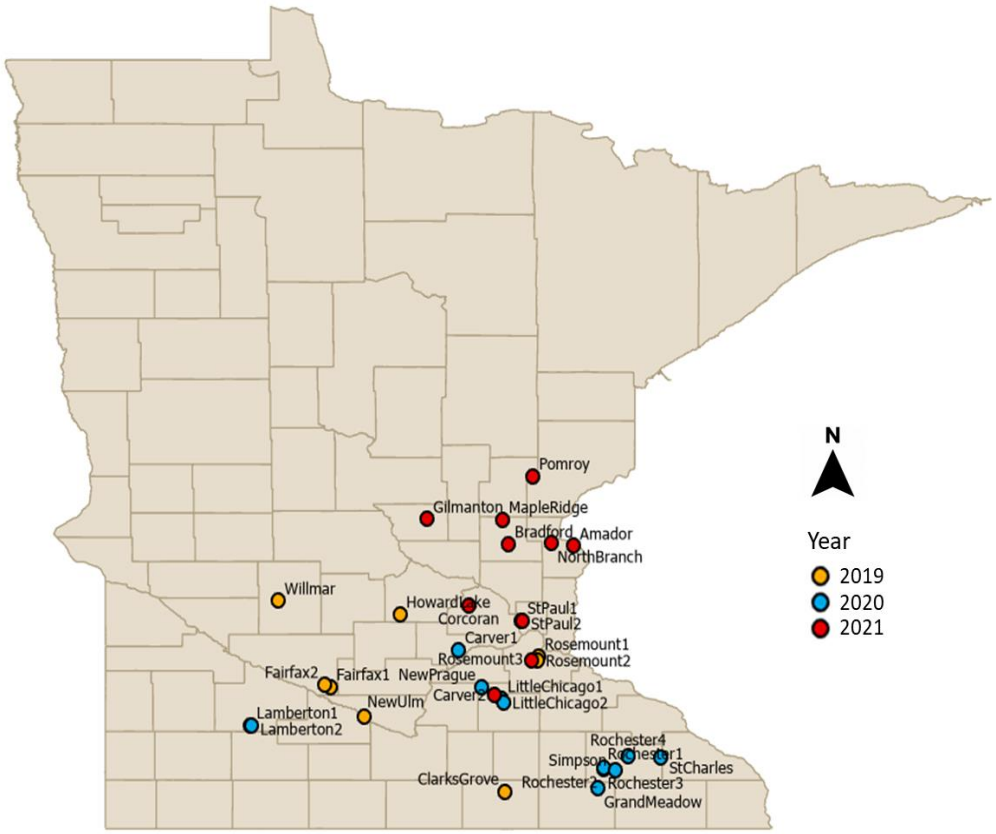


Fig 1.1. Field-collected *Aphis glycines* populations from Minnesota-US in the years 2019, 2020, or 2021.

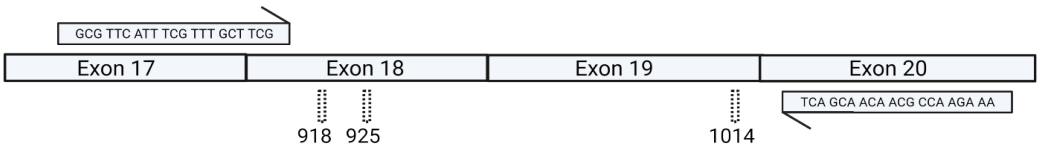


Fig 1.2. Representation of exon 17 to exon 20 of the VGSC-II in the coding sequence of the soybean aphid (GenBank accession number KAE9541230.1), showing the pyrethroid resistance-associated loci at amino acid positions 918, 925, and 1014, as wells as, a forward exon-exon junction primer bridging exons 17 and 18, and a reverse primer in exon 20. The forward primer sequence: AglyVGSC-17-18-F: 5-'GCGTTCATTTCGTTTGCTTCG-3', and the reverse primer sequence: AglyVGSC-20-R: 5'-TCAGCAACAACGCCAAGAAA-3'.

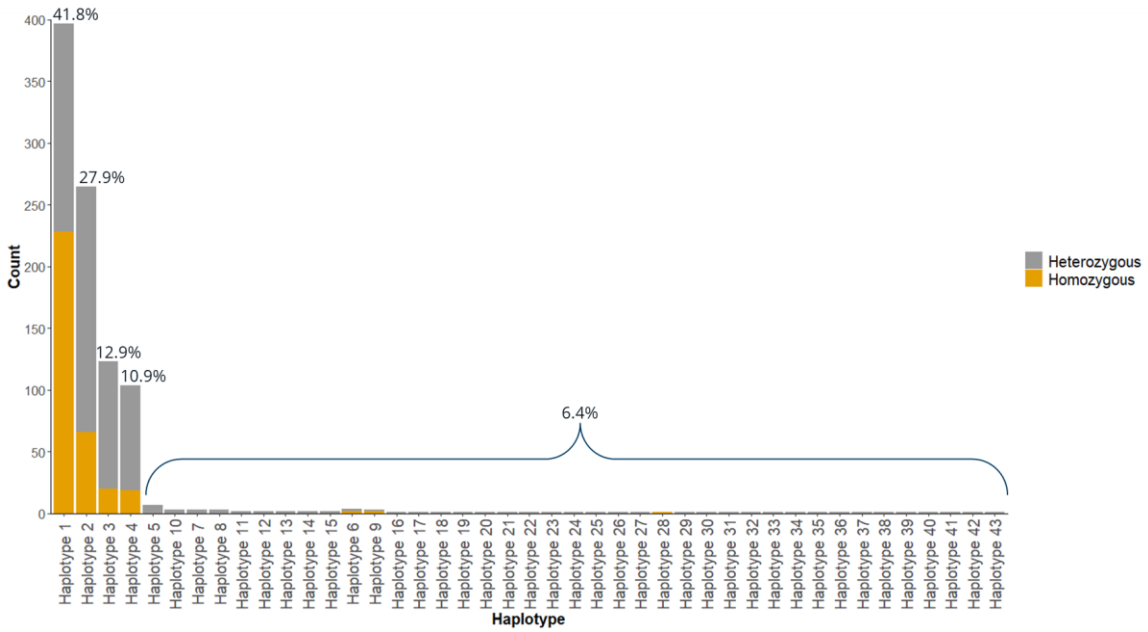


Fig 1.3. Unique VGSC-II haplotypes found in 32 field populations of *Aphis glycines* in Minnesota-US from 2019 to 2021. Haplotype percentages across the 3 years are the top of each bar.

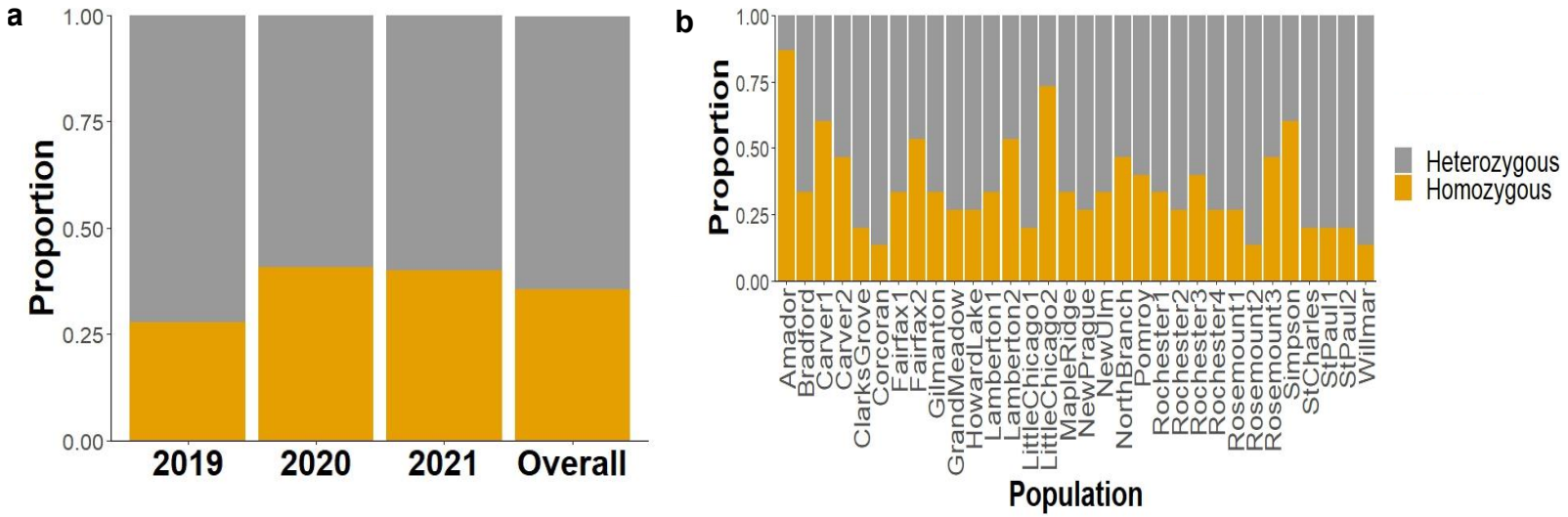


Fig 1.4. Proportion of VGSC-II homozygous and heterozygous in *Aphis glycines* individuals from 32 field populations (a) by year (2019 to 2021) and overall (three years combined), and (b) by populations in Minnesota-US across the three years.

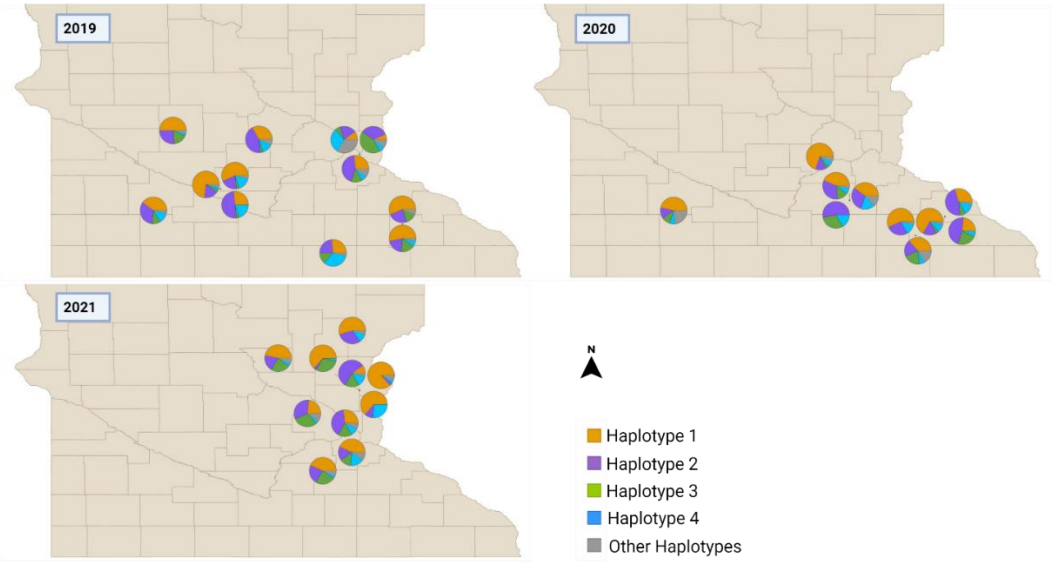


Fig 1.5. Proportion of the VGSC-II haplotypes in *Aphis glycines* from 32 field populations in their approximate sampled locations from 2019 to 2021 in the southern half of Minnesota, US. Each slice in the pie chart represents a haplotype.

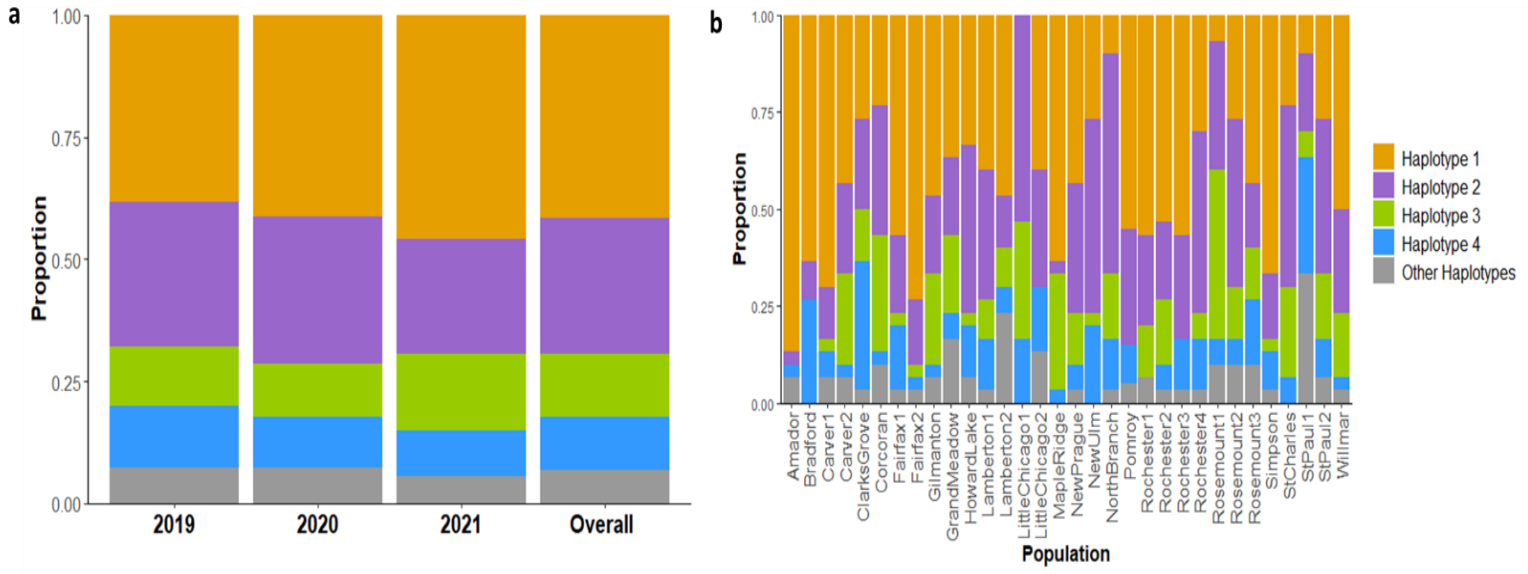


Fig 1.6. Proportion of VGSC-II haplotypes in *Aphis glycines* from 32 field populations a) by year (2019 to 2021), overall (three years combined), and b) by populations collected in Minnesota-US across the three years.

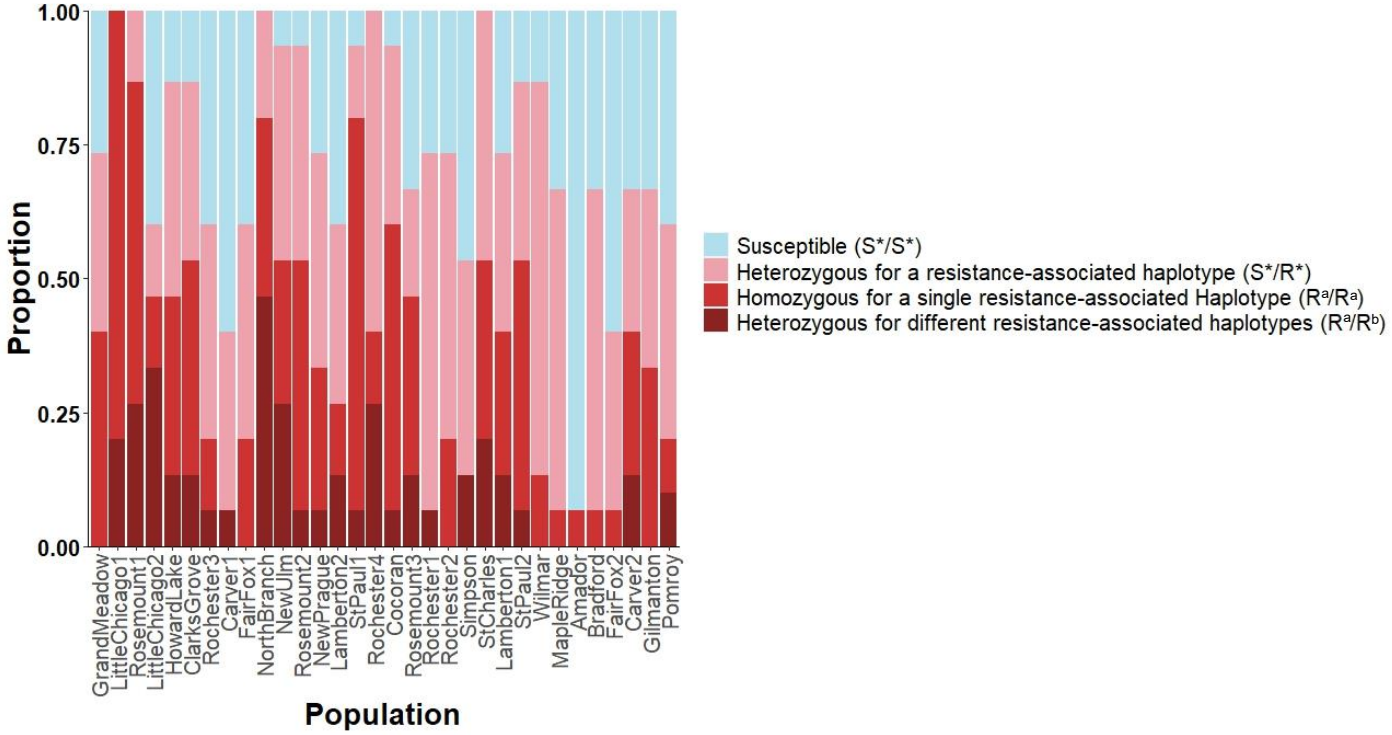


Fig 1.7. Proportion of *Aphis glycines* individuals that were susceptible for pyrethroid (S*/S*), heterozygous for a pyrethroid resistance-associated haplotype (S*/R*), homozygous for a single pyrethroid resistance-associated haplotype (R^a/R^a), heterozygous for different pyrethroid resistance-associated haplotypes (R^a/R^b), or with any two pyrethroid resistance-associated haplotypes, regardless of zygosity (R^*/R^*) across 32 field populations collected in Minnesota-US from 2019 to 2021.

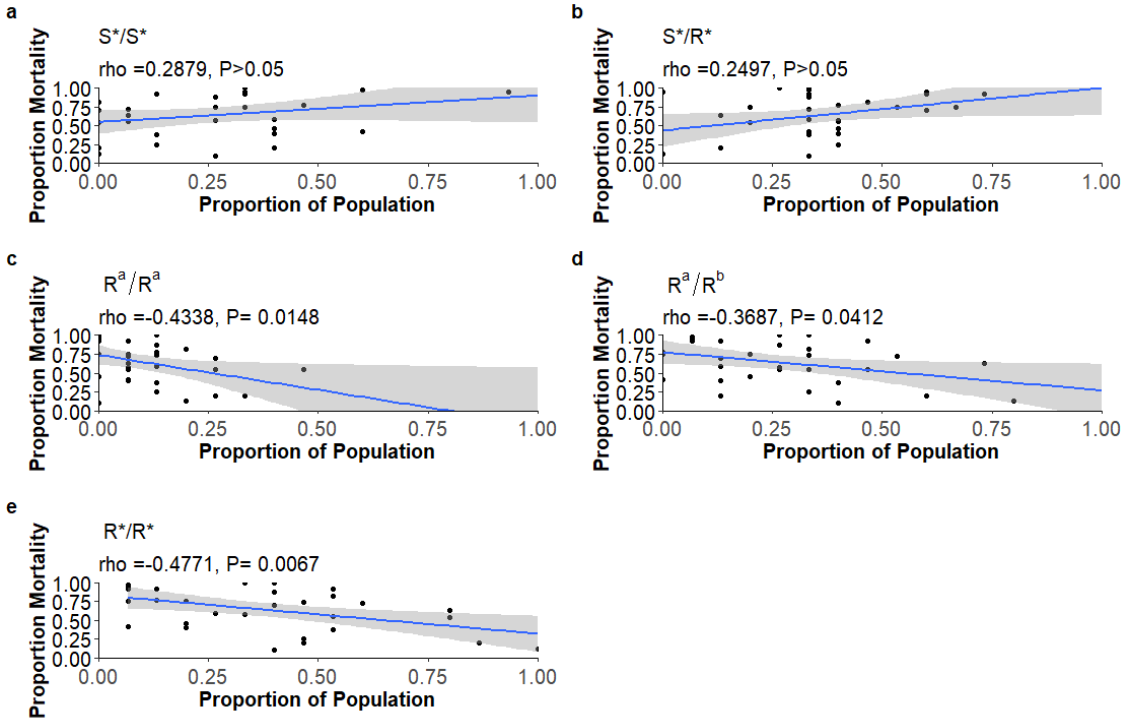


Fig 1.8. Correlation between the proportion mortality from exposure to bifenthrin and the proportion of each *Aphis glycines* population that was a) susceptible (S*/S*); b) heterozygous for a resistance-associated haplotype (S*/R*); c) homozygous for a single resistance-associated haplotype (R^a/R^a); d) heterozygous for different resistance-associated haplotypes (R^a/R^b); or e) with any two resistance-associated haplotypes, regardless of zygosity (R^*/R^*) across 32 field populations collected in Minnesota-US from 2019 to 2021.

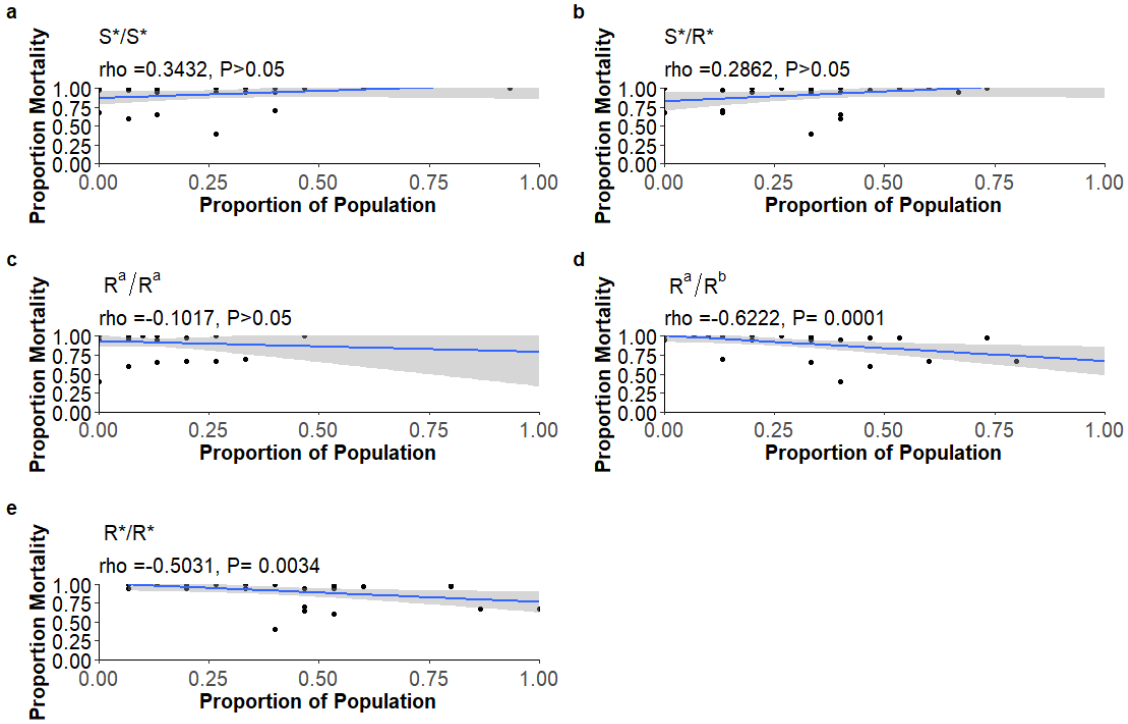


Fig 1.9. Correlation between the proportion mortality from exposure to lambda cyhalothrin and the proportion of each *Aphis glycines* population that was a) susceptible (S*/S*); b) heterozygous for a resistance-associated haplotype (S*/R*); c) homozygous for a single resistance-associated haplotype (R^a/R^a); d) heterozygous for different resistance-associated haplotypes (R^a/R^b), or e) with any two resistance-associated haplotypes, regardless of zygosity (R^*/R^*) across 32 field populations collected in Minnesota-US from 2019 to 2021.

Chapter II: Validation of Reference Genes Across Populations of *Aphis Glycines* (Hemiptera : Aphididae) for RT-qPCR Analysis of Gene Expression Related to Pyrethroid Detoxification

Rosa E. Lozano, Débora P. Paula, David A. Andow, Robert L. Koch

Abstract

Metabolic detoxification is a common mechanism of insecticide resistance, in which detoxifying enzyme genes are overexpressed. *Aphis glycines* Matsumura (Hemiptera: Aphididae) is one of the major soybean pests in the United States and has developed resistance to pyrethroid insecticides after almost two decades of use. To date, there are no validated reference genes to normalize expression of detoxification genes for pyrethroid resistance in *A. glycines*. From a literature review, a list was compiled of genes from 36 gene families (68 sequences) frequently used as reference genes in gene expression analysis in Hemiptera. Exon–exon junction primers were designed for the best alignment matches to a draft *A. glycines* genome and were assayed in a three-phase screening. The first screen eliminated nonamplifying primers. The second screen used nine *A. glycines* populations varying in resistance to pyrethroids and eliminated primers with inconsistent amplification or low amplification efficiency, and quantitatively assessed the stability of expression in the 14 remaining candidates using NormFinder and a generalization of BestKeeper. The third screen quantitatively validated these results on the best candidates. Six genes were identified with the greatest stability across technical and biological

replication and the nine populations. The genes identified as the most suitable reference genes for the study of detoxifying enzymes related to pyrethroid resistance in soybean aphid were: actin, RPL9 (ribosomal protein L9), RPS9 (ribosomal protein S9), AK (arginine kinase), RNAPol2 (RNA polymerase II), and RPL17 (ribosomal protein L17). Our findings will support studies related to insecticide resistance in *A. glycines.*

Introduction

Soybean, *Glycine max* (L.) Merrill, is an important commodity crop in the U.S. economy. The major insect pest affecting soybean in the North Central Region of the United States and southeast Canada is soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae) (Ragsdale et al. 2011). Since the detection of *A. glycines* in 2000 in the United States (Hartman et al. 2001), chemical control has been the most widely used control tactic (Hodgson et al. 2012, Menger et al. 2020). Reliance on the few insecticide groups available, primarily pyrethroids and organophosphates, favored the selection of *A. glycines* resistance to pyrethroids (Hanson et al. 2017, Koch et al. 2018, Menger et al. 2020). Several mechanisms of resistance to insecticides have been documented in insects. Among these mechanisms is metabolic resistance (Feyereisen 1995), which results from the overexpression of detoxification enzymes, such as cytochrome P450 monooxygenases, glutathione-S-transferases, and esterases (E4 and CES) (Coppin et al. 2012, Panini et al. 2016). For *A. glycines*, there is

evidence of metabolic resistance to pyrethroids in China (Xi et al. 2015) and in the United States (Paula et al. 2020). Metabolic resistance is the most common mechanism of insecticide resistance, and it can present a considerable challenge in insecticide resistance management (IRAC 2023b). Therefore, the monitoring of metabolic resistance is imperative to improve integrated pest management (IPM) programs (Li et al. 2016) for a pest such as *A. glycines* with a pyrethroid resistance history.

Real-time reverse transcription–quantitative polymerase chain reaction (RT-qPCR) is a sensitive, practical, and low-cost tool for expression analysis of detoxification genes (Bansal et al. 2012, Kozera and Rapacz 2013). An essential component of gene expression analysis by RT-qPCR is the selection and validation of appropriate reference genes (Huggett et al. 2005, Koramutla et al. 2016). Reference genes serve as endogenous or internal controls to normalize variability in the RT-qPCR signal across samples, usually introduced in the RNA extraction, complementary DNA (cDNA) synthesis, or PCR stages (Huggett et al. 2005, Kozera and Rapacz 2013), because they have constitutive expression that is not affected by the experimental conditions or treatments (Yang, Pan, et al. 2014) or the population to which the organism belongs (Sun et al. 2010, Lu et al. 2013). They are generally housekeeping genes (HKGs), so called because they are responsible for basic cell metabolism, among other functions (Thellin O. et al.

1999, Butte et al. 2001). Nevertheless, since it has been demonstrated that expression of HKGs may vary in certain circumstances in response to diverse biotic or abiotic factors (Thellin O. et al. 1999), it became advisable to use multiple reference genes (Vandesompele et al. 2002, Radonić et al. 2004, Lü et al. 2018).

According to Lü et al. (2018), the most common reference genes used in gene expression analysis in insects are: actin (main groups: alpha, beta, and gamma), ribosomal protein L (RPL), tubulin, elongation factor 1 alpha (EF1a), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), ribosomal protein S (RPS), TATA-box binding protein (TBP), 18S ribosomal RNA (18S), heat shock protein (HSP), and succinate dehydrogenase subunit A (SDHA). However, their common use does not assure their validity as reference genes as their expression stability may vary in different insect species and experimental conditions (Gutierrez et al. 2008). Several reference genes were validated for many gene expression analyses in aphids, e.g., *Acyrthosiphon pisum* (Harris) (Yang, Pan, et al. 2014), Aphis gossypii Glover (Ma et al. 2016), and *Myzus persicae* (Sulzer) (Kang et al. 2017), including *A. glycines* (Bansal et al. 2012). However, reference genes have not yet been identified for the study of expression of detoxification genes related to pyrethroid resistance in *A. glycines*.

To accurately quantify and monitor the incidence of metabolic resistance to pyrethroids in field populations of *A. glycines*, this work aimed to identify

reference genes across nine populations varying in resistance to pyrethroids. This assessment was performed as a three-phase screening for stability of expression of candidate reference genes from 36 gene families (68 sequences). Our results will provide a fundamental base for additional study of gene expression analysis in *A. glycines* under different levels of pyrethroid resistance.

Materials and Methods

Soybean aphids

Nine populations of *A. glycines* were studied. The populations were: (1) a laboratory susceptible control population (Biotype 1, previously shown to be insecticide susceptible [Hanson et al. 2017]) and field-collected populations from (2) Sutherland (IA), (3) Lamberton (MN), (4) Howard Lake (MN), (5) Rosemount (MN), (6) Fairfax (MN), (7) St. Paul (MN), (8) Rochester (MN) in 2019, and (9) Hancock (MN) in 2018. The field-collected populations from 2019 were collected from one infested soybean plant at each of five locations per field, with locations spaced at least 20 m apart. The Hancock population was collected in 2018 from a few plants within 0.5 m of each other at one location in the field. The pyrethroid (lambda-cyhalothrin) resistance of each field-collected population was characterized by a LC99 glass-vial bioassay (Menger et al. 2020). Clonal populations were started from survivors of the initial bioassays for each field-collected population, except for Howard Lake. All these populations were

maintained in a greenhouse (University of Minnesota) in 60-cm2 cages containing healthy soybean plants (SD01-76R cultivar) at 25 ± 2°C, 18–22% relative humidity, and 16:8 h (L:D) photoperiod. For each clonal population, the level of pyrethroid (lambda-cyhalothrin) resistance was assessed with the abovementioned LC99 glass-vial bioassay. Tukey's test (P, 0.05) was performed to compare the mean proportion mortality among populations. From the clonal populations, apterous adult aphids were collected from the plants in the colonies and preserved for further work. For the Howard Lake population, apterous adult aphids were collected and preserved directly from the initial bioassay (i.e., a clonal population was not developed for this population). Three biological replicates from each of the nine populations (27 A. glycines) were studied for gene expression. Prior to preservation, aphids were inspected with a dissecting microscope, using RNase-free materials, to select only intact apterous adults. Individuals were transferred to 1.5-ml microtubes, flash-frozen in liquid nitrogen, submerged in RNAlater, and stored at –80°C.

Selection of the candidate reference genes and primer design

The literature was searched for candidate reference genes for RT-qPCR studies, and 36 gene families (68 sequences) were selected (Table 2.1) from nine aphids and one psyllid species: *Aphis craccivora* Koch (Yang et al. 2015), *A. glycines* (Bansal et al. 2012), *A. gossypii* (Ma et al. 2016), *Diaphorina citri*

Kuwayama (Bassan et al. 2017), *Diuraphis noxia* (Mordvilko) (Sinha and Smith 2014), *Lipaphis erysimi* (Kaltenbach) (Koramutla et al. 2016), *Megoura viciae* Buckton (Cristiano et al. 2016), *Myzus persicae* (Kang et al. 2017), *Rhopalosiphum padi* (L.) (Wu et al. 2014), and *Toxoptera citricida* (Kirkaldy) (*Aphis citricidus* (Kirkaldy)) (Shang et al. 2015). Using their GenBank accession numbers, the nucleotide sequences were retrieved and used as queries to search for orthologous *A. glycines* sequences within the database consisted of contigs from the "Genome Assembly v1.0 of *Aphis glycines*, Biotype 4 (Ag_bt4)" (Wenger et al. 2017) available in the "*A. glycines* blast server" supported by the Bioinformatics Platform for Agroecosystem Arthropods (bipaa. genouest.org/sp/aphis_glycines/blast). For the search, BLASTx 2.6.0+ (Altschul et al. 1997) was used with default parameters. Considering only the best alignment matches, 32 gene candidate sequences were obtained (from 26 gene families) (Table 2.2). Exon and intron positions were identified using AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission) to design exon–exon junction primer-pairs. Two exon–exon junction primer-pairs were designed for almost all of them, totaling 58 primer-pairs, using IDT's PrimerQuest® Tool 2012 (https://www.idtdna.com/pages/tools/primerquest) with the parameters: melting temperature 58°C (minimum), 60°C (ideal), and 63°C (maximum); GC content 35% (minimum), 48% (ideal), and 50% (maximum); primer length 18 bp (minimum), 22 bp (ideal), and 25 bp (maximum); amplicon length 80 bp

(minimum), 130 bp (ideal), and 200 bp (maximum); and to target exon–exon junctions (3′–5′). The GenBank accession numbers of the 32 *A. glycines* nucleotide sequences and the 58 primer-pair sequences are presented in Table 2.2.

RNA extraction and cDNA synthesis

Single apterous adult aphids were individually transferred to 2.0-ml screw-cap tubes containing two 5-mm borosilicate beads and 100 ll of RNeasy Lysis Buffer (RNeasy Mini Kit Qiagen, Venlo, Netherlands). Aphids were homogenized at 4 m/s for 20 s in a FastPrep-24™ homogenizer (MP Biomedicals, Irvine, CA). Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer instructions. The RNA yield was measured using Qubit™ RNA HS Assay Kit and Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Twenty nanograms of RNA from each sample was used to synthesize first-strand cDNA using SuperScript® IV RT (Invitrogen–ThermoScientific, Waltham, MA), according to the manufacturer instructions. A rough estimate of the cDNA obtained per sample was performed in a NanoDrop 2000 spectrometer (Thermo Fisher Scientific) to normalize the amount of cDNA across samples. A single 20-fold dilution was performed for each cDNA sample with nuclease-free water for the RT-qPCR analysis.

RT-qPCR

The RT-qPCR was performed in a LightCycler® 480 Instrument II (Roche, Basel, Switzerland) using Maxima SYBR Green/ROX qPCR (Thermo Fisher Scientific) and 384-well plates. Each sample had three technical replicates and each primer-pair had no template controls (NTCs). The RT-qPCR program for all the primers consisted of: one cycle of initial denaturation at 95°C for 10 min, followed by 45 cycles of two-step amplification process (denaturation at 95°C for 10 s, annealing and extension at 60°C for 60 s), and subsequent melting curve with temperature increase of 1°C/s starting at 40°C for 1 min and going to 95°C, then final cooling at 40°C for 10 s. We performed three-phase screening with RT-qPCR analysis. In the first screening, we used cDNA from the three biological replicates from the Hancock population to test primer-specificity and efficiency, as well as to check for the possibility of nonexpressing genes (nonfunctional copies or pseudogenes), for the 58 primer-pairs of the 32 candidate reference genes (Table 2.2). In the second screening, genes/primer-pairs retained from the first screening (Table 2.2) had expression tested in the three biological replicates from the nine A. glycines populations to select genes with more stable expression across replicates and populations. In the third screening, the most promising candidate reference genes from the second screening results were analyzed again as to validate the results of the second screening (Table 2.2).

Stability of gene expression

The raw fluorescence from each well was used to generate melting curves using the MBmca package (Nucleic Acid Melting Curve Analysis on Microbead Surfaces) (Rödiger et al. 2014) and the function diffQ to calculate the melting temperatures (Tm) from the first derivative (Rödiger et al. 2014). After verification of presence of single peaks and no amplification in the NTCs, we used LinRegPCR version 2014.5 (Ruijter et al. 2009) to analyze the RT-qPCR raw fluorescence data and estimate Log10 (N0), which is equivalent to efficiency-corrected Cq (quantification cycle) for all samples. In the first screening, primers with no amplification, nonspecific primers with multiple peaks in the melting curve, and primers with low amplification efficiency were eliminated. In the second screening, primers that did not amplify any one of the biological replicates or averaged more than one nonamplifying technical replicate were disregarded, leaving 14 candidate reference genes for quantitative analysis. Missing values were imputed for subsequent analysis using multilevel multiple imputation (50 times) with the jomo package (Quartagno and Carpenter 2019) in R. For each imputation, we calculated five measures of gene stability and the Pearson correlation coefficient for all pair-wise gene expression levels across the populations and biological and technical replicates. The results from each imputation were averaged across the 50 imputations. To represent the clustering of candidate reference gene groups a principal components analysis (PCA) was performed (Pearson 1901) using the average correlation matrix. This reveals

potential reference genes that provide complementary measures of stability and correlated genes that reinforce each other. Three of the stability measures were calculated from a generalization of the BestKeeper method (Pfaffl et al. 2004). The BestKeeper method uses the standard deviation of gene expression across samples to estimate stability, with the smallest standard deviation being the most stable. Our data were structured to be able to estimate variation among technical replicates, biological replicates (individual aphids), and populations. We estimated the standard deviation (SD) among technical replicates within aphids for each gene (pooled across aphids within a population), the SD among aphids (biological replicates) within a population for each gene (pooled across populations), and the SD among populations for each gene as three measures of stability. We also used NormFinder version 2015 (Andersen et al. 2004) to estimate the stability of expression for individual genes and for pairs of genes. We did not use geNorm (Vandesompele et al. 2002), because, as shown by Andersen et al. (2004) in their supplementary information, geNorm assumes independence of expression among candidate genes and selects the gene that is most similar to the other genes tested and, therefore, can give erroneous results. The five measures of stability were normalized by transforming each to standard normal deviates across the candidate genes. To determine the most stable genes, the five normalized measures were averaged and the genes with the lowest values were selected as the most stable genes. Finally, using the results

from the PCA, we selected genes that were independently expressed to have multiple measures of the reference genes, as well as correlated genes (similar to BestKeeper) to reduce variation in expression associated with one gene.

Results

Pyrethroid susceptibility among soybean aphid populations.

The soybean aphids from Biotype 1 (susceptible control), Sutherland, and Lamberton showed the highest mean mortalities (high susceptibility) of 1.00, 0.82, and 0.97, respectively (Fig 2.1). Soybean aphids from Howard Lake and Hancock presented an intermediate susceptibility (0.60 and 0.45, respectively) and those from St. Paul, Rochester, Rosemount, and Fairfax were the least susceptible (0.15, 0.02, 0.02, and 0.00, respectively) (Fig 2.1).

Amplification of the candidate reference genes (Screen 1)

We chose the best primer-pair for each of the candidate reference genes based on the amplification efficiency showing a single peak in the melt curve analysis, and the R2 of the regression to estimate Cq (Schmittgen and Livak 2008) in RT-qPCR. Across all 58 primer-pairs (from 32 candidate reference genes) assessed in the initial screening, amplification efficiency ranged from 0.000 to 2.071 and the R2 of the regression to estimate Cq ranged from 0.499 to 1.000. A total of 30 primer-pairs for 30 sequences of the candidate reference genes were selected from the first RT-qPCR screening to proceed to the next screening (Table 2.2). The primer-pairs selected to advance to the second

screening had amplification efficiencies ranging from 1.720 to 1.982 and R2 ranging from 0.683 to 0.987. The candidate reference genes helicase and RPL27 were eliminated in the first screening.

Expression stability (Screen 2)

Out of the 30 sequences of the candidate reference genes analyzed in the second screening (Table 2.2), 16 were removed from consideration because they had at least one biological replicate with nonamplification or averaged more than one nonamplifying technical replicate. The remaining 14 candidate sequences, with an average 12.08% nonamplification of technical replicates, were statistically analyzed for stability (Fig 2.2). The NormFinder single gene stability analysis indicated that β-actin, AK (arginine kinase), RPL17 (ribosomal protein L17), RNAPol2 (RNA polymerase II), and RPL5 (ribosomal protein L5) had the highest stability (Fig 2.3). Better stability (<0.25) was obtained with NormFinder paired gene stability analysis. Actin, RPS18 (ribosomal protein S18), RPL5, β-actin, AK, RPL9 (ribosomal mitochondrial protein L9), RPL17, and RPS9 (ribosomal protein S9) contributed to the highest stability in gene pairs (Fig 2.4). With BestKeeper, stable candidate reference genes exhibit a standard deviation <1 (Sundaram et al. 2019). The most stable candidate reference genes from the generalized BestKeeper analysis differed by source of variation (i.e., technical, biological, and between-population variation) (Fig 2.5). In terms of technical variation, actin, RPS18, β-actin, AK, and RPL5 were the most stable genes. In terms of biological

variation (i.e., variation among aphids within populations), AK, actin, RPL9, RNAPol2, and RPL17 were the most stable genes. In terms of variation among populations, actin, β-tubulin, α-tubulin, RPL9, and AK were the most stable genes. The average of the normalized values of the five stability measures (Table 2.3) indicated that AK, actin, β-actin, and RPL9 were the most stable genes. In addition, RNAPol2, RPL5, and RPL17 were more stable than the average candidate reference genes.

Two groups of genes were identified based on the PCA of the 14 sequences of the candidate reference genes (Fig 2.6). The first PC axis (PC1) explained 88.68% of the variance in expression and the first two axes explained 94.05% of the variation. PC1 separated the candidate reference genes into two groups. The first group of four genes was actin, β-tubulin, α-tubulin, and RPL9. Three of these, actin, β-tubulin, and α-tubulin, were highly correlated with each other (0.70–0.85), and RPL9 was less correlated with them (0.67–0.71). These four genes were not highly correlated with the genes in the second group (0.004–0.60). Of these four genes, actin and RPL9 had the highest expression stability (Table 2.3) and were selected for third level of screening (i.e., validation analysis). The other two genes, α-tubulin and β-tubulin, had poor stability and were disregarded from further consideration as a reference gene.

The second group comprised 10 highly correlated candidate reference genes. The six genes forming the core of this group, RPS18, RPS9, β-actin,

RPL5, RPL7, and 60S, were highly correlated (0.50–0.60) with stabilities ranging from 0.79 to 0.97. Of these, β-actin was the most stable (Table 2.3) and was retained for validation. RPS9 and 60S were the most unrelated to other candidates in this core group (Table 2.4), and as RPS9 had higher stability than 60S, it was retained for validation. The remaining four genes in this group were less correlated with the core group (0.52–0.82), and therefore could provide partially complementary information to the core. RNAPol2 and RPL17 had the highest stability and were selected for validation. AK was weakly associated with the core group (0.52–0.76) and was retained because it might provide the most complementary information to the core. These four genes could provide complementary information to the core group and to each other.

In summary, the second screening of candidate reference genes resulted in seven genes for validation: actin, β-actin, RPL9, RPS9, AK, RNAPol2, and RPL17. These genes were distributed throughout the PCA space, as would be expected for complementary gene expression.

Validation of the selected candidate reference genes (Screen 3)

In the third level of screening, β-actin had >50% of wells without amplification and, therefore, this gene was disregarded as a reference gene. The candidate reference genes actin, RPL9, and RPS9 had better stability characteristics than in the second screening; however, AK and RNAPol2 had poorer stability, and RPL17 had similar stability (Tables 3, 5). Correlation

analysis at this level of screening showed that actin and RPL9 were highly correlated, but they were only moderately correlated in the second level of screening. The other genes were not highly correlated, and most were less correlated than in the previous screening, indicating that they may provide complementary expression.

Even though there were differences in the collection methods and in the level of resistance of the *A. glycines* populations (different phenotypes), we found good stability in the gene expression of the candidate reference genes (Table 2.6). Finally, after the third level of screening, six genes (actin, RPL9, RPS9, AK, RNAPol2, and RPL17) were validated as suitable candidate reference genes for gene expression analysis in different *A. glycines* populations with contrasting levels of pyrethroid resistance.

Discussion

The accuracy of the RT-qPCR data analysis depends on an adequate selection of reference genes (Everaert et al. 2011). It has been found that for trustworthy results more than one reference gene should be used in an experiment because together they improve stability among samples (Vandesompele et al. 2002, Radonić et al. 2004). Reference genes that are highly but not perfectly correlated provide measures of the same or similar biological process. Having multiple highly correlated reference genes would reduce the influence of methodological and other sources of technical variation

but would not reduce variation associated with biological replication or among populations. To reduce error associated with these sources of variation, the expression profiles of the candidate genes should not be highly correlated. Reference genes that are not highly correlated provide complementary information and will reduce the influence of variation in reference gene expression across biological replicates and populations. Our introduction of the correlation matrix and PCA for the selection of appropriate reference genes provides a statistical method for identifying complementary reference genes. This allowed us to select reference genes that were individually stable and complementary.

It is also important to evaluate gene expression stability across populations or treatments (Mamidala et al. 2011, Lu et al. 2013, Zhai et al. 2014). Both NormFinder and BestKeeper, two of the most commonly used programs for identifying best reference genes, do not clearly address this issue, because they only consider stability for a single source of variation. Our data were highly structured, which allowed us to partition the observed variation in expression and estimate stability for three sources of variation, technical variation, biological replication, and among populations. This allowed us to generalize BestKeeper and required the best reference genes to be stable for all three sources of variation.

Adequate reference genes have been identified in many insect species (Van Hiel et al. 2009, Mamidala et al. 2011, Bansal et al. 2012, Paim et al. 2012, Lu et al. 2013, Zhai et al. 2014, Rodrigues et al. 2014, Yang, Pan, et al. 2014, Cristiano et al. 2016, Ma et al. 2016, Chang et al. 2017). For our work, nine aphids and one psyllid (*A. craccivora*, A. glycines, *A. gossypii*, *Diaphorina citri*, *Diuraphis noxia*, *L. erysimi*, *Megoura viciae*, *Myzus persicae*, *R. padi*, and *T. citricida* [*Aphis citricidus*]) served as the source of potential reference genes for *A. glycines* for analysis of detoxification gene expression. From our three-phase screening, the HKGs identified as suitable reference genes for pyrethroid resistance expression were actin, RPL9, RPS9, RPL17, AK, and RNAPol2. Of the 10 most frequently used reference genes in insects (i.e., Actin, RPL, Tubulin, GAPDH, RPS, 18S, EF1α, TATA, HSP, and SDHA) (Lü et al. 2018), our work includes three of these genes (Actin, RPL, and RPS). Other studies on aphid species also validated these three genes as robust reference genes as detailed below (Bansal et al. 2012, Sinha and Smith 2014, Koramutla et al. 2016, Kang et al. 2017).

Actin plays many roles in cell function (Perrin and Ervasti 2010) and was identified to have good expression stability in our study. Actin also showed good stability in other aphid species such as *Diuraphis noxia* under exposure to host plant resistance (Sinha and Smith 2014), *R. padi* with viral infection (Wu et al. 2014), and *Myzus persicae* across different tissues, wing dimorphism,

photoperiod, and temperature (Kang et al. 2017). Additionally, actin was validated in other insects under different experimental conditions such as in *Apis mellifera* L. subjected to bacterial infection (Scharlaken et al. 2008), *Drosophila melanogaster* Meigen under virus challenge, heat stress, and various diets (Ponton et al. 2011), Anopheles sinensis Wiedemann in different life stages, tissues, and levels of insecticide (neonicotinoid) resistance, and *Liriomyza trifolii* (Burgess) in different development stages and temperatures (Chang et al. 2017). Jiang et al. (2010) and Yang, Li, et al. (2014) also found actin to be suitable as a reference gene for *Liposcelis bostsrychophila* Badonnel under pyrethroid stress and immatures of *Locusta migratoria* (L.). However, actin was found to have low stability under different biotic and abiotic conditions in *Drosophila suzukii* (Matsumura) (Zhai et al. 2014), suggesting that even commonly used HKGs must be validated on a case-by-case basis.

Ribosomal proteins are involved in multiple processes in the genome (Plocik and Guthrie 2012), and translation is one of the most important (Hoffman et al. 1996). We found three ribosomal genes with good stability. The gene RPL9 had the best stability across populations of *A. glycines* with varying levels of insecticide resistance. It has been reported as a good reference gene for different nymphal stages and tissues of other insects (He et al. 2014, An et al. 2016). The gene RPS9 has been established as a suitable reference gene in *A. glycines* and *Diabrotica virgifera virgifera* LeConte subjected to host plant

resistance and exposure to dsRNA (Bansal et al. 2012, Rodrigues et al. 2014), Rodrigues et al. 2014). Our results demonstrate the stability of this gene across populations of *A. glycines* with a range of pyrethroid resistance. The gene RPL17 was established as a good reference gene in our work. This gene also showed stable expression in *T. citricida* undergoing temperature variation (Ma et al. 2016) and Myzus persicae under different photoperiods, temperatures, and levels of insecticide susceptibility (Kang et al. 2017).

The enzyme AK catalyzes phosphorylation in cells (Zhou et al. 1998) and was stable in our experiment. Expression of this gene was found to be stable across different body tissues in *Bombus terrestris* (L.) (Horňáková et al. 2010); insecticide-induced stress in *Spodoptera litura* (Fabricius) (Lu et al. 2013); different populations, developmental stages, and tissue in *Drosophila suzukii* (Zhai et al. 2014).

The multiprotein RNAPol2 transcribes DNA into mRNA (Hirose et al. 1999). RNAPol2 showed the highest levels of stability across populations of *A. glycines* in our study. The expression of RNAPol2 was also found to be stable under starvation and UV irradiation stress treatments in other insects (Shang et al. 2015).

Interestingly, some genes (e.g., TBP, EF1α, GAPDH, β-Actin, and RPS18) that are widely used in studies related to pyrethroid resistance were among the

least stable across our populations of *A. glycines* with different level of pyrethroid resistance. The genes TBP and EF1α have been used as reference genes in various insect studies, including *Schistocerca gregaria* (Forskal) (Van Hiel et al. 2009), Drosophila melanogaster (Ponton et al. 2011), *A. glycines* (Bansal et al. 2012), *Plutella xylostella* (L.) (Fu et al. 2013), *T. citricida* (Shang et al. 2015), and A. gossypii (Ma et al. 2016). In the aforementioned studies, TBP and EF1α were found to be stable across different experimental conditions. Additionally, EF1α was found to be stable between populations of *Bemisia tabaci* (Gennadius) varying in neonicotinoid resistance (Li et al. 2013) and between groups of *Ctenocephalides felis* (Bouché) exposed and not exposed to avermectin (McIntosh et al. 2016). However, we did not find that these genes were expressed consistently across *A. glycines* populations in this study. GAPDH, which is involved in energy production (Nicholls et al. 2012), is commonly used in insect species as a reference gene under disease stress, different tissues, temperature fluctuations, and insecticide exposure (avermectin) (Scharlaken et al. 2008, Van Hiel et al. 2009, Paim et al. 2012, Ma et al. 2016, McIntosh et al. 2016, Chang et al. 2017). However, GAPDH did not show adequate stability for *A. glycines* populations with different levels of pyrethroid resistance. The genes β-Actin and RPS18 have been used several times as reference gene in insects, being mostly successful in *Rhodnius prolixus* Stål in experiments with different tissues (Paim et al. 2012), *T. citricida* under heat stress and across various life

stages (Shang et al. 2015), A. gossypii undergoing different developmental studies, geographical distribution and feeding conditions for β-Actin, and temperature oscillation for RPS18 (Ma et al. 2016), and *Tetranychus cinnabarinus* (Boisduval) strains with different levels of resistance to insecticides in different developmental stages for RPS18 (Sun et al. 2010). Although at the initial screening stages of our study β-Actin and RPS18 showed good stability characteristics when paired with other genes, their stability was not good by themselves. Then the stability of these two genes decreased in the second level of screening; therefore, they were not chosen to be final candidate reference genes. These results demonstrated that even commonly used reference genes need to be validated in specific species and experimental condition combinations.

This is the first study that sought to assess stability of candidate reference genes across *A. glycines* populations for examination of expression of detoxification genes related to pyrethroid resistance. To understand if differences in pyrethroid susceptibility are due to overexpression of detoxification genes in important agricultural pests, such as *A. glycines*, it is imperative to have adequate reference genes to normalize the expression across populations. Furthermore, the work presented here brings three innovations to the process of mining the best reference genes for gene expression analysis. First, a three-phase screening process was used to identify potential genes based on

expression and stability, and to validate the identified genes. Second, the commonly used software for selecting reference genes, BestKeeper, generalized to handle nested data structures and independently evaluate sources of variation in expression. Third, PCA was used to identify nonredundant genes. PCA provided an objective method to select a stable set of genes. The methods and results of this robust study should contribute to gene expression studies not only in *A. glycines*, but also to other aphid species.

Acknowledgments

We are grateful to Amelia Lindsey for providing a review of an earlier version of this paper, Lisa Behnken and Bruce Potter for helping to locate fields with soybean aphid infestations, and James Menger, Arthur Vieira, Mads Bartz, and Pheylan Anderson for assistance in the field and laboratory. This work was supported by the Minnesota Rapid Agricultural Response Program (RARF) and the USDA National Institute of Food & Agriculture (NIFA) (award no. 2019-68008-29892).

Tables

Table 2.1. Candidate genes initially considered to screen/mine reference genes for the study of gene expression of detoxifying enzymes associated with pyrethroid resistance in *Aphis glycines*. Except *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), all the other species are aphids.

Species	Gene	GenBank accession number	Reference
Aphis craccivora	12S ribosomal RNA	GAJW01000011	(Yang et al. 2015)
	18S ribosomal RNA	GAJW01000254	
	70 heat shock protein	GAJW01000112	
	Elongation factor 1 α	KC897473	
	NADH-ubiquinone oxidoreductase	GAJW01000104	
	Ribosomal protein L11	GAJW01000099	
	Ribosomal protein L14	GAJW01000046	
	Ribosomal protein S8	GAJW01000269	
	Ribosomal protein S23	GAJW01000179	

	Vacuolar type H^+-ATPase	GAJW01000023	
Aphis glycines	Elongation factor 1 α	JQ654778	(Bansal et al. 2012)
	Glyceraldehyde-3-phosphate dehydrogenase	JQ654777	
	Helicase	JQ654779	
	Ribosomal protein S9	JQ654782	
	Stromal cell-derived factors	JQ654783	
	TATAbox binding protein	JQ654781	
Aphis gossypii	18S ribosomal RNA	KF018922.1	(Ma et al. 2016)
	28S ribosomal RNA	KC796354.1	
	α-Tubulin	KP676379	
	β-Actin	KF018928.1	
	Elongation factor 1 α	EU019874.1	
	Glyceraldehyde-3-phosphate dehydrogenase	KP676380	
	Ribosomal protein L7	KP676382	
	TATA box binding protein	AGT79997.1	
Diaphorina citri	Ribosomal protein L17	NM_001297694.1	(Bassan et al. 2017)

Diuraphis noxia	Actin-5C	XM_015517697	(Sinha and Smith 2014)
	Ribosomal protein L5	AB914563	
	Ribosomal protein L9	AB914565	
	Ribosomal protein L27	AB914564	
	Elongation factor 1 α	AB914566	
Lipaphis erysimi	16S ribosomal RNA	FJ411411	(Koramutla et al. 2016)
	18S ribosomal RNA	NM_001126217.2	
	β-Tubulin	NM_001190398	
	Actin	NM_001126200	
	Arginine kinase	XM_008187305.1	
	Elongation factor 1 α	XM_008184147.1	
	Glyceraldehyde-3-phosphate dehydrogenase	NM_001293474.1	
	Ribosomal protein L13	XM_001949594.3	
	Ribosomal protein L27	NM_001126221.2	
	Ribosomal protein L29	XM_001943721.3	
	Succinate dehydrogenase B	NM_001162436	
Megoura viciae	β-Tubulin	XM_008191981.1	(Cristiano et al. 2016)

	Actin	NM_001142636.1	
	Nicotinamide adenine dinucleotide	XM_001946205.2	
	Ribosomal protein L32	NM_001126210.2	
	Ribosomal protein S9	XM_001945492.3	
	TATAbox binding protein	NM_00162717.2	
	Ubiquinone	XM_001950304.2	
	Ubiquitin	NM_001126205.2	
Myzus persicae	18S ribosomal RNA	AF487712.1	(Kang et al. 2017)
	β-Tubulin	XM_022309483.1	
	Actin	XM_022309797.1	
	Acetylcholinesterase-like	XM_022319367.1	
	Glyceraldehyde-3-phosphate dehydrogenase	XM_022315441.1	
	Ribosomal protein L7	XM_022312633.1	
	Ribosomal protein L27	XM_022325621.1	
	Ribosomal protein L32	XM_022324450.1	
	Elongation factor 1 α	EU358933	Liu et al. unpublished

Rhopalosiphum padi	18S ribosomal RNA	KJ612093	(Wu et al. 2014)
	Actin 1	KJ612090	
	Elongation factor 1 α	KJ612092	
	Glyceraldehyde-3-phosphate dehydrogenase	KJ612091	
Toxoptera citricida (*Aphis citricidus*)	18S ribosomal RNA	AY216697.1	(Shang et al. 2015)
	α-Tubulin	KP260944	
	β-Actin	KP260943	
	Elongation factor 1 α	EU358941.1	
	Glyceraldehyde-3-phosphate dehydrogenase	KP260945	
	RNA polymerase II	KP260942	

Table 2.2. Primer-pairs used to select candidate reference genes for the expression analysis of detoxification enzymes in *Aphis glycines*. These genes were selected based on other house-keeping genes used in other studies, mainly with aphids (Table 2.1). The primer-pairs highlighted in bold (*n*=30) are the ones selected for the second screening phase by RT-qPCR. *

Gene and GenBank accession number	**Primer name**	**Efficiency (%)**	**R^2**	**Primer forward (5'-> 3')**	**Primer reverse (5'-> 3')**
60S variant 1: MT332164	**Agl_60S_a_v1**	1.877	0.894	TCACGTCGTGGT GTTTGT	GGTCCTTAAGG CTAACCTCTTT
	Agl_60S_b_v1	1.952	0.593	GCCAAGTCAAAG AATCATACC	TTTGACAAACA CCACGAC
60S variant 2: MT332185	**Agl_60S_a_v2**	1.938	0.888	GAAGAACTGTCC GTGGTATG	CTCATGGCCAA AGGTACAA
	Agl_60S_b_v2	1.901	0.953	ACTGTGATGTTG GTCGTTTATC	GGCTTCTCTGG TCAATTTATGT
α-Tubulin variant 1: MT332192	**Agl_α-Tubulin_a _v1**	1.865	0.706	GAGCAACTGATT ACTGGCAA	CACCGAACGAG TGGAATATC

	Agl_α-Tubulin_b_v1	1.926	0.499	AGACGACAGTTT CAACACG	TCCTTGCCAGT AATCAGTTG
α-Tubulin variant 2: MT332196	**Agl_α-Tubulin_a_v2**	1.886	0.937	GAGGACCCAGA ATTACAAATGA	GTCCTGGTGAA GTTTGTTCT
β-Actin: MT332180 †	**Agl_β-Actin_a †**	1.916	0.904	GAAGTAGCTGCA TTGGTAGTAG	AACCATGACTC CCTGATGA
	Agl_β-Actin_b	1.877	0.899	CCATTGGAAATG AAAGATTCCG	TGGTACCTCCA GACAATACA
β-Tubulin variant 1: MT332169	Agl_Tubulin-β1_a_v1	1.902	0.879	TAGACGTAGTCA GGAAAGAGG	GTCATAATTCTG TCCGGGTATT
	Agl_Tubulin-β1_b_v1	1.811	0.973	CTCTGGTATGGG AACACTTATG	TCGGATACCTT GGGTGAA
β-Tubulin variant 2: MT332183	Agl_β-Tubulin_a_v2	0.000	0.746	GGCCCATTCGG TCAATTAT	CCTCAGCTTCT TTCCTAACTAC
	Agl_β-Tubulin_b_v2	1.882	0.916	GTAGTTAGGAAA GAAGCTGAGG	CATAATGCGGT CTGGGTAAT
γ-Tubulin: MT332197	Agl_γ-Tubulin_b	1.869	0.969	ATATGCAATTCG GGAGTCTATG	TGGCGTTAAGT CAGGTTTAC

	Agl_*γ*-Tubulin_v2	1.869	0.969	ATATGCAATTCG GGAGTCTATG	TGGCGTTAAGT CAGGTTTAC
AChE: MT332191	Agl_AChE_a	2.013	0.895	CCAACGGCCGA AGAAATA	AAATTCAGATCT CCGTCTATGG
	Agl_AChE_b	1.997	0.808	TACGGCCATTTC AAAGAGTATG	CACAATCGTCC AAACCGATAA
Actin: MT332161 †	Agl_Actin_a	1.763	0.799	CGACGATTCGAC AAGGATTT	ACTGTAAACGG ACTTTGCG
	Agl_Actin_b †	1.824	0.909	CTCGATCACGAT CGCAAGTTTA	CGACTACCAAA GCTGCTACATC
AK: MT332182 †	**Agl_Arginine-Kinase_a †**	2.041	0.915	CGATTTCTACAG GCTGCTAATG	CCTCCTTGTTG CATCGATATG
	Agl_Arginine-Kinase_b	1.909	0.764	GCTGCGGACAA AGCTAAA	CTCAGTCAAAC CCATTCTTCTC
EF1α variant 1: MT332174	**Agl_EF1_a_v1**	1.928	0.974	ACTCCAGGACGT CTACAAA	TGCATCTCCAC GGACTTA
	Agl_EF1_b_v1	1.897	0.833	CATGGTTCAAGG GATGGAATG	TTTGTAGACGT CCTGGAGTG

Alpha-tubulin N-acetyltransferase 1-like isoform X2: MT332187	Agl_ATAT1_a_X2	1.904	0.971	ATCGATGAGATGGGCAAAG	CACTGAAATGTTCTGGTTTATCG
	Agl_ATAT1_B_X2	1.946	0.976	CGATAAACCAGAACATTTCAGTG	TCCATAACCTTTACGCTGTTT
EF2: MT332165	**Agl_EF2_a**	1.873	0.957	TGCAGTAGGAGGTATCTACAG	ATCAGCAGTGAATCCGAAAG
	Agl_EF2_b	1.846	0.931	CGCTCTTGGTGTTAAGAACT	GGTCCCATAATACGAGCTTTC
GAPDH3 variant 1: MT332189	Agl_GAPDH3_a_v1	1.864	0.908	GGTGATACCCACTCATCAATC	TCATTGTCGTACCATGAGATAAG
	Agl_GAPDH3_b_v1	1.877	0.861	GTTGTTGATTTGACTGTAAGACTTG	GAGTGGGTATCACCAATGAAAT
GAPDH3 variant 2: MT332190	**Agl_GAPDH3_a_v2**	1.944	0.720	GCATCAACGATCCATTCATTAG	TGGACAGCACTTGTAATCAG

	Agl_GAPDH3_b_v2	1.866	0.947	TGATTACAAGTGCTGTCCAA	GAAACATTAGCAACTGGAACTC
Helicase: MT332181	Agl_Helicase_a	1.685	0.539	GGGAAATCCACAGGAAAGAA	GCTTCGTCGAGAACCAAATA
	Agl_Helicase_b	1.881	0.958	CGCCTTAAACGAGCAGATT	AAAGGAGGAAGTTGTGTTGG
HSP70: MT332198	**Agl_HSP70**	1.849	0.890	ATGGGTAATGATGTAGCAGAAA	GGTGACATACGGAGAAGTTG
RPL9: MT332163 †	Agl_RPL9_a	1.841	0.953	TCAACAGTTCAACAGGACTTTA	CGGACTTTCCTCAACATCATAG
	Agl_RPL9_b †	1.886	0.963	TGTTGAAGGATTAGGTCATCAAG	CTAGGTTTCTCTTCGTCTTTGG
RNAPol2 variant 1: MT332167	**Agl_RNAPol2_v1**	1.906	0.909	GGACCATTGCCAGAACAATA	CTTCTCGTGAATATCACCTAACC
RNAPol2 variant 2: MT332186 †	**Agl_RNAPol2_a_v2** †	1.925	0.926	GTTAGCCCTACTCATCCTAAAG	CTAGTTCATCACCCTCCTTCAC

	Agl_RNAPol2_b	1.829	0.936	CTGGTGCTGCTA CACATATT	TTGCATTGCTCT TGGTAGG
RPL5: MT332193	Agl_RPL5_a	2.071	0.805	GTCGCCGATGAT TTGGAA	TTAGCGTTCCA GCGTTTAG
	Agl_RPL5_b	1.979	0.757	GTTGCTCGCCG ATTATTGA	TCTAGATTGCA ACGGAAAGC
RPL7: MT332184	Agl_RPL7_a	1.851	0.877	CGGTAGTCAGTA CTAACCTCAA	TTGGAGCATTG TCATCAACC
	Agl_RPL7_b	1.871	0.956	GCGTATTCGTGG TGTGAAT	CCCAAGTCACA TATGGTTCA
RPL14: MT332170	**Agl_RPL14_a**	1.914	0.952	TCCACTTGACCA AGTTTAAGAT	AAAGATGCACG CTTCTCC
	Agl_RPL14_b	1.897	0.998	GGCCCGTAAAC ACGTA AA	GTTTAGCTTCAA CTCCAACTTT
RPL17: MT332188 †	**Agl_RPL17_a** †	1.928	0.869	CACTTCAAGAAC ACGAGAGAG	GCCATTGAACC TCCTGAAA
	Agl_RPL17_b	1.911	0.860	GTCGTATTAACC CGTACATGAG	CCTCATCCTTG GGAACTTTAG

RPL27: MT332162	Agl_RPL27_a	1.920	0.919	CCGCGAATACTTGGCATTT	ATTGTGCCGATTTGTGCT
	Agl_RPL27_b	1.892	0.960	CAGCACAAATCGGCACAATG	TTGTCTTTCCGCTGTACCTTC
RPS8: MT332194	**Agl_RPS8_a**	1.865	0.843	TGCCAAATTGACCGAAGT	ACGTCCGGTCATGAATTG
	Agl_RPS8_b	1.909	1.000	CCTCAGAAAGAAGAGGAAGTT	GTACGAACTGTGTGGATTCT
RPS9: MT332178 †	**Agl_RPS9_a** †	2.004	0.799	GGAGAGTATGGTCTGAGAAATAAG	CGCAACAAAGCATTACCTTC
	Agl_RPS9_b	0.000	0.831	GATTGGCCAAATCCATTCATC	TTTGCGCACACAGTATCC
RPS18: MT332176	**Agl_RPS18_a**	1.960	0.934	TCTCAACTTACCTCTAGTACCC	CACCAACAGTTCTTCCTCTAC
	Agl_RPS18_b	1.868	0.924	GGAGAATGTACCGACGAAGA	GGGTACTAGAGGTAAGTTGAGAG

Syntaxin 1: MT332177	**Agl_Syntaxin1**	1.978	0.933	GAAATAACGGGA AGAACAACTAC	TGCCTGGCTTC AATATCAG
TATAbox: MT332168	Agl_TATAbox_a	1.859	0.936	TGGCCAGTTTAG CAGTTATG	GCACCTGTCAG TACAACTTT
	Agl_TATAbox_b	1.872	0.918	CTCACTCATGGC CAGTTTAG	TACTTTGGCAC CTGTCAGTA
V-ATPaseA: MT332179	**Agl_V-ATPaseA**	1.892	0.791	CTACGAATAATC TGGGCTGTT	ACTCCTTATTCA AGGGATTCAT

* All primer-pairs were used at 0.1 µM and were annealed and extended at 60°C for 60 s. Amplicon length varied from 80 to 200 bp. Agl: *Aphis glycines*; 60S: Ribosomal Protein 60S; AChE: Acetylcholinesterase; AK: Arginine kinase; EF: Elongation Factor; GAPDH3: Glyceraldehyde-3-Phosphate Hydrogenase; HSP: Heat Shock Protein; RPL: Ribosomal Protein L; RM: Ribosomal Mitochondrial Protein; RNAPoI2: RNA Polymerase II; RPS: Ribosomal Protein S; V-ATPaseA: Vacuolar ATPase; R^2: coefficient of determination.
† Genes (*n*=7) with expression analyzed in the third screening phase by RT-qPCR (validation).

Table 2.3. Standard normal deviates for measures of stability of expression of candidate reference genes for expression analysis of detoxification genes in 27 apterus adults of *Aphis glycines* from populations with different pyrethroid susceptibility. ‡

Gene	**Actin**	**RPL9**	**EF2**	**RPS18**	**RPS9**	**β-Actin**	**AK**	**β-Tubulin**	**RPL7**	**60S**	**RNA Pol2**	**RPL17**	**α-Tubulin**	**RPL5**
NormFinder single	0.55	0.47	0.23	0.09	-0.19	-1.66	-1.43	1.27	0.64	0.79	-0.84	-0.94	1.70	-0.68
NormFinder pairs	-1.07	-1.00	0.73	0.19	0.07	-0.91	-1.81	1.32	0.78	1.32	-0.26	-0.31	1.32	-0.40
Technical repl, pooled	-1.61	0.35	1.63	-0.89	0.37	-1.11	-0.70	-0.47	1.10	0.75	1.31	0.58	-0.47	-0.85
Biological repl, pooled	-1.12	-0.71	0.45	1.55	0.05	0.84	-2.26	0.81	0.61	0.51	-1.03	-0.39	0.16	0.51
Among populations	-1.56	-1.22	0.24	1.25	0.69	0.70	-0.81	-1.33	0.80	0.88	0.04	0.67	-1.20	0.86
Average	**-0.96**	**-0.42**	0.66	0.44	0.20	**-0.43**	**-1.40**	0.32	0.79	0.85	**-0.16**	**-0.08**	0.30	**-0.11**

‡ Negative values are better than the average and the smallest negative values are best. 60S: Ribosomal Protein 60S subunit; AK: Arginine kinase; EF: Elongation Factor; RPL: Ribosomal Protein L; RNAPoI2: RNA Polymerase II; RPS: Ribosomal Protein S.

Table 2.4. Pearson correlation coefficients between candidate reference genes of the second core group of the second screening for expression level analysis of detoxification genes in 27 apterus adults of *Aphis glycines* from populations with different pyrethroid susceptibility that could provide independent or complementary information. [§]

Candidate	Gene comparison						
	Actin	**α-Tubulin**	**RPL9**	**AK**	**RNAPol2**	**RPL17**	**Average**
RPS18	0.2373	0.2043	0.3677	0.6783	0.8231	0.7869	**0.5754**
RPS9	0.1234	0.0585	0.2759	0.6549	0.7755	0.7288	**0.5052**
β-Actin	0.3062	0.2606	0.4370	0.6576	0.7817	0.8134	**0.5946**
RPL5	0.2144	0.1979	0.4377	0.7184	0.8208	0.8237	**0.6013**
RPL7	0.1503	0.1402	0.3455	0.7555	0.7703	0.7244	**0.5482**
60S	0.1172	0.0081	0.2737	0.6676	0.8297	0.6769	**0.5021**

[§] AK: Arginine kinase; RPL: Ribosomal Protein L; RNAPol2: RNA Polymerase II

Table 2.5. Standard normal deviates for measures of stability of expression of candidate reference genes of detoxification genes in 27 apterus adults of *Aphis glycines* from populations with different pyrethroid susceptibility. **

	Actin	**RPL9**	**RPS9**	**β-Actin**	**AK**	**RNAPol2**	**RPL17**
NormFinder single	-1.20	-1.58	0.44	0.57	0.38	0.01	0.17
NormFinder pairs	-0.78	-0.76	0.24	0.53	0.16	0.41	0.18
Technical repl, pooled	-2.06	-2.45	-0.57	1.47	0.17	1.67	-0.39
Biological repl, pooled	-1.85	-1.73	-0.52	-0.56	-1.20	-0.68	-1.69
Among populations	-1.50	-1.35	0.68	-0.96	-0.13	0.18	1.26
Average	**-1.48**	**-1.57**	**0.06**	**0.21**	**-0.12**	**0.32**	**-0.09**

** Negative values are better than the average and smaller negative values are better. AK: Arginine kinase; RPL: Ribosomal Protein L; RNAPol2: RNA Polymerase II; RPS: Ribosomal Protein S.

Table 2.6. Gene pairs with best expression stability (NormFinder) for expression analysis of detoxification genes in apterus adults of *Aphis glycines* in pyrethroid resistance studies. ††

		Stability	
Gene1	**Gene2**	**Mean**	**SD**
RPL9	RNAPol 2	0.220	0.014
Actin	RPL17	0.225	0.018
RPL9	RPS9	0.234	0.017
Actin	RNAPol 2	0.237	0.016
Actin	RPS9	0.240	0.018
RPL9	RPL17	0.248	0.016

†† SD= standard deviation. RPL: Ribosomal Protein L; RNAPoI2: RNA Polymerase II; RPS: Ribosomal Protein S.

Figures

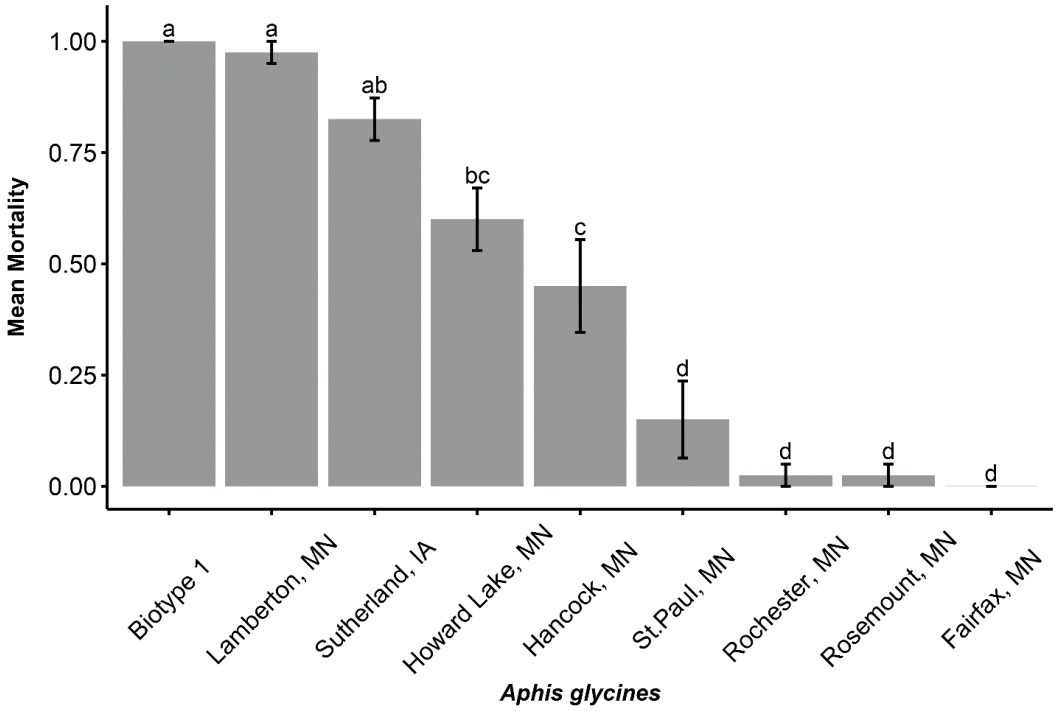


Fig 2.10. Mean mortality of *Aphis glycines* in the LC99 glass-vial pyrethroid (Lambda-cyhalothrin) bioassay to estimate level of pyrethroid resistance. Different letters indicate significant differences in population mortality by Tukey's test ($p<0.05$).

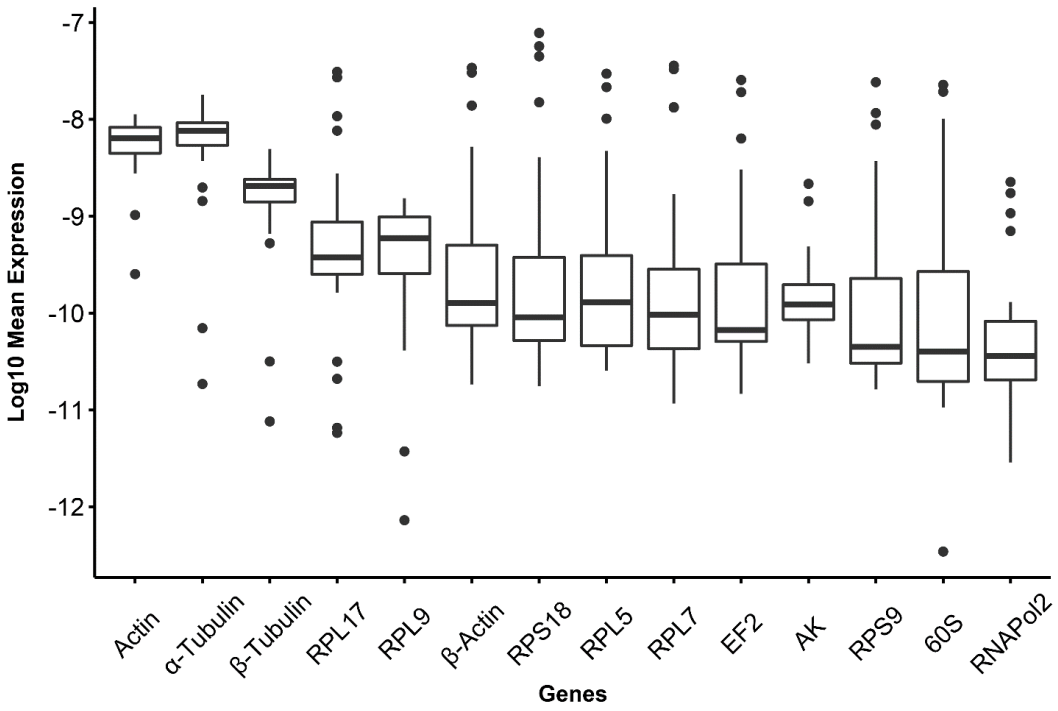


Fig 2.11. Expression profile of the candidate reference genes (Log_{10} (N_0)) from *Aphis glycines* populations with different pyrethroid resistance levels. *60S*: Ribosomal Protein 60S; *AK*: Arginine kinase; *EF*: Elongation Factor; *RPL*: Ribosomal Protein L; *RNAPoI2*: RNA Polymerase II; *RPS*: Ribosomal Protein S.

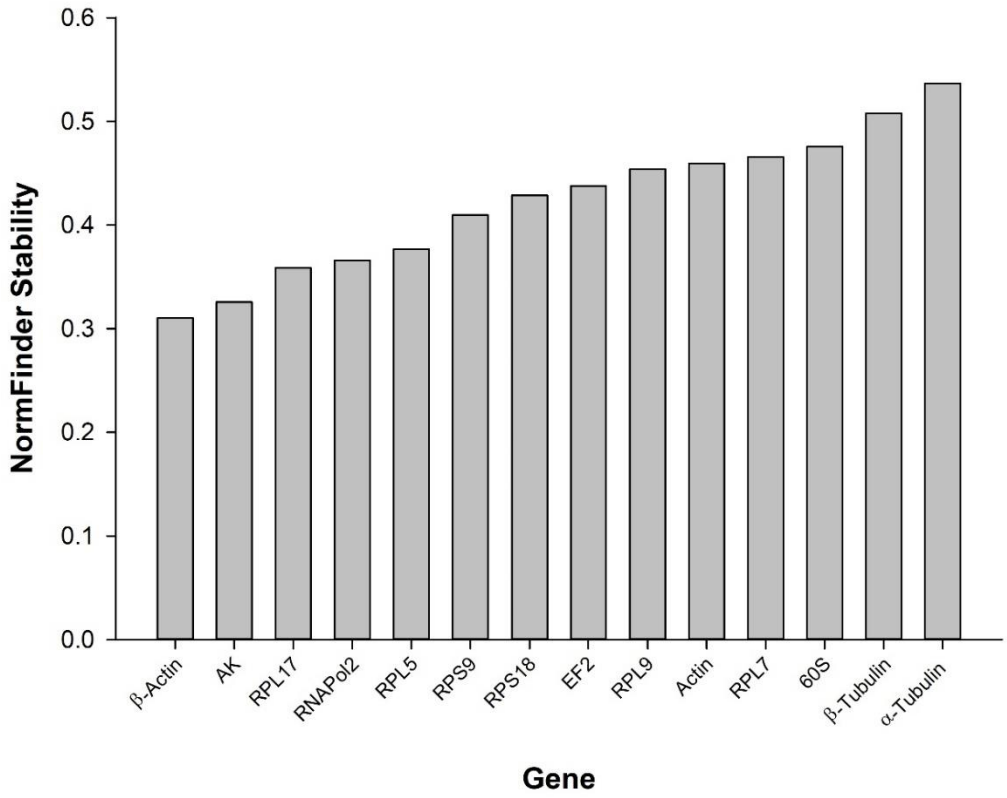


Fig 2.12. Single gene expression stability according to NormFinder. The lower stability values indicate a more stable expression. *60S*: Ribosomal Protein 60S subunit; *AK*: Arginine kinase; *EF*: Elongation Factor; *RP*L: Ribosomal Protein L; *RNAPoI2*: RNA Polymerase II; *RPS*: Ribosomal Protein S.

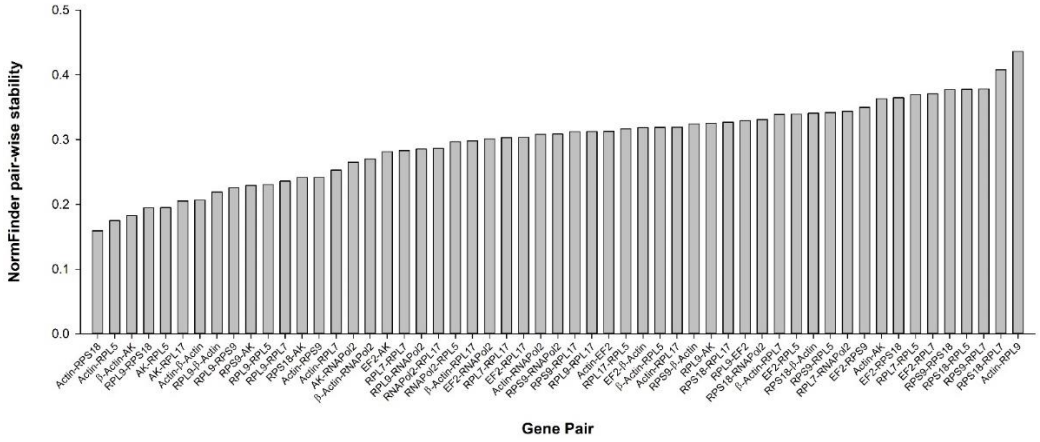


Fig 2.13 Gene pair expression stability according to NormFinder. Gene pairs with poor stability are not shown. *AK*: Arginine kinase; *EF*: Elongation Factor; *RPL*: Ribosomal Protein L; *RM*: Ribosomal Mitochondrial Protein; *RNAPoI2*: RNA Polymerase II; *RPS*: Ribosomal Protein S.

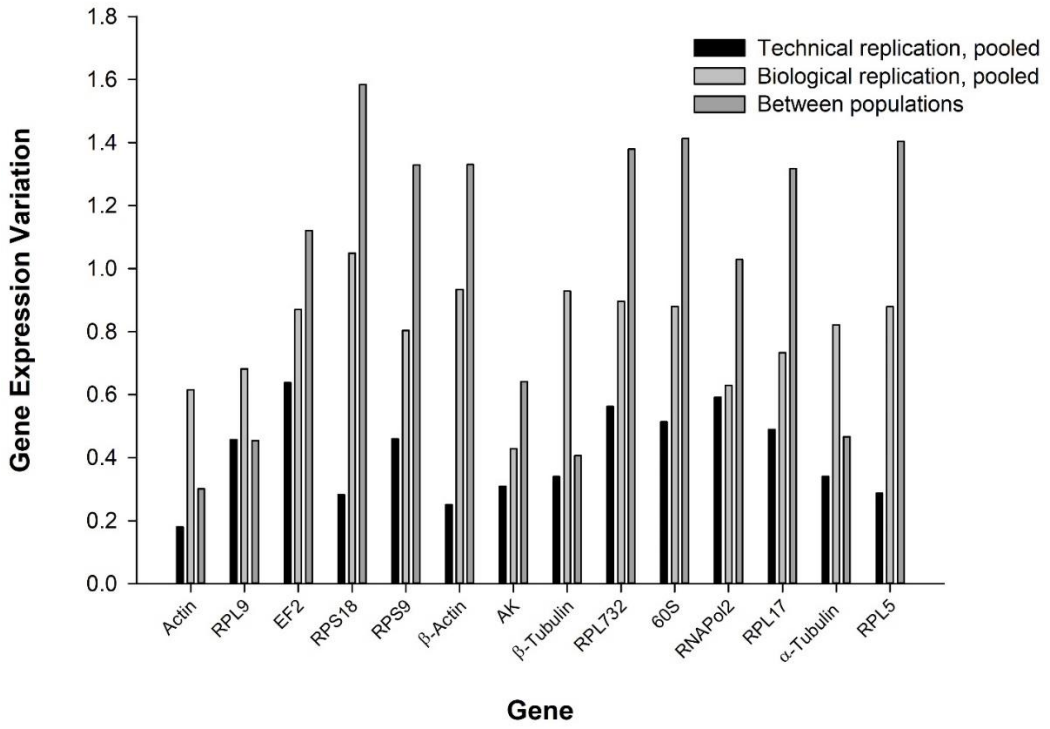


Fig 2.14. Estimated standard deviations for gene expression variation in technical and biological replicates and among *Aphis glycines* populations with different pyrethroid resistance level. 60S: Ribosomal Protein *60S* subunit; *AK*: Arginine kinase; *EF*: Elongation Factor; *RPL*: Ribosomal Protein L; *RNAPoI2*: RNA Polymerase II; *RPS*: Ribosomal Protein Small Subunit.

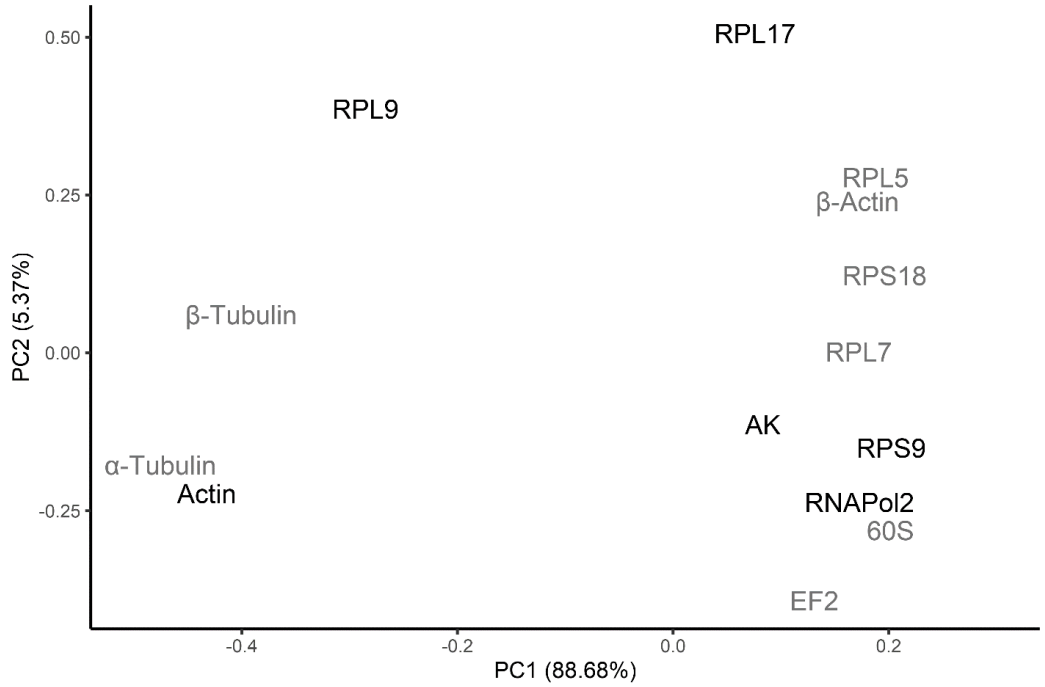


Fig 2.15. Principal components analysis of the correlations between the expression of the candidate reference genes. The validated reference genes for pyrethroid resistance analysis were actin, *RPL9*, *RPS9*, *RPL17, AK*, and *RNAPol2* and are represented in black. The genes in gray were not selected.

Chapter III: Variability of expression of detoxifying enzyme genes among populations of Soybean Aphid (*Aphis glycines* Matsumura)

Rosa E. Lozano, Amelia R. I. Lindsey, Débora P. Paula, David A. Andow, and Robert L. Koch

Abstract

Soybean is an economically and nutritionally important crop that plays a vital role in food security. However, soybean aphid is a major pest that can cause significant damage to soybean crops, leading to yield losses and economic impacts. In recent years, the soybean aphid has developed resistance to commonly used insecticides, such as pyrethroids, making control of this pest more challenging. Insects have evolved enzymatic detoxification mechanisms to counteract the toxic effects of various compounds they encounter in their environment. One of the primary ways insects detoxify pyrethroids is through enzymatic breakdown. Aphids produce specific enzymes, including cytochrome P450s, esterases, and glutathione S-transferases, which can metabolize insecticides into less toxic or non-toxic compounds. In this study we quantified the expression of nine detoxifying enzymes (AglyCYP4C1-like variant 8, AglyCYP4C1-like variant 11, AglyCYP6A13 variant 3, AglyCYP49A1, AglyCYP6A14-like variant 1, AglyCYP303A1-like variant 2, AglyCYP303A1-like variant 3, AglyCYP306A1, and Venom CES-6-like) across four field-collected soybean aphid populations. We found no strong evidence that enzyme-mediated detoxification is a major mechanism of pyrethroid resistance in these Minnesota

soybean aphid populations. Understanding the molecular mechanisms of insecticide resistance is crucial for effective pest management and sustainable agriculture. It helps in developing new insecticides, optimizing pesticide use, and mitigating the environmental impact of pesticide applications.

Introduction

Soybean is an important crop worldwide that provides a valuable source of protein and oil for both human and animal consumption (Ragsdale et al. 2011). However, soybean plants are vulnerable to attack by various pests, including the soybean aphid (*Aphis glycines* Matsumura), which feeds on the plant's sap and can cause significant yield losses (Tilmon et al. 2011). Arthropods such as the soybean aphid have evolved mechanisms to overcome pyrethroids insecticides (Khan et al. 2020). Pyrethroids are a common class of insecticides used to control soybean aphids in agricultural settings (Koch et al. 2019), but their effectiveness is often reduced by various insecticide resistance mechanisms (Hodgson et al. 2012, Koch et al. 2018). One of the mechanisms that can confer resistance to insecticides is metabolic detoxification which includes enzyme-mediated detoxification (Simon 2011).

Metabolic detoxification enzymes, such as cytochrome P450 monooxygenases (CYP), glutathione-S-transferases (GST) and esterases (E4 and CES) play a crucial role in detoxifying insecticides and other xenobiotic compounds by breaking down and eliminating these toxic compounds (Panini et

al. 2016). Specifically, CYP450 enzymes play a vital role in metabolizing a wide range of toxic compounds, including insecticides. These enzymes catalyze the oxidation of these compounds, making them more easily excreted from the insect's body (Nelson 2018, Nauen et al. 2022) For instance, in the cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuoidae) P450 enzymes are involved in the detoxification of pyrethroid insecticides (Yang et al. 2006, Tossou et al. 2019). GSTs are responsible for conjugating toxins with glutathione, making them more water-soluble and easier to eliminate from the insect's body (Yu 1996). For example, in the diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae), GSTs confer resistance to various insecticides, such as pyrethroids and organophosphates (Dukre et al. 2009). Insects possess a group of esterases known as carboxylesterases (CES), which are involved in the hydrolysis of various ester-containing compounds, including insecticides and plant toxins (Yan et al. 2009). For instance, carboxylesterases in the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) can metabolize insecticides such as cyhalothrin (Lü et al. 2015). Repeated exposure to insecticides can select for soybean aphids with increased metabolic activity, allowing them to survive and reproduce despite the application of insecticides, making control more challenging (Panini et al. 2016, Paula et al. 2020). In soybean aphid populations, the overexpression of CYP enzymes is commonly correlated with pyrethroid resistance (Xi et al. 2015, Paula et al. 2020).

Insecticide resistance presents a significant challenge for farmers attempting to control soybean aphid populations and protect their crops. The use of integrated pest management approaches that incorporate both chemical and non-chemical control methods (da Silva Queiroz et al. 2020, Koch et al. 2020) can help reduce the use of insecticides, slow down the development of insecticide resistance, and promote sustainable soybean production. Therefore, to elucidate the potential of metabolic detoxification as a mechanism for pyrethroid resistance in soybean aphid field-collected populations, we quantified expression of nine detoxification enzymes in aphids after a pyrethroid bioassay.

Materials and Methods

Aphid populations

Four soybean aphid field populations were collected in Minnesota in 2019 and 2020. The samples were collected from non-insecticide treated fields: 1) Carver1, 2) GrandMeadow, 3) LittleChicago2, and 4) Rochester1 (Table 3.1). Aphids were collected from five 1m2 sites in each field that were at least 20 meters apart from each other. Soybean aphids were kept live on branches carefully inserted in florist foam to maintain moisture and later proceed with bioassays within 24 hours of collection (Lozano et al. *in progress*: Chapter I). In short, we used a diagnostic-concentration bioassay (Menger et al. 2020) to quantify susceptibility to pyrethroids (bifenthrin and lambda cyhalothrin) in the soybean aphid populations, including an insecticide-susceptible laboratory

population (i.e., Biotype 1), with 4 replications (10 soybean aphids per replication). After 4 h of exposure to the pyrethroid insecticides (bifenthrin and lambda cyhalothrin) soybean aphids were scored as live if they survived or dead if they were dead or moribund. All aphids were flash frozen in liquid nitrogen, submerged in RNAlater, and preserved at -80ºC.

Individual RNA extractions and cDNA synthesis

Aphids from the pyrethroid bioassays (Lozano et al. *in progress*: Chapter I) were individually homogenized in RNase/DNase-free 1.1 ml microtubes (National Scientific Supply) with 2 1/8 inches carbon steel balls and 200 µl of RNeasy Lysis Buffer from the RNeasy Mini Kit (Qiagen). These tubes were placed in a 96-tube rack with lid, padded on the outside with foam and cardboard, and placed in a 5G-HD Harbil 5-gallon heavy-duty shaker for sample homogenization for 30 seconds. Total RNA was isolated from the homogenate with the RNeasy® 96 Kit (Qiagen) following the manufacturer's protocol. A NanoDrop 2000 spectrophotomer (Thermo Fisher Scientific) was used to measure RNA concentration and 20 ng of each sample was used for complementary DNA (cDNA) conversion with the SuperScript IV First-Strand Synthesis System for RT-PCR kit (ThermoFisher Scientific) according to the manufacturer’s protocol.

Real-time quantitative PCR (qPCR) for gene expression

The expression levels of nine detoxifying enzyme genes that were previously found to be constitutively overexpressed in pyrethroid-resistant clonal

soybean aphid laboratory populations (Table 3.2) (Paula et al. 2020) and two internal reference genes that were found the be the most suitable for the study of detoxifying enzymes related to pyrethroid resistance in soybean aphid (Lozano et al. 2022) were compared between four field soybean aphid populations from Minnesota.

Real-time quantitative PCR (qPCR) was performed using QuantStudio 3 Applied Biosystems instrument (Thermo Fisher Scientific) and Luna® Universal qPCR Master Mix 2X (New England Biolabs) in a 20 µl reaction with 0.5µM of each primer and 1 µl of cDNA template. The qPCR cycling protocol was as follows: a cycle of initial denaturation at 95ºC for 1 minute, followed by 40 cycles of two-step amplification (denaturation at 95ºC for 15 seconds, annealing and extension at 60ºC for 60 seconds), and subsequent melting curve and dissociation step cycle for 10 seconds at 95ºC, 1 minute at 60ºC, and 15 seconds at 95ºC. There were 6 biological replicates per condition, run in technical duplicates. The primer sequences are listed in Table 3.2.

Relative Gene Expression and Statistical Analysis

Relative gene expression (RGE) was calculated using the Vandesompele method (Vandesompele et al. 2002) which accounts for multiple housekeeping genes. In short, all technical replicates were averaged, and Delta cycle threshold (ΔCt) was determined for each target gene by calculating: (1) relative quantity (RQ) values for each target, (2) the geometric mean of the reference genes RQ values, and finally (3) the RGE values. Significant differences in gene expression

were assessed separately for each combination of gene and treatment (bifenthrin or lambda cyhalothrin) (a total of 18 models) using linear regression models. For each model, the dependent variable was log(RGE). Independent variables were status, location, and the interaction between status and location. The significance of the independent variables was examined using two-way analysis of variance (Anova) (package: car, code: Anova). To understand the nature of significant interactions, means of status by location were compared with Tukey's test (package: lsmeans, function: lsmeans). Significance was defined at $\alpha = 0.05$. For aphids treated with lambda-cyhalothrin, Carver1 and Rochester1 were excluded from the analysis because there were no live individuals.

Results

For bifenthrin, status and the interaction between status and location was only significant ($p < 0.05$) for AglyCYP4C1-like variant 8 (Table 3.3, Fig 3.1f). The expression of AglyCYP4C1-like variant 8 in live aphids was 1.7 times lower than dead individuals at Carver (Fig 3.1f). The effect of location was significant ($p < 0.05$) for seven detoxifying enzyme genes: AglyCYP303A1-like variant 2 (Fig 3.1a), AglyCYP303A1-like variant 3 (Fig 3.1b), AglyCYP306A1 (Fig 3.1c), AglyCYP49A1 (Fig 3.1d), AglyCYP6A13 variant 3 (Fig 3.1g), AglyCYP6A14-like variant 1 (Fig 3.1h), and Venom CES-6-like (Fig 3.1i) ($p < 0.05$) (Table 3.3).

For lambda-cyhalothrin, the interaction between status and location was significant ($p < 0.05$) for AglyCYP4C1-like variant 11 and AglyCYP4C1-like

variant 8. The expression of AglyCYP4C1-like variant 11 in live aphids at GrandMeadow was 4.4 times higher than dead individuals (Fig 3.2e). The expression of AglyCYP4C1-like variant 8 in live aphids at LittleChicago2 was 2.6 times lower than dead individuals (Fig 3.2f). The effect of location was only significant for four detoxifying enzyme genes: AglyCYP303A1-like variant 2 (Fig 3.2a), AglyCYP303A1-like variant 3 (Fig 3.2b), AglyCYP6A14-like variant 1 (Fig 3.2h), and Venom CES-6-like (Fig 3.2i) ($p < 0.05$) (Table 3.3).

Discussion

Arthropods, including soybean aphids, have been shown to have a high level of metabolic detoxification capability. In a recent study (Paula et al. 2020), researchers found that soybean aphids had a high expression of cytochrome P450 genes, which can be involved in the detoxification of insecticides. The selection for overexpressed detoxification genes allows insects to quickly detoxify insecticides, leading to insecticide resistance (Liu et al. 2015). However, another group of researchers used synergists to inhibit detoxification enzymes (CYP, E4, and CES), and did not find a significant change in mortality of soybean aphid (Valmorbida et al. 2022b). Similarly, we found no evidence that the detoxifying enzyme genes evaluated have a role in soybean aphid pyrethroid detoxification in these Minnesotan populations. Insecticide resistance mechanisms in soybean aphid are complex and can vary depending on the population and the specific insecticide involved.

Soybean aphid resistance to pyrethroid insecticides may be due to the involvement of other mechanisms of pyrethroid resistance, such as target site insensitivity (Paula et al. 2021, Valmorbida et al. 2022b, Lozano et al. *in progress*: Chapter I). Additionally, the specific functionality of the detoxifying enzyme genes can be a factor, for instance, the CYP genes analyzed were from the CYP2 clan (AglyCYP303A1-like variant 2, AglyCYP303A1-like variant 3, and AglyCYP306A1), CYP3 clan (AglyCYP6A13 variant 3 and AglyCYP6A14-like variant 1), CYP4 clan (AglyCYP4C1-like variant 8 and AglyCYP4C1-like variant 11), and mitochondrial CYP clan (AglyCYP49A1). CYP2 clan and mitochondrial CYP clan have been associated with metabolizing endogenous compounds (Niwa and Niwa 2014). Conversely, CYP3 clan and CYP4 clan are associated with defense mechanisms mostly related to the detoxification of xenobiotics (Feyereisen 2012). CYP4, subfamily CYP4G is mainly linked with the biosynthesis of cuticular compounds (Feyereisen 2020). Therefore, all the genes evaluated have different functions, nonetheless, when upregulated can contribute to resistance against xenobiotics (Nauen et al. 2022). Halloween genes such as CYP303A1 are responsible for steps in ecdysone synthesis (Rewitz et al. 2006) and not to be directly related to insecticide detoxification. This agrees with the results in this study, where overexpression of these genes was absent (AglyCYP303A1-like variant 2, AglyCYP303A1-like variant 3). The gene CYP4C1 is expressed in the midgut of *Cydia pomonella* Linnaeus (Lepidoptera: Tortricidae) suggesting a role in detoxification (Dai et al. 2022). Similarly, the

expression of the gene CYP4C1 has been suggested to be also related to fatty acid metabolism (fat body is a detoxification-related tissue) in the in the cockroach *Blaberus discoidalis* Audinet-Serville (Blattodea: Blaberidae) (Lu et al. 1996). However, in this study it is not clear if this gene plays a role in detoxification. For instance, AglyCYP4C1-like variant 8 had lower expression in soybean aphids that survived after the exposure of either bifenthrin or lambda cyhalothrin. Conversely, AglyCYP4C1-like variant 11 was overexpressed in aphids that survived after the exposure of lambda cyhalothrin. The genes CYP6A13 and CYP6A14 were overexpressed in lambda-cyhalothrin soybean aphid resistant individuals (Xi et al. 2015). However, in this study overexpression of these genes (AglyCYP6A13 variant 3 and AglyCYP6A14-like variant 1) was not found. Venom CES-6-like was overexpressed in clonal soybean aphid populations resistant to pyrethoids (Paula et al. 2020). However, this was not the case for the populations screened in this study. Furthermore, the fact that the populations in the different studies are different can play a role in the variation of the expression levels.

Overall, while some studies have found that CYP and CES gene expression is higher in pyrethroid resistant insect populations (Xi et al. 2015, Paula et al. 2020), there are also studies that have found gene expression to be lower in resistant insect populations (Bass et al. 2011, Sun et al. 2017). We did not find evidence of enzyme-mediated detoxification in soybean aphid resistance to pyrethroid insecticides in the populations surveyed across Minnesota.

Acknowledgements

Thank you to Arthur Vieira for the statistical advice. Also, thank you to Lisa Behnken, Bruce Potter and Jonathan Dregni for helping to locate fields with soybean aphid infestations, and James Menger for assistance with bioassays and keeping the soybean aphid colonies. This work was supported by the Minnesota Rapid Agricultural Response Program (RARF), the USDA National Institute of Food & Agriculture (NIFA) (award no. 2019-68008-29892), the Department of Entomology Lugger-Radcliffe Summer Fellowship, and a University of Minnesota Doctoral Dissertation Fellowship.

Tables

Table 3.1. Field-collected *Aphis glycines* populations from the state of Minnesota-US.

Population	County	Date of collection (MM/DD/YYYY)	Latitude (decimal degrees)	Longitude (decimal degrees)
Rochester1	Olmsted	8/15/2019	43.933079	-92.537685
Carver1	Carver	7/31/2020	44.777051	-93.67496
LittleChicago2	Rice	8/17/2020	44.402686	-93.322886
GrandMeadow	Mower	8/26/2020	43.791315	-92.581083

Table 3.2. Primer-pairs used to validate the potential of metabolic detoxification as a mechanism for pyrethroid resistance in soybean aphid field populations.

GenBank accession number	Gene Name	Primer Forward (5'>3')	Primer Reversed (5'>3')
AG013703	AglyCYP4C1-like variant 8	TTCCGAGTGGACTGTTTATG	CTCTATGATCTGTTGTGGTGTA
G016855	AglyCYP4C1-like variant 11	CCAGATGTGTTTGAGGAATTG	AGGAGGGAACAATCGTAATG
AG005793	AglyCYP6A13 variant 3	TGCTGCACAACCTTTACC	CAGTATGTGCCCTATTAGTTCC
AG012883	AglyCYP49A1	CCAGCAAATACATTGGTGATG	AAAGGTAACGAAGCGTAAGG
AG005036	AglyCYP6A14-like variant 1	CTACGTTCAGCTCTGGTAAA	GATGGTTGCTTCCATGTATTC
AG015634	AglyCYP303A1-like variant 2	CAACGTGGATTGAACAGAGAAA	GCCATTCTCACATTTGTTCGT
AG009673	AglyCYP303A1-like variant 3	CACGAAGAGGTCTTCTACTAAC	GACTAACTGAACTGCTTCTTCT
AG005942	AglyCYP306A1	GCAAGAACATGGTCTGATATTT	GTCGGCAATAAGTAGTTGTAAG
AG011922	Venom CES-6-like	GAGATTTCAGGAACCTCAACC	CATAAAGGCAATCCTCGTCTC
MT332161	Actin	CTCGATCACGATCGCAAGTTTA	CGACTACCAAAGCTGCTACATC

MT332163	RPL9	TGTTGAAGGATTAGGTCATCAAG	CTAGGTTTCTCTTCGTCTTTGG

Table 3.3. Summary of Anova results for the log(RGE) comparison among locations and status in soybean aphid field populations. Significance was defined at α= 0.05 and are indicated in bold.

		Bifenthrin			**Lambda Cyhalothrin**		
Gene Nomenclature	**Variable**	***F***	**df**	***p-value***	***F***	**df**	***p-value***
AglyCYP4C1-like variant 8	Status	5.2336	1,37	**0.02797**	3.9749	1,20	0.06000
	Location	2.2704	3,37	0.09643	0.0045	1,20	0.94707
	Status:Location	3.0528	3,37	**0.04038**	8.1014	1,20	**0.00998**
AglyCYP4C1-like variant 11	Status	0.7128	1,38	0.40380	0.6282	1,20	0.43732
	Location	1.0825	3,38	0.36810	0.0016	1,20	0.96841
	Status:Location	2.1873	3,38	0.10540	4.7330	1,20	**0.04175**
AglyCYP6A13 variant 3	Status	0.3577	1,36	0.55354	0.8332	1,20	0.37220
	Location	3.8285	3,36	**0.01772**	0.2099	1,20	0.65180
	Status:Location	1.1514	3,36	0.34167	0.0143	1,20	0.90610
AglyCYP49A1	Status	1.4397	1,37	0.23782	0.0848	1,20	0.77380

	Location	6.9862	3,37	**0.00077**	0.9686	1,20	0.33680
	Status:Location	0.5224	3,37	0.66954	1.6831	1,20	0.20930
AglyCYP6A14-like variant 1	Status	3.0411	1,36	0.08972	5.97780	1,20	**0.02387**
	Location	2.9085	3,36	**0.04773**	5.82090	1,20	**0.02556**
	Status:Location	2.6953	3,36	0.06038	0.07030	1,20	0.79366
AglyCYP303A1-like variant 2	Status	0.0027	1,37	0.95917	0.0743	1,20	0.78791
	Location	3.1139	3,37	**0.03776**	10.2816	1,20	**0.00443**
	Status:Location	0.1404	3,37	0.93514	1.1970	1,20	0.28692
	Status	0.0092	1,36	0.92396	0.0492	1,20	0.82674

AglyCYP303A1-like variant 3	Location	8.5022	3,36	**0.00021**	20.7400	1,20	**0.00019**
	Status:Location	0.9135	3,36	0.44413	2.4303	1,20	0.13469
AglyCYP306A1	Status	0.5657	1,35	0.45699	1.0814	1,20	0.3108
	Location	4.1286	3,35	**0.01316**	0.6268	1,20	0.4378
	Status:Location	1.8250	3,35	0.16060	1.3614	1,20	0.2570
Venom CES-6-like	Status	0.0180	1,37	0.89394	5.8122	1,20	**0.02566**
	Location	7.4779	3,37	**0.00049**	26.9964	1,20	**4.389e-05**
	Status:Location	1.7650	3,37	0.17076	2.9995	1,20	0.09869

Figures

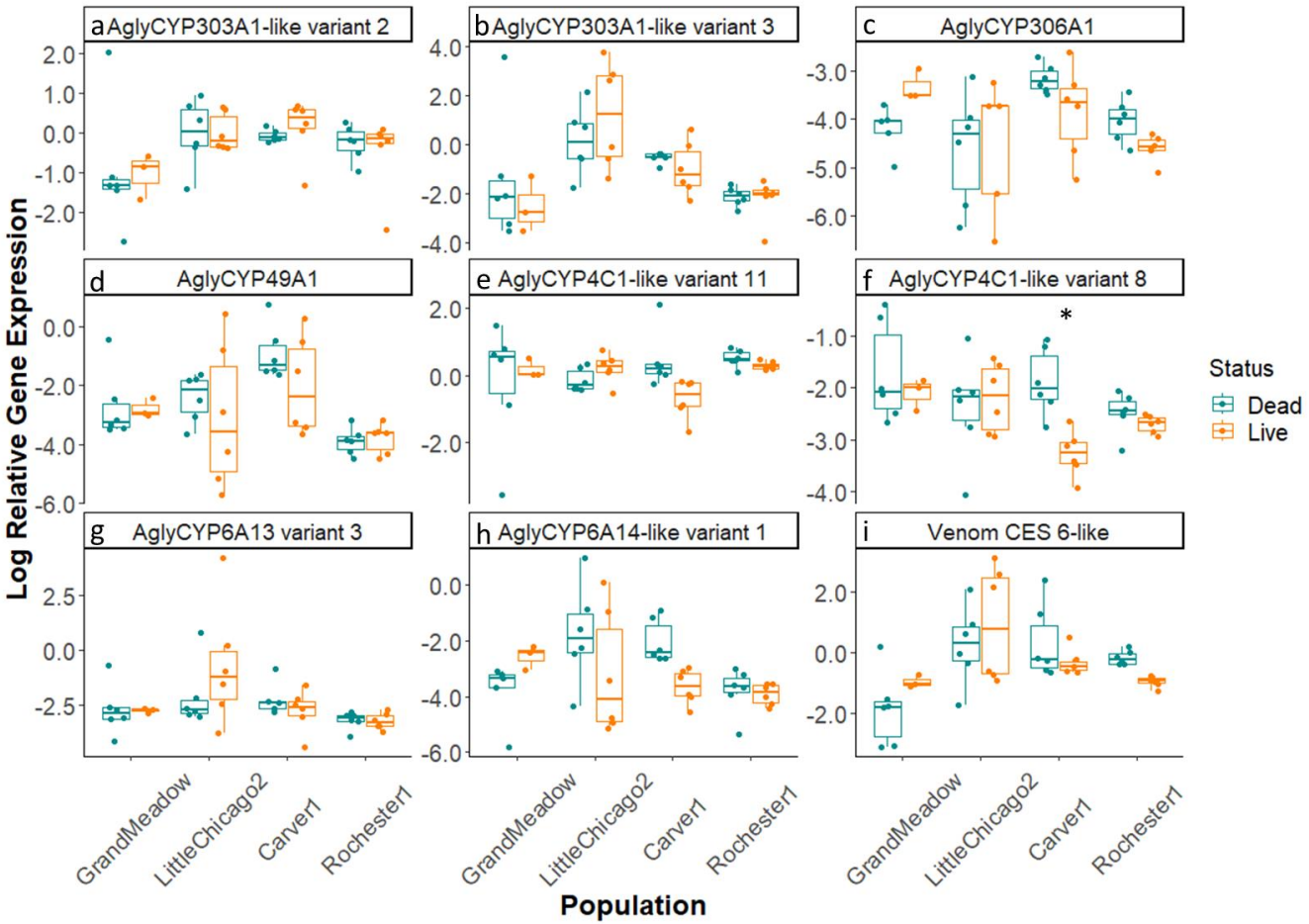

a AglyCYP303A1-like variant 2
b AglyCYP303A1-like variant 3
c AglyCYP306A1
d AglyCYP49A1
e AglyCYP4C1-like variant 11
f AglyCYP4C1-like variant 8
g AglyCYP6A13 variant 3
h AglyCYP6A14-like variant 1
i Venom CES 6-like
*
Log Relative Gene Expression
Population
GrandMeadow
LittleChicago2
Carver1
Rochester1
Status
Dead
Live

Fig 3.1. Log relative gene expression (normalized to the average Ct) among nine detoxifying enzyme genes (AglyCYP4C1-like variant 8, AglyCYP4C1-like variant 11, AglyCYP6A13 variant 3, AglyCYP49A1, AglyCYP6A14-like variant 1, AglyCYP303A1-like variant 2, AglyCYP303A1-like variant 3, AglyCYP306A1, and Venom CES-6-like) in four field collected soybean aphid populations preserved after insecticide bifenthrin bioassays. The status of the individuals after the bioassays were categorized as live and dead. Significant differences between groups are represented with black stars.

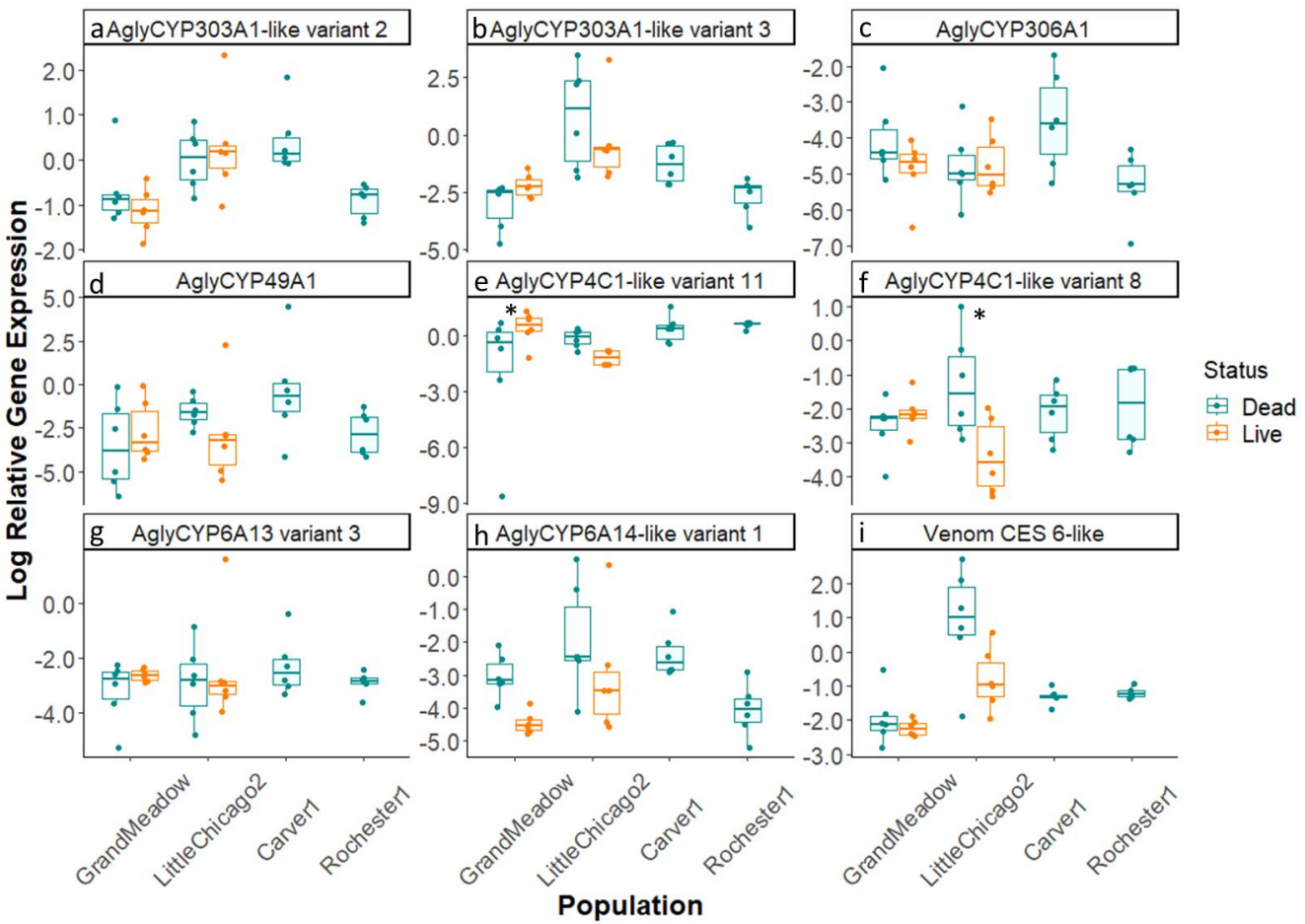

a AglyCYP303A1-like variant 2
b AglyCYP303A1-like variant 3
c AglyCYP306A1
d AglyCYP49A1
e AglyCYP4C1-like variant 11
f AglyCYP4C1-like variant 8
g AglyCYP6A13 variant 3
h AglyCYP6A14-like variant 1
i Venom CES 6-like
Log Relative Gene Expression
Population
GrandMeadow
LittleChicago2
Carver1
Rochester1
Status
Dead
Live

Fig 3.2. Log relative gene expression (normalized to the average Ct) among nine detoxifying enzyme genes (AglyCYP4C1-like variant 8, AglyCYP4C1-like variant 11, AglyCYP6A13 variant 3, AglyCYP49A1, AglyCYP6A14-like variant 1, AglyCYP303A1-like variant 2, AglyCYP303A1-like variant 3, AglyCYP306A1, and Venom CES-6-like) in four field collected soybean aphid populations preserved after insecticide lambda-cyhalothrin bioassays. The populations with filled boxplots (Carver1 and Rochester1) were excluded from the analysis because there were no live individuals. The status of the individuals after the bioassays were categorized as live and dead. Significant differences between groups are represented with black stars.

Chapter IV: Soybean Aphid Management and Perceptions on Insecticide Resistance

Rosa E. Lozano, Terrance M. Hurley, Janet Knodel, David Andow, and Robert L. Koch

Abstract

Soybean, one of the most important crops in the US, is threatened by soybean aphids causing significant economic losses. The repeated use of insecticides within the same group has led to the development of insecticide resistance in soybean aphids. Implementing integrated pest management (IPM) and insecticide resistance management (IRM) strategies can help farmers reduce the frequency of insecticide applications and delay the evolution of soybean aphid resistance while reducing production costs, maintaining yields, and protecting the environment. Farmers play a crucial role in implementing such practices, therefore, to understand farmers' practices for management of soybean aphid and their perceptions of insecticide resistance, a survey of soybean farmers in Minnesota and North Dakota was conducted in 2021. Overall, the reported decision-making sources were similar for both farmers who changed and those who did not change their aphid management due to insecticide resistance; these were crop consultants and agricultural retailers. The threshold used by farmers in both groups to determine whether to use a foliar insecticide was lower than the research-based threshold for soybean aphid, and

farmers were aware that this could be a practice that can contribute to insecticide resistance. Additionally, farmers who changed soybean aphid management were more concerned about insecticide resistance and were implementing good practices such as scouting, using label application rates, and rotating insecticide modes of action. However, more education is necessary to effectively manage insecticide resistance. The results of this research can help inform refinement of integrated pest management and insecticide resistance management programs as well as extension education efforts.

Introduction

Soybean is a crucial crop for United States agriculture, economy, and food security. Minnesota and North Dakota rank 3rd and 8th, respectively, for soybean production in the United States, with a combined total of 5.3 million hectares of soybean planted in 2022 (USDA- NASS 2023a). Soybean aphid (*Aphis glycines* Matsumura) is an invasive insect in North America and has become a major pest of soybean (Ragsdale et al. 2004, 2011). Over the years, implementation of integrated pest management (IPM) strategies to control soybean aphid have decreased due to the increase in the use of short-term strategies (non-IPM) that can maintain profitability (Hurley and Sun 2019, Bueno et al. 2021).

As with other field crop pests, management of soybean aphid in the U.S. relies largely on seed-applied and foliar-applied insecticides (Hurley and Mitchell 2020). Preventative use of insecticide (neonicotinoid) seed treatments on

soybean are widespread (Tooker et al. 2017) but provide control of soybean aphid for only a limited duration (McCornack and Ragsdale 2006, Seagraves and Lundgren 2012, Krupke et al. 2017). Foliar applications of insecticides can be effective for management of soybean aphid (Hodgson et al. 2012, Koch et al. 2016). In the context of integrated pest management (IPM) for this pest, the application of foliar insecticides should be based on scouting and the research-based treatment threshold of 250 aphid per plant, with more than 80% of the plants infested per field, and populations increasing over time (Ragsdale et al. 2007, Koch et al. 2016). Alternatively, binomial sequential sampling ('Speed Scouting') based on the number of soybean plants with more than 40 aphids, can be used to determine when to apply insecticides (Hodgson et al. 2004). However, adoption of this recommended level of IPM is often minimal in field crops like soybean (Bueno et al. 2021).

From a broader IPM perspective, non-chemical management strategies have received considerable research attention, but are not widely implemented in conventional soybean production. For example, aphid-resistant soybean varieties have proven effective for suppressing soybean aphid infestations (Tilmon et al. 2011, Hesler et al. 2013, McCarville and O'Neal 2013) but availability and use of aphid-resistant soybean varieties have been minimal (Tilmon et al. 2021). Cultural control options for soybean aphid are limited (Hodgson et al. 2012). However, relay cropping soybean into a winter cover crop of rye has shown potential to reduce soybean aphid infestations of soybean (Koch et al. 2012), but

adoption of such cover crops in northern states like Minnesota remains below 4% of corn and soybean acres (Lee and McCann 2019, EWG 2022). Exotic parasitoids have been evaluated and released for classical biological control of soybean aphid, but establishment of these parasitoids was apparently unsuccessful (Heimpel and Asplen 2011). Selective insecticides (e.g., afidopyropen and sulfoxaflor) hold promise for improving conservation biological control of soybean aphid (Aita et al. 2019, Koch et al. 2020), but the extent of use of these insecticides by farmers remains unknown.

The general lack of non-chemical management strategies for soybean aphid has resulted in long-term reliance (20+ years) on a limited number of insecticide groups, which in-turn has resulted in development of insecticide resistance (Koch et al. 2018). Soybean aphid resistance to pyrethroids was confirmed with laboratory bioassays (Hanson et al. 2017, Menger et al. 2020). Additionally, decreased efficacy of field applications of pyrethroids for soybean aphid (i.e., practical resistance) was documented (Menger et al. 2022a). The resistance mechanisms identified in soybean aphid are target-site insensitivity (Paula et al. 2021, Valmorbida et al. 2022b) and potentially metabolic detoxification (Paula et al. 2020).

The emergence of resistance to pyrethroids in soybean aphids necessitates insecticide resistance management (IRM) strategies to slow down or prevent the development of resistance to these and other insecticides. Insecticide resistance management for soybean aphid incorporates the use of a

combination of tactics such as rotating insecticides with different modes of action, limiting the use of pesticides to only when necessary (i.e., reliance on IPM tactics described above), and applying those insecticides properly (Koch et al. 2018), as well as, monitoring for resistance (Roush and Miller 1986, Raghavendra et al. 2017). Such IRM practices could help preserve the effectiveness of insecticides and protect the environment.

The success of IPM and IRM programs depends on farmer decision making and proper implementation of practices (Leach et al. 2019, Hurley et al. 2023). Surveys have been used to improve researcher's understanding of aspects of farmers practices in several different cropping systems and led to adaptation of extension education programming, identification of knowledge gaps and future research priorities, and improvement of IPM and IRM strategies (Jabbour and Noy 2017, Hoidal and Koch 2021, Penn et al. 2021, Cass et al. 2022, Lane et al. 2023). Since the emergence of insecticide resistance in soybean aphid, farmers' practices for management of soybean aphid and perceptions of insecticide resistance are not well understood. To address this knowledge gap, a survey was conducted of soybean farmers in Minnesota and North Dakota in 2021 where insecticide resistant soybean aphids is established. Results of this survey can help inform refinement of IPM and IRM programs, applied research and Extension education efforts.

Materials and Methods

Farmers information

Authorization from the Institutional Review Board (IRB) was obtained prior to contacting farmers. Farmers' names and addresses were purchased from a marketing company (Data Transmission Network and Dataline: DTN). Data Transmission Network and Dataline maintains a proprietary database of over two million farm operators and owners covering more than 95% of all U.S. farm operations. The database is regularly updated and audited using information from the U.S. Department of Agriculture and state and local governments, as well as other private sources. DTN provided randomly selected samples of 2300 Minnesota and 691 North Dakota farm operators or owners from this database with a positive record for soybean acreage.

Survey Development and Distribution

The survey was composed of 28 questions divided into 5 sections. Section 1 focused on general information about their farms. Section 2 focused on soybean aphid management. Section 3 focused specifically on the use of foliar insecticides for soybean aphid management. Section 4 focused on farmer perceptions of insecticide resistance. Section 5 focused on farmer demographics. The complete survey is included in the supplemental materials (File SF 1). The survey was distributed in Minnesota and North Dakota in 2021 in a four-step process (Dillman et al. 2014). On 3 November 2021, postcards were used to notify farmers that they would soon receive the survey. On 11 and 12 November 2021, the survey was mailed to the farmers. On 18 November 2021, a reminder

to complete the survey was sent to farmers. Finally, on 20 December 2021, a second copy of the survey was sent to farmers who had not yet returned the completed survey.

Data Analysis

Data were analyzed using linear regression with the response to a particular question as the dependent variable. The independent variable was whether the farmers reported that they changed their soybean aphid management in response to insecticide resistance (yes or no in response to question 4.7 from the survey) (File SF 1). The analyses were weighted to account for differences in the numbers of farmers in different farm size classes (large vs. small) relative to the actual numbers in those size classes (USDA NASS 2022). Large farms were those greater than 1000 and 2000 acres in MN and ND, respectively.

Results

Survey Response and Demographics

The net response rate was 16.6% for Minnesota and 7.3% for North Dakota, for an overall net response rate of 14.5% with good geographic coverage across the two states (Fig 4.1). Most respondents were male (90.7%) and their mean age across both groups (farmers that changed and those that did not change their soybean aphid management) was 64.5 years old. Across both groups, 100% of the respondents had a high school diploma (GED) or higher

education level. Respondents indicated that they have been farming on average for 42 years across both groups.

Soybean Aphid Management

Fewer than one in four (21.6%) ($P<0.0001$) farmers reported changing their soybean aphid management over the five-year period (2017-2021), due to insecticide concerns. To better understand what aspects of soybean aphid management and perceptions on insecticide resistant soybean aphid were different between those that changed and those that did not change their soybean aphid management, responses to the remaining questions were analyzed to compare differences between these two groups.

Farmers relied on several sources of information to manage soybean aphid in 2021 (Fig 4.2). More than 50% of farmers from both groups (change and no change soybean aphid management) reported that they relied on crop consultants (55.1%) and agricultural retailers (57.4%) when deciding how to manage soybean aphid (Fig 4.2). Among the potential sources of information, farmers who changed soybean aphid management relied more on seed or chemical company representatives ($P=0.0332$) and extension specialists, educators, or county agents ($P<0.0001$) than did farmers who did not change their soybean aphid management practices (Fig 4.2). Farmers who changed practices relied on more sources of information than those who did not change.

Farmers used several practices to manage soybean aphids in 2021 (Fig 4.3). Over 60% of farmers scouted fields to manage soybean aphids in both

groups. Among the potential practices, farmers who changed soybean aphid management reported more use of insecticide-treated soybean seed (P<0.0001) and soybean with resistance or tolerance to soybean aphid (P=0.0042) than farmers who did not change soybean aphid management.

Foliar Insecticides Used to Manage Soybean Aphid Over a Five-Year Period (2017-2021)

In the five-year period, the percentage of farmers using insecticides to manage soybean aphid was significantly greater for those who changed soybean aphid management (79.6%) compared to those who did not change soybean aphid management (62.8%) (P<0.0001). Out of the five-year period, farmers who changed soybean aphid management applied foliar insecticides to manage soybean aphids in more years (2.9 out of 5 years) than did farmers who did not change soybean aphid management (2.6 out of 5 years) (P=0.001). However, over the past five-year period, the number of years farmers used foliar insecticides more than once per year to manage soybean aphid was similar (2.6 out of 5 years) for those who changed and those who did not change soybean aphid management (P>0.05).

Farmers were influenced by several factors when deciding to apply foliar insecticides to manage soybean aphids (Fig 4.4). On average, the percentage of farmers influenced by agricultural retailers or chemical company representatives (34.9%) and crop consultant recommendation (33.5%) was highest in both groups. Among the factors that influenced farmers, farmers who changed

soybean aphid management were influenced more by time of year or calendar date (P=0.0044), soybean growth stage (P=0.0071), extension reports of soybean aphid activity (P=0.0006), and other factors (P<0.0001), than were farmers who did not change. Farmers who changed practices were influenced by more factors than those who did not change.

Over the five-year period, the percentage of farmers who made foliar insecticide applications based on scouting was similar for farmers who changed (97.3%) and did not change (97.5%) soybean aphid management (P>0.05). Across the two groups, more than 50% of the farmers scouted fields between two to five times per year and this did not differ between farmers who changed and did not change soybean aphid management (P>0.05). Scouting was performed by different individuals. Farmers who changed soybean aphid management were more likely to scout their fields themselves (P=0.0217), or to have it performed by a crop consultant (P=0.0225), or by an extension specialist, educator, or county agent (P=0.0004) compared to farmers who did not change soybean aphid management (Fig 4.5).

Over the five-year period, the mean percentage of farmers using a threshold (based on the average number of soybean aphids per plant) to determine whether to use a foliar insecticide was 74.0% and did not differ between the groups of farmers (P>0.05). The mean number of aphids used to determine whether a foliar insecticide should be used was 168 aphids per plant and did not differ between the two groups of farmers (P>0.05). The mean

percentage of farmers that used speed scouting to determine when to apply foliar insecticides was 41.6% and did not differ between the two groups of farmers (P>0.05).

Farmers used a variety of foliar insecticides to control soybean aphids. Pyrethroids and organophosphates were the most reported insecticides either as products with a single active ingredient or as formulated mixtures of more than one active ingredient. Among the insecticide products with single active ingredients, farmers who changed soybean aphid management were more likely to have used pyrethroids (P=0.0001) and organophosphates (P<0.0001) than did farmers who did not change soybean aphid management (Fig 4.6a). Within the pyrethroids, farmers used primarily lambda cyhalothrin and bifenthrin (Fig 4.7a). Within the organophosphates, chlorpyrifos was the only active ingredient used (Fig 4.7b). Among the insecticide products with a formulated mixture of more than one active ingredients, farmers who changed soybean aphid management were more likely to have used a combination of pyrethroids than did farmers who did not change soybean aphid management (P=0.0004) (Fig 4.6b).

Farmers used different practices related to their foliar insecticide applications. On average, 49.8% of farmers reported the use of labeled application rates (Fig 4.8). Among the different practices, farmers who changed soybean aphid management were more likely to have used labeled application rates (P=0.0001), used products with a single insecticide group number/mode of action (P=0.0064), changed or rotated insecticide group number/mode of action

(P=0.0002), tank-mixed two or more insecticides with different insecticide group numbers/modes of action (P=0.0035), and used a premix of two or more insecticides with different insecticide group numbers/modes of action (P=0.0009) compared to farmers who did not change soybean aphid management.

Thoughts About Insecticide Resistance

Farmers had different thoughts about which soybean aphid management practice contributed the most to causing insecticide resistant soybean aphids (Fig 4.9). On average 75.4% of farmers in both groups reported that the use of the same insecticide group number/mode of action repeatedly is the practice that contributed the most. Among the different thoughts on the causes of insecticide resistance, farmers who changed soybean aphid management reported that the practice that contributed the most was the use of treatment thresholds that are too low when scouting compared to farmers who did not change soybean aphid management (P=0.0019).

Farmers had similar levels of confidence that various solutions will keep insecticide resistant soybean aphids from becoming a problem for their farm. Overall, more than 60% of farmers in both groups were somewhat confident that practices such as new soybean aphid resistant or tolerant seed varieties, new insecticide group numbers/modes of action, and individual farmer use of best soybean aphid management will keep insecticide resistant soybean aphids from becoming a problem on their farm, and this did not differ between groups of farmers (P>0.05) (Fig 4.10). Lastly, farmers expressed different levels of concern

about insecticide resistant soybean aphids. Farmers who changed managements practices reported to be on average (mean of 6.9 on a scale from 0 (not at all concerned) to 10 (very concerned)) more concerned about insecticide resistant soybean aphids compared to farmers who did not change (mean of 5.2 on the same scale) ($P<0.0001$).

Discussion

In response to challenges, such as insecticide resistance, climate, and technological advancements, farmers are making significant changes to their management practices to ensure the continued productivity and profitability of farms (Van den Berg et al. 2022). The success in the implementation of IPM and IRM strategies depends largely on farmer decision-making and their willingness to adopt these strategies (Ehler 2006, Bueno et al. 2021). These practices are essential to reduce the use of pesticides, making agriculture more environmentally friendly and cost-effective in the long term (Phillips et al. 1989, Peterson et al. 2018).

Farmers receive information from various sources. Agricultural advisors serve crucial roles as information brokers between scientists and farmers and help farmers with agricultural management decision making (Haigh et al. 2015). In the present study, crop consultants and agricultural retailers were the most important sources of information and decision making reported by farmers in both groups (change and no change soybean aphid management) (Fig 4.2). Similarly,

Hurley and Mitchell (2020) reported that U.S. soybean farmers typically relied primarily on these two groups. Additionally, farmers who changed soybean aphid management relied on company representatives, and extension staff more frequently than farmers who did not change soybean aphid management (Fig 4.2). By relying on company representatives and extension staff, farmers can get updated information on research findings about new pest management technologies and strategies that can enable them to make more informed decisions about their pest management practices, reducing the risk of resistance development (Ford and Babb 1989).

Current management recommendations for soybean aphid focus on scouting and threshold-based application of foliar insecticides (Koch et al. 2016, 2018). However, to determine when to apply foliar insecticides, farmers from both groups used a lower threshold (farmers who changed 72.1%, and farmers who did not change 76.0), average of 168 aphids per plant, than the research-based recommended economic threshold (ET) of an average of 250 aphids per plant (Ragsdale et al. 2007). Previously, Olson et al. (2008) found that 50% of growers from the Midwest (including Iowa, Michigan, Minnesota, and Wisconsin) used the university-recommended ET, which is a higher percentage than the value observed in this study. Currently, the use of a lower threshold may be due to the lack of trust reported by farmers on the university-determined ET saying that it is too high (Hoidal and Koch 2021). The use of insecticides when the pest populations are lower than the economic threshold may cause unnecessary

exposure of the pest to the insecticide, which increases the likelihood of resistance development (Bortolotto et al. 2015, Koch et al. 2018).

Chemical control of soybean aphid has relied mainly on pyrethroids and organophosphates (Hodgson et al. 2012). Farmers who changed soybean aphid management are using more pyrethroids and pyrethroid mixes than farmers who did not change soybean aphid management (Fig 4.6). The over-reliance on a single chemical family most likely increased the risk of insecticide resistance by selecting for resistant individuals in the population leading to a decline in the effectiveness of the insecticide (Hodgson et al. 2012, Koch et al. 2016). Also, the use of mixtures is not recommended for IRM, unless it is used in rotation with other modes of action (IRAC 2012, Koch et al. 2018). Additionally, another of the main active ingredients to control soybean aphid reported in this study, chlorpyrifos, is no longer available (EPA 2022), leaving pyrethroids as the main insecticide to control soybean aphid in 2021. Education efforts can focus on reducing pyrethroid usage and informing farmers about other non-chemical alternatives to reduce the selection for resistant soybean aphid populations.

Non-chemical strategies to manage soybean aphid populations include insecticide seed treatments and host plant resistance (Clifton et al. 2018). Farmers who changed soybean aphid management used insecticide seed treatments and aphid resistant soybean varieties more frequently than farmers who did not change soybean aphid management (Fig 4.3). Aphid-resistant soybean varieties are an important component of IPM programs that help

maintain beneficial insects by reducing insecticide use (Sharma 2007). Even though aphid-resistant soybean varieties have proven to be effective, they are not widely used (McCarville and O'Neal 2013, Tilmon et al. 2021) due to limited varieties with shorter maturity for northern states and other breeder issues. Education efforts can be focused on promoting the adoption of aphid-resistant varieties, including cost-benefit assessments that can help farmers make informed decisions about investing in this alternative. On the other hand, seed treatments have been reported by farmers as the primary seed technology in soybean (Hurley and Mitchell 2020). Systemic insecticidal seed treatments such as neonicotinoids are usually no longer effective when peak aphid colonization happens (Hodgson et al. 2012, Seagraves and Lundgren 2012). However, there are a few scenarios where seed treatments could be beneficial such as early-season infestations, early planting, and resistance management (using different modes of action than foliar insecticides) (McCarville and O'Neal 2013, Esquivel et al. 2019). Considering that seed treatments are not effective in most situations, this is another area where education efforts can be focused, providing farmers with knowledge to account for factors such as historical aphid pressure, real time data on current infestations and severity, local environmental conditions, and economics before deciding to use seed treatments for soybean aphid management.

Furthermore, IRM strategies such as the use of labeled rates of insecticides and alternate insecticides modes of action are critical to slow the

evolution of insecticide resistance (Sparks and Nauen 2015). The aforementioned IRM strategies were reported to be implemented by the farmers who changed their soybean aphid management and can benefit from the delay of resistance of soybean aphid populations (Fig 4.8). The implementation of IRM and IPM approaches can lead to more efficient and sustainable pest control, helping to reduce the pest's ability to adapt, reducing the reliance on chemical treatments (Head and Savinelli 2008). Ultimately, the implementation of soybean aphid management will depend on the individual farmer's situation and goals, but there is potential for changes in management practices based on new research and technology.

Overall, farmer decision making is crucial for the successful implementation of IPM and IRM strategies. We found that farmers who changed soybean aphid management due to insecticide resistance concerns are in general implementing good practices to delay insecticide resistance (e.g., host plant resistance, use of labeled rates of insecticides, and alternate insecticides modes of action). However, increasing awareness of the extent of insecticide resistance to modulate farmer concerns would be useful. More education and outreach programs can be implemented to provide farmers and agricultural advisors with the information and training needed to effectively manage insecticide resistance. Also, the ability and readiness of farmers to successfully change their management practices is improved by education and training (Kilpatrick 2000). These programs can include Farmer Field Schools (FFS),

workshops, training sessions, and educational materials that explain the risks and consequences of insecticide resistance, as well as the best practices for managing soybean aphid populations. Additionally, area-wide approaches that involve the collaboration of multiple farmers can be beneficial, as they can help to reduce the likelihood of resistance evolution across an entire region (Zalucki et al. 2009). In conclusion, providing farmers with education on managing insecticide resistance is crucial to protect the sustainability of our agricultural system and ensure the availability of effective pest control options for future generations.

Acknowledgments

Thank you to Heidi Hoffman, Celia Studenski, Nick Seitzer, and Abby Poser for their assistance entering data. This work was supported by the Minnesota Rapid Agricultural Response Program (RARF), the USDA National Institute of Food & Agriculture (NIFA) (award no. 2019-68008-29892), the MN Agricultural Experiment Station Project MIN-14-120, the Minnesota Soybean Research and Promotional Council, the North Dakota Soybean Council, the Department of Entomology Lugger-Radcliffe Summer Fellowship to REL, and a University of Minnesota Doctoral Dissertation Fellowship to REL.

Figs

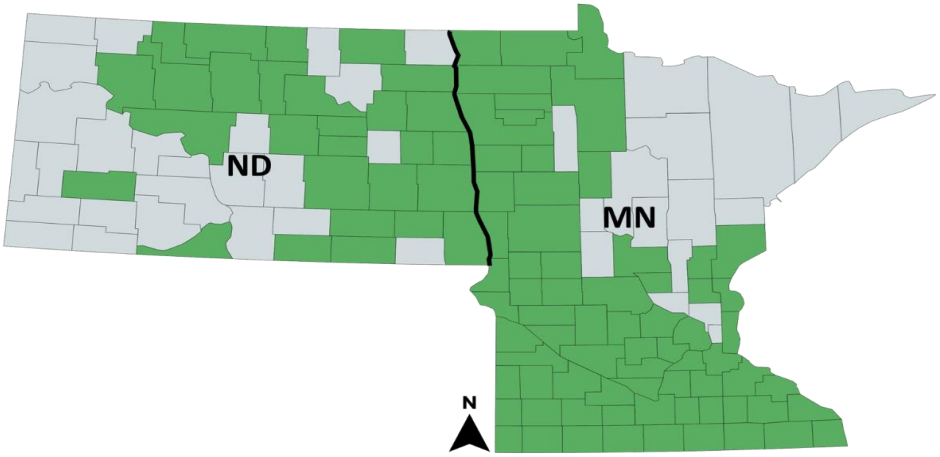


Fig 4.1.Survey response representation across Minnesota (MN) and North Dakota (ND) in 2021.

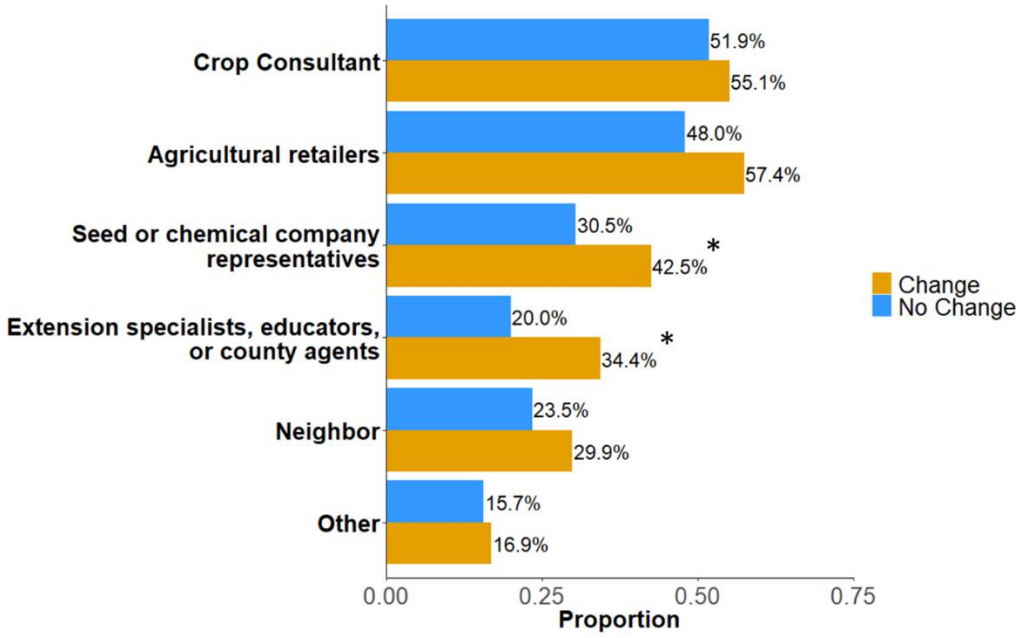


Fig 4.2. Percentages of sources of information that farmers relied on when deciding how to manage soybean aphid in Minnesota and North Dakota in 2021. Respondents could select more than one option. Significant differences between groups are represented with black stars. Significance was defined at α= 0.05

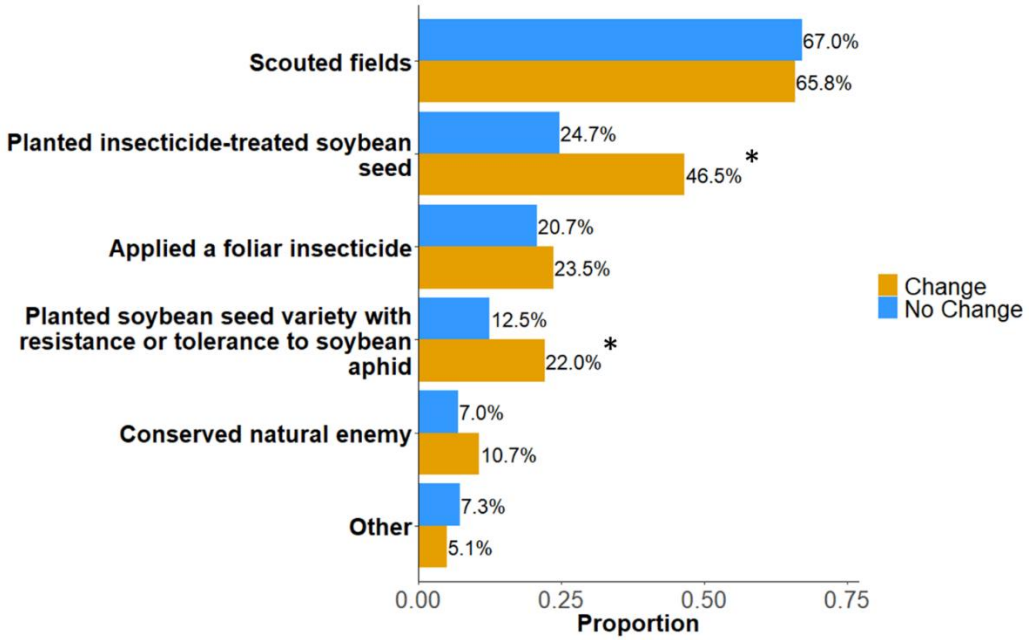


Fig 4.3. Percentages of practices used by farmers to manage soybean aphids in Minnesota and North Dakota in 2021. Respondents could select more than one option. Significant differences between groups are represented with black stars. Significance was defined at α= 0.05.

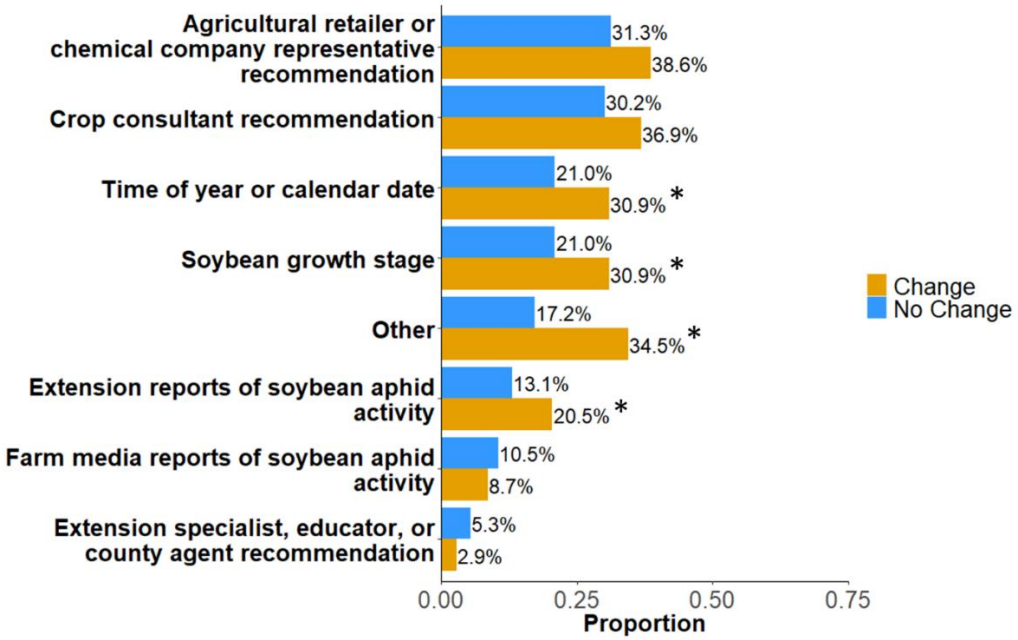


Fig 4.4. Percentages of factors that influenced farmers decision to apply foliar insecticides to manage soybean aphids in Minnesota and North Dakota in the five-year period (2017-2021). Respondents could select more than one option. Significant differences between groups are represented with black stars. Significance was defined at α= 0.05.

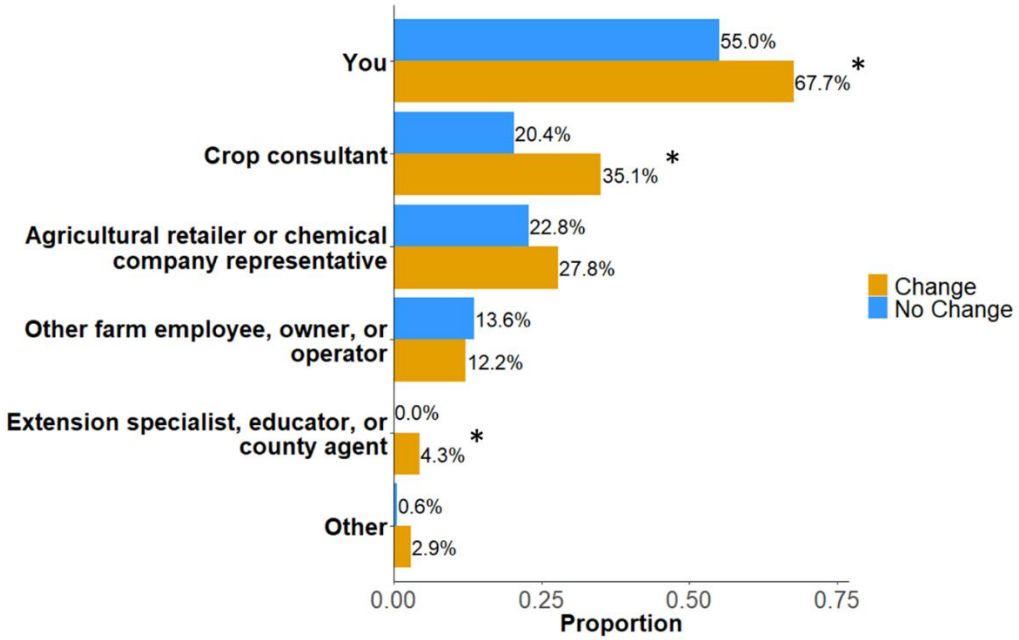


Fig 4.5. Person who scouted fields for soybean aphids in the five-year period (2017-2021). Respondents could select more than one option. Significant differences between groups are represented with black stars. Significance was defined at α= 0.05.

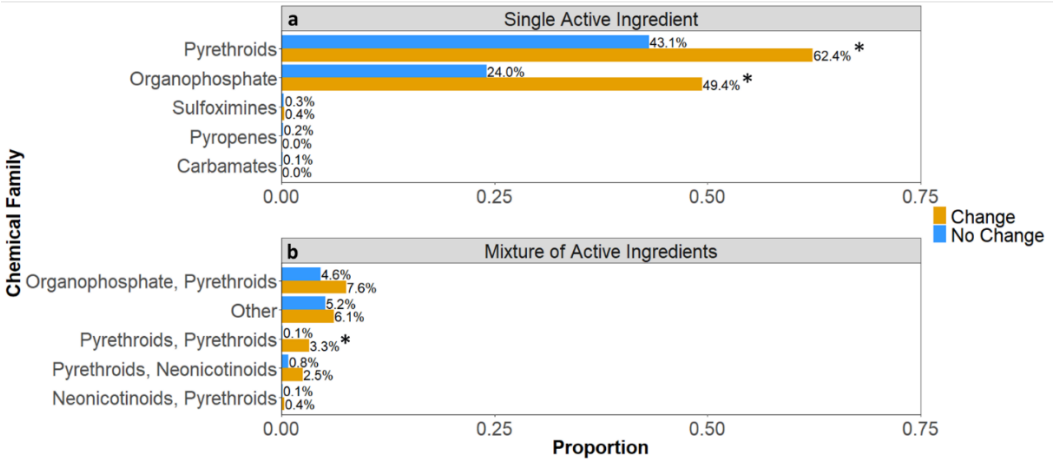


Fig 4.6. Percentages of farmers that used chemical insecticide families to control soybean aphids in Minnesota and North Dakota in the five-year period (2017-2021). Respondents could select more than one option. Insecticide options with zero response from farmers are not shown (single active ingredients: butenolides, diamides, flonicamid, neonicotinoids, and spinosyns; and mixtures of active ingredients: pyrethroids + diamides, pyrethroids + neonicotinoids + pyrethroids, and pyrethroids + pyropenes). Significant differences between groups are represented with black stars. Significance was defined at $\alpha = 0.05$.

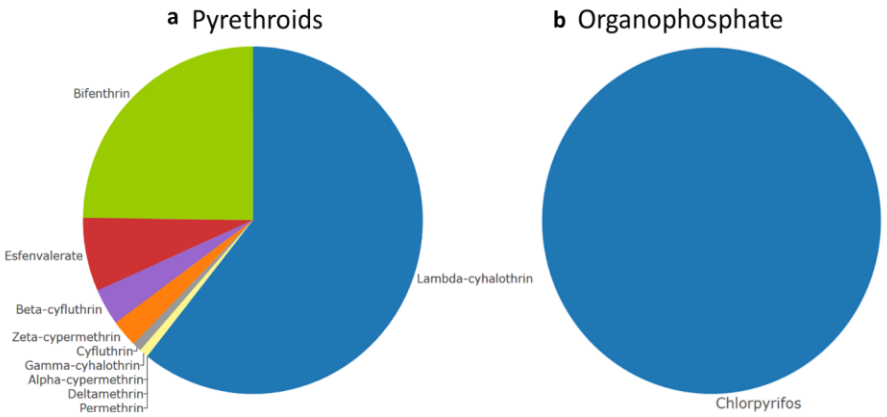


Fig 4.7. Percentages of foliar insecticides used to control soybean aphids reported by farmers in Minnesota and North Dakota in the five-year period (2017-2021).

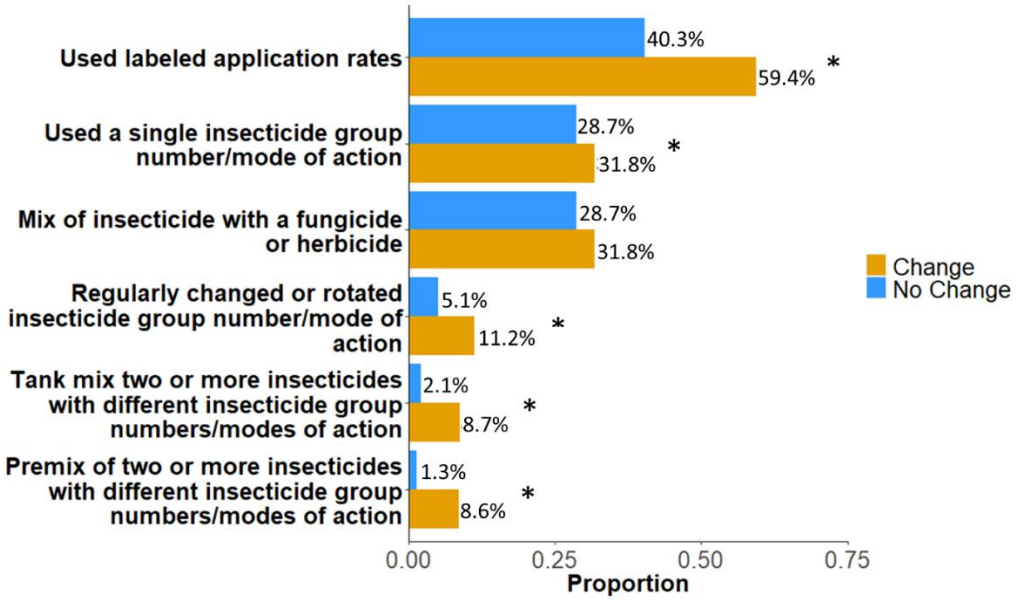


Fig 4.8. Percentages of practices used with foliar insecticide applications to control soybean aphids in Minnesota and North Dakota in the five-year period (2017-2021). Respondents could select more than one option. Significant differences between groups are represented with black stars. Significance was defined at α= 0.05.

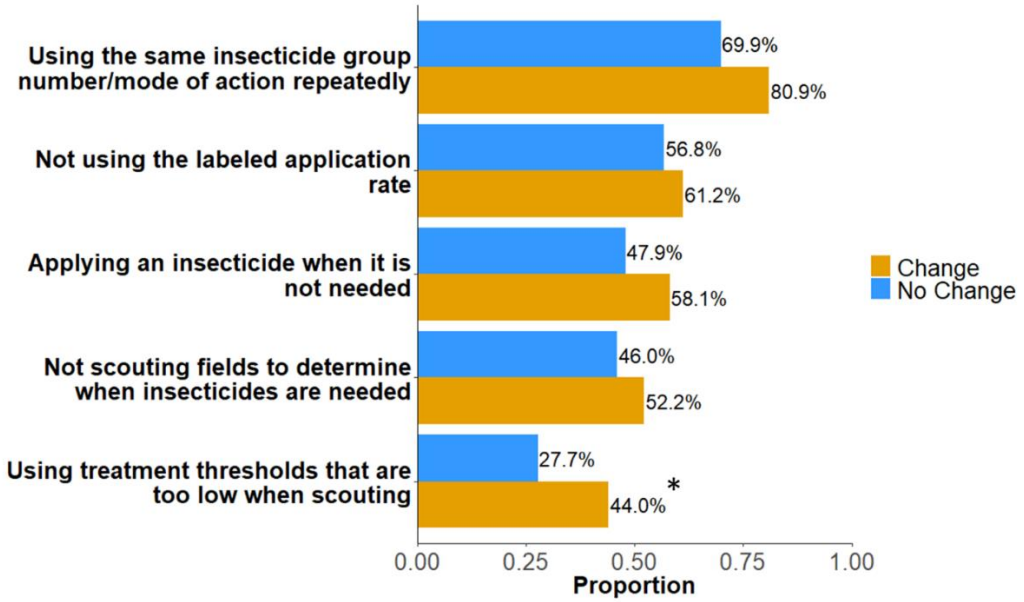


Fig 4.9. Percentage of the soybean aphid management that farmers thought contributed the most to causing insecticide resistant soybean aphids in Minnesota and North Dakota in 2021. Respondents could select more than one option.

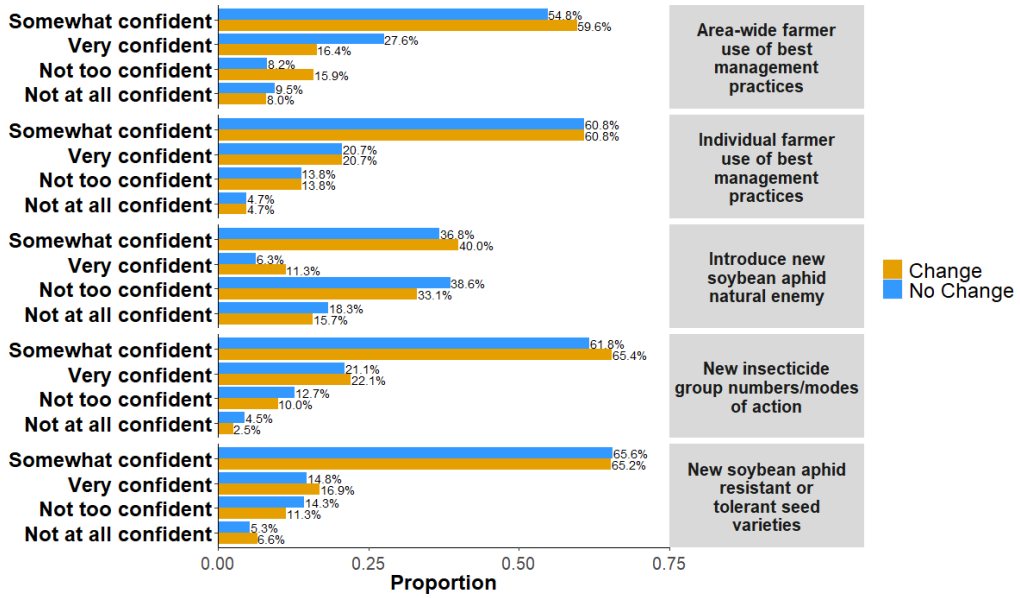


Fig 4.10. Percentage of the confidence level of different solutions that can keep insecticide resistant soybean aphids from becoming a problem on farms in Minnesota and North Dakota in 2021.

References Cited

Aita, R.C., A. Vieira Ribeiro, and R.L. Koch. **2019**. Efficacy of Foliar Insecticides for Management of Soybean Aphid, 2019. Arthropod Manag. Tests. 44: 1–2.

Alleman, R., C. Grau, and D. Hogg. **2002**. Soybean aphid host range and virus transmission efficiency. Proc. Wisconsin Fertil. Agline Pest Manag. Conf. 1–3.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. **1997**. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Am. J. Bot. 25: 3389–3402.

An, X.K., M.L. Hou, and Y. Di Liu. **2016**. Reference gene selection and evaluation for gene expression studies using qRT-PCR in the white-backed planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae). J. Econ. Entomol. 109: 879–886.

Andersen, C.L., J.L. Jensen, and T.F. Ørntoft. **2004**. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64: 5245–5250.

Bansal, R., P. Mamidala, M.A.R. Mian, O. Mittapalli, and A.P. Michel. **2012**. Validation of reference genes for gene expression studies in *Aphis glycines* (Hemiptera: Aphididae). J. Econ. Entomol. 105: 1432–1438.

Basit, M. 2019. Status of insecticide resistance in Bemisia tabaci: resistance, cross-resistance, stability of resistance, genetics and fitness costs. Phytoparasitica. 47: 207–225.

Bass, C., R.A. Carvalho, L. Oliphant, A.M. Puinean, L.M. Field, R. Nauen, M.S. Williamson, G. Moores, and K. Gorman. **2011**. Overexpression of a cytochrome P450 monooxygenase, CYP6ER1, is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*. Insect Mol. Biol. 20: 763–773.

Bass, C., A.M. Puinean, C.T. Zimmer, I. Denholm, L.M. Field, S.P. Foster, O. Gutbrod, R. Nauen, R. Slater, and M.S. Williamson. **2014**. The evolution of insecticide resistance in the peach potato aphid, Myzus persicae. Insect Biochem. Mol. Biol. 51: 41–51.

Bassan, M.M., J. Angelotti-Mendonca, G.R. Alves, P.T. Yamamoto, and F.D.A.A. Mouraõ Filho. **2017**. Selection of reference genes for expression studies in *Diaphorina citri* (Hemiptera: Liviidae). J. Econ. Entomol. 110: 2623–2629.

Beckendorf, E.A., M.A. Catangui, and W.E. Riedell. **2008**. Soybean aphid feeding injury and soybean yield, yield components, and seed composition. Agron. J. 100: 237–246.

Bolger, A.M., M. Lohse, and B. Usadel. **2014**. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 30: 2114–2120.

Bortolotto, O.C., A. Pomari-Fernandes, R.C.O. de F. Bueno, A. de F. Bueno, Y.K.S. da Cruz, A. Sanzovo, and R.B. Ferreira. **2015**. The use of soybean integrated pest management in Brazil: a review. Agron. Sci. Biotechnol. 1: 25–32.

Brito, L.P., J.G.B. Linss, T.N. Lima-Camara, T.A. Belinato, A.A. Peixoto, J.B.P. Lima, D. Valle, and A.J. Martins. **2013**. Assessing the Effects of Aedes aegypti kdr Mutations on Pyrethroid Resistance and Its Fitness Cost. PLoS One. 8.

Browning, B.L., X. Tian, Y. Zhou, and S.R. Browning. **2021**. Fast two-stage phasing of large-scale sequence data. Am. J. Hum. Genet. 108: 1880–1890.

Bueno, A.F., A.R. Panizzi, T.E. Hunt, P.M. Dourado, R.M. Pitta, and J. Gonçalves. **2021**. Challenges for Adoption of Integrated Pest Management (IPM): the Soybean Example. Neotrop. Entomol. 50: 5–20.

Bustin, S.A., and R. Mueller. **2005**. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin. Sci. 109: 365–379.

Busvine, J.R. 1951. Mechanism of resistance to insecticide in Houseflies. Nature. 168: 193–195.

Butte, A.J., V.J. Dzau, and S.B. Glueck. **2001**. Further defining housekeeping, or “maintenance,” genes Focus on “a compendium of gene expression in normal human tissues.” Physiol Genomics. 7: 95–96.

Byrne, F.J., K.J. Gorman, M. Cahill, I. Denholm, and A.L. Devonshire. **2000**. The role of B-type esterases in conferring insecticide resistance in the tobacco whitefly, *Bemisia tabaci* (Genn). Pest Manag. Sci. 56: 867–874.

Carlson, S.M., C.J. Cunningham, and P.A.H. Westley. **2014**. Evolutionary rescue in a changing world. Trends Ecol. Evol. 29: 521–530.

Cass, R.P., E.W. Hodgson, M.E. O’Neal, A.L. Toth, and A.G. Dolezal. **2022**. Attitudes About Honey Bees and Pollinator-Friendly Practices: A Survey of Iowan Beekeepers, Farmers, and Landowners. J. Integr. Pest Manag. 13.

Chang, C.C., C.C. Chow, L.C.A.M. Tellier, S. Vattikuti, S.M. Purcell, and J.J. Lee. **2015**. Second-generation PLINK : rising to the challenge of larger and richer datasets. 1–16.

Chang, Y.-W., J.-Y. Chen, M.-X. Lu, Y. Gao, Z.-H. Tian, W.-R. Gong, W. Zhu, and Y.-Z. Du. **2017**. Selection and validation of reference genes for

quantitative real-time PCR analysis under different experimental conditions in the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae). Sci. Hortic. (Amsterdam). 225: 195–203.

Charaabi, K., S. Boukhris-Bouhachem, M. Makni, B. Fenton, and I. Denholm. **2016**. Genetic variation in target-site resistance to pyrethroids and pirimicarb in Tunisian populations of the peach potato aphid, Myzus persicae (Sulzer) (Hemiptera: Aphididae). Pest Manag. Sci. 72: 2313–2320.

Clark, T.L., B. Puttler, and W.C. Bailey. **2006**. Is horsenettle, Solanum carolinense L. (Solanaceae), an alternate host for soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae)? J. Kansas Entomol. Soc. 79: 380–383.

Clifton, E.H., G.L. Tylka, J. Gassmann, and W. Erin. **2018**. Interactions of effects of host plant resistance and seed treatments on soybean aphid (*Aphis glycines* Matsumura) and soybean cyst nematode (*Heterodera glycines* Ichinohe). Pest Manag. Sci. 74: 992–1000.

Coppin, C.W., C.J. Jackson, T. Sutherland, P.J. Hart, A.L. Devonshire, R.J. Russell, and J.G. Oakeshott. **2012**. Testing the evolvability of an insect carboxylesterase for the detoxification of synthetic pyrethroid insecticides. Insect Biochem. Mol. Biol. 42: 343–352.

Cristiano, G., G. Grossi, A. Scala, P. Fanti, J.J. Zhou, S.A. Bufo, L. Palazzo, and P. Falabella. **2016**. Validation of reference genes for qRT-PCR analysis in *Megoura viciae* (Hemiptera Aphididae). Bull. Insectology. 69: 229–238.

Dai, W.T., J. Li, and L.P. Ban. **2022**. Genome-wide selective signature analysis revealed insecticide resistance mechanisms in cydia pomonella. Insects. 13.

Dai, Z., and J. Fan. **1991**. Effects of Aphid Population Dynamics and Damage Period on Soybean Yield. J. Shenyang Agric. Univ. 22: 135–139.

Danecek, P., A. Auton, G. Abecasis, C.A. Albers, E. Banks, M.A. Depristo, R.E. Handsaker, G. Lunter, G.T. Marth, S.T. Sherry, G. Mcvean, R. Durbin, G. Project, and T. Vcf. **2011**. The variant call format and VCFtools. 27: 2156–2158.

David, J., H.M. Ismail, A. Chandor-proust, M. John, I. Paine, M. John, and I. Paine. **2013**. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. Philos. Trans. R. Soc. B Biol. Sci. 368: 1612.

Davies, T., and M. Williamson. **2009**. Interactions of pyrethroids with the voltage-gated sodium channel. Pyrethroid Sci. Forum 2009. 62: 159–178.

Deming, R., P. Manrique-Saide, A. Medina Barreiro, E.U.K. Cardenã, A. Che-Mendoza, B. Jones, K. Liebman, L. Vizcaino, G. Vazquez-Prokopec, and A. Lenhart. **2016**. Spatial variation of insecticide resistance in the dengue vector Aedes aegypti presents unique vector control challenges. Parasites and Vectors. 9: 1–10.

Denholm, I., and G. Devine. **2013**. Insecticide Resistance. Encycl. Biodivers. Second Ed. 4: 298–307.

Dillman, D.A., Smyth, J.D. and Christian, L.M., 2014. Internet, phone, mail, and mixed-mode surveys: The tailored design method. John Wiley & Sons. New Jersey, US.

Dong, K., Y. Du, F. Rinkevich, Y. Nomura, P. Xu, L. Wang, K. Silver, and B.S. Zhorov. **2014**. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem. Mol. Biol. 50: 1–17.

Donnelly, M.J., A.T. Isaacs, and D. Weetman. **2016**. Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors. Physiol. Behav. 32: 197–206.

Dukre, A.B., M.P. Moharil, B.S. Ghodki, and N.G. V Rao. **2009**. Role of glutathione-s-transferase in imparting resistance to pyrethroids in Plutella xylostella (L .). J. Integr. Biol. 6: 17–21.

Ehler, L.E. 2006. Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. Pest Manag. Sci. 62: 787–789.

Eleftherianos, I., S.P. Foster, M.S. Williamson, and I. Denholm. **2008a**. Characterization of the M918T sodium channel gene mutation associated with strong resistance to pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer). Bull. Entomol. Res. 98: 183–191.

Eleftherianos, I., S.P. Foster, M.S. Williamson, and I. Denholm. **2008b**. Inheritance of L1014F and M918T sodium channel mutations associated with pyrethroid resistance in Myzus persicae. Biol. Lett. 4: 545–548.

Elliott, M., and N.F. Janes. **1978**. Synthetic pyrethroids - A new class of insecticide. Chem. Soc. Rev. 7: 473–505.

Esquivel, C.J., C.M. Ranger, L. Phelan, E.J. Martinez, W.H. Hendrix, L.A. Canas, and P. Andrew. **2019**. Weekly Survivorship Curves of Soybean Aphid Biotypes 1 and 4 on Insecticidal Seed-Treated Soybean. J. Econ. Entomol. 112: 712–719.

Everaert, B.R., G.A. Boulet, J.P. Timmermans, and C.J. Vrints. **2011**. Importance of suitable reference gene selection for quantitative real-time PCR: special reference to mouse myocardial infarction studies. PLoS One. 6: e23793.

Fassinou, A.J.Y.H., C.Z. Koukpo, R.A. Ossè, F.R. Agossa, B.S. Assogba, A. Sidick, W.T. Sèwadé, M.C. Akogbéto, and M. Sèzonlin. **2019**. Genetic structure of *Anopheles gambiae* s.s populations following the use of

insecticides on several consecutive years in southern Benin. Trop. Med. Health. 47: 1–10.

Fenton, B., J.T. Margaritopoulos, G.L. Malloch, and S.P. Foster. **2010**. Micro-evolutionary change in relation to insecticide resistance in the peach-potato aphid, Myzus persicae. Ecol. Entomol. 35: 131–146.

Feyereisen, R. 1995. Molecular biology of insecticide resistance. Toxicol. Lett. 82–83: 83–90.

Feyereisen, R. 2005. Insect Cytochrome P450. Compr. Mol. Insect Sci. 4–6: 1–77.

Feyereisen, R. 2012. Insect CYP genes and P450 enzymes, Insect Mol. Biol. Biochem. Elsevier B.V.

Feyereisen, R. 2020. Origin and evolution of the CYP4G subfamily in insects, cytochrome P450 enzymes involved in cuticular hydrocarbon synthesis. Mol. Phylogenet. Evol. 143: 106695.

Fontaine, S., L. Caddoux, C. Brazier, C. Bertho, P. Bertolla, A. Micoud, and L. Roy. **2011**. Uncommon associations in target resistance among French populations of Myzus persicae from oilseed rape crops. Pest Manag. Sci. 67: 881–885.

Ford, S.A., and E.M. Babb. **1989**. Farmer Sources and Uses of Information. Agribusiness. 5: 465–476.

Foster, S.P., I. Denholm, G.M. Poppy, R. Thompson, and W. Powell. **2011**. Fitness trade-off in peach-potato aphids (Myzus persicae) between insecticide resistance and vulnerability to parasitoid attack at several spatial scales. Bull. Entomol. Res. 101: 659–666.

Foster, S.P., V.L. Paul, R. Slater, A. Warren, I. Denholm, L.M. Field, and M.S.

Williamson. **2014**. A mutation (L1014F) in the voltage-gated sodium channel of the grain aphid, Sitobion avenae, is associated with resistance to pyrethroid insecticides. Pest Manag. Sci. 70: 1249–1253.

Freitas, F.C.P., T.S. Depintor, L.T. Agostini, D. Luna-lucena, F.M.F. Nunes, M.M.G. Bitondi, Z.L.P. Simões, and A.P. Lourenço. **2019**. Evaluation of reference genes for gene expression analysis by real- time quantitative PCR (qPCR) in three stingless bee species (Hymenoptera: Apidae: Meliponini). Sci. Rep. 9: 17692.

Fu, W., W. Xie, Z. Zhang, S. Wang, Q. Wu, Y. Liu, X. Zhou, X. Zhou, and Y. Zhang. **2013**. Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). Int. J. Biol. Sci. 9: 792–802.

Gohl, D., D.M. Gohl, A. MacLean, A. Hauge, A. Becker, D. Walek, and K.B. Beckman. **2016**. An optimized protocol for high-throughput amplicon-based microbiome profiling. Protoc. Exch.

Gutierrez, L., M. Mauriat, S. Guénin, J. Pelloux, J.F. Lefebvre, R. Louvet, C. Rusterucci, T. Moritz, F. Guerineau, C. Bellini, and O. Van Wuytswinkel. **2008**. The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol. J. 6: 609–618.

Haddi, K., M. Berger, P. Bielza, D. Cifuentes, L.M. Field, K. Gorman, C. Rapisarda, M.S. Williamson, and C. Bass. **2012**. Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*). Insect Biochem. Mol. Biol. 42: 506–513.

Haddi, K., W.R. Valbon, L.O. Viteri Jumbo, L.O. de Oliveira, R.N.C. Guedes,

and E.E. Oliveira. **2018**. Diversity and convergence of mechanisms involved in pyrethroid resistance in the stored grain weevils, *Sitophilus spp.* Sci. Rep. 8: 1–15.

Haigh, T., L.W. Morton, M.C. Lemos, C. Knutson, L.S. Prokopy, Y.J. Lo, and J. Angel. **2015**. Agricultural Advisors as Climate Information Intermediaries: Exploring Differences in Capacity to Communicate Climate. Weather. Clim. Soc. 7: 83–93.

Hanson, A.A., J. Menger-Anderson, C. Silverstein, B.D. Potter, I. V. Macrae, E.W. Hodgson, and R.L. Koch. **2017**. Evidence for soybean aphid (Hemiptera: Aphididae) resistance to pyrethroid insecticides in the upper Midwestern United States. J. Econ. Entomol. 110: 2235–2246.

Hardstone, M.C., and J.G. Scott. **2010**. A review of the interactions between multiple insecticide resistance loci. Pestic. Biochem. Physiol. 97: 123–128.

Hartman, G.L., L.L. Domier, L.M. Wax, C.G. Helm, D.W. Onstad, J.T. Shaw, L.F. Solter, D.J. Voegtlin, C.J. D'Arcy, M.E. Gray, K.L. Steffey, S.A. Isard, and P.L. Orwick. **2001**. Occurrence and distribution of *Aphis glycines* on soybeans in Illinois in 2000 and its potential control. Plant Heal. Prog. 2: 17.

He, X. ting, C. cheng Liu, Z. qun Li, Z. Zhang, G. qing Li, F. Li, and S. lin Dong. **2014**. Validation of reference genes for quantitative real-time PCR in *Laodelphax striatellus*. J. Integr. Agric. 13: 811–818.

Head, G., & Savinelli, C. 2008. Adapting insect resistance management programs to local needs. Insect resistance management: biology, economics and prediction, ed. by Onstad DW. Academic Press of Elsevier Ltd, London, UK. pp. 89–106

Heimpel, G.E., and M.K. Asplen. **2011**. A ' Goldilocks ' hypothesis for dispersal

of biological control agents. 441–450.

Heimpel, G.E., L.E. Frelich, and D.A. Landis. **2010**. European buckthorn and Asian soybean aphid as components of an extensive invasional meltdown in North America. Biol. Invasions. 12: 2913–2931.

Hemming-Schroeder, E., S. Strahl, E. Yang, A. Nguyen, E. Lo, D. Zhong, H. Atieli, A. Githeko, and G. Yan. **2018**. Emerging pyrethroid resistance among *Anopheles arabiensis* in Kenya. Am. J. Trop. Med. Hyg. 98: 704–709.

Henderson, C.F., and E.W. Tilton. **1955**. Tests with Acaricides against the Brown Wheat Mite. J. Econ. Entomol. 48: 157–161.

Hesler, L.S., M. V. Chiozza, M.E. O'Neal, G.C. MacIntosh, K.J. Tilmon, D.I. Chandrasena, N.A. Tinsley, S.R. Cianzio, A.C. Costamagna, E.M. Cullen, C.D. Difonzo, B.D. Potter, D.W. Ragsdale, K. Steffey, and K.J. Koehler. **2013**. Performance and prospects of Rag genes for management of soybean aphid. Entomol. Exp. Appl. 147: 201–216.

Van Hiel, M.B., P. Van Wielendaele, L. Temmerman, S. Van Soest, K. Vuerinckx, R. Huybrechts, J. Vanden Broeck, and G. Simonet. **2009**. Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. BMC Mol. Biol. 10: 56.

Hirose, Y., R. Tacke, and J.L. Manley. **1999**. Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. Genes Dev. 13: 1234–1239.

Hodgson, E.W., E.C. Burkness, W.D. Hutchison, and D.W. Ragsdale. **2004**. Enumerative and binomial sequential sampling plans for soybean aphid (Homoptera: Aphididae) in soybean. J. Econ. Entomol. 97: 2127–2136.

Hodgson, E.W., B.P. McCornack, K. Tilmon, and J.J. Knodel. **2012**. Management Recommendations for Soybean Aphid (Hemiptera: Aphididae) in the United States. J. Integr. Pest Manag. 3: 1–10.

Hoffman, D.W., C.S. Cameron, C. Davies, S.W. White, and V. Ramakrishnan. **1996**. Ribosomal protein L9: a structure determination by the combined use of X-ray crystallography and NMR spectroscopy. J. Mol. Biol. 264: 1058–1071.

Hoidal, N., and R.L. Koch. **2021**. Perception and Use of Economic Thresholds among Farmers and Agricultural Professionals: A Case Study on Soybean Aphid in Minnesota. J. Integr. Pest Manag. 12.

Holsinger, K.E., and B.S. Weir. **2009**. Genetics in geographically structured populations: Defining, estimating and interpreting FST. Nat. Rev. Genet. 10: 639–650.

Homem, R.A., B. Buttery, E. Richardson, Y. Tan, L.M. Field, M.S. Williamson, and T.G. Emyr Davies. **2020**. Evolutionary trade-offs of insecticide resistance — The fitness costs associated with target-site mutations in the nAChR of Drosophila melanogaster. Mol. Ecol. 29: 2661–2675.

Horňáková, D., P. Matoušková, J. Kindl, I. Valterová, and I. Pichová. **2010**. Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. Anal. Biochem. 397: 118–120.

Huggett, J., K. Dheda, S. Bustin, and A. Zumla. **2005**. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. 6: 279–284.

Hurley, T.M., and P.D. Mitchell. **2020**. The value of insect management to US maize, soybean and cotton farmers. Pest Manag. Sci. 76: 4159–4172.

Hurley, T.M., P.D. Mitchell, and H. Sun. **2023**. Insect resistance management: adoption and compliance, Insect Resist. Manag. Elsevier Ltd.

Hurley, T.M., and H. Sun. **2019**. Softening Shock and Awe Pest Management in Corn and Soybean Production with IPM Principles. J. Integr. Pest Manag. 10.

Insecticide Resistance Action Committee (IRAC). 2012. Interactive MoA classification. https://irac-online.org/documents/irac-mixture-statement/. Accessed: 2023-07-25.

Insecticide Resistance Action Committee (IRAC). 2023a. Interactive MoA classification. https://irac-online.org/modes-of-action/. Accessed: 2023-07-25.

Insecticide Resistance Action Committee (IRAC). 2023b. Resistance mechanisms. https://irac-online.org/about/resistance/mechanisms/. Accessed: 2023-07-25.

IRAC, I.R.A.C. 2020. Resistance Mechanisms. (https://irac-online.org/about/resistance/mechanisms/).

Jabbour, R., and S. Noy. **2017**. Wyoming producer priorities and perceptions of alfalfa insect pests. J. Integr. Pest Manag. 8: 26.

Jiang, H.B., Y.H. Liu, P.A. Tang, A.W. Zhou, and J.J. Wang. **2010**. Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of *Liposcelis bostsrychophila* (Psocoptera: Liposcelididae). Mol. Biol. Rep. 37: 1019–1029.

Kandel, E.R. 1991. Nerve Cells and Behavior. 18–34.

Kang, Z.W., F.H. Liu, H.G. Tian, M. Zhang, S.S. Guo, and T.X. Liu. **2017**. Evaluation of the reference genes for expression analysis using quantitative

real-time polymerase chain reaction in the green peach aphid, *Myzus persicae*. Insect Sci. 24: 222–234.

Khalfan, M. 2020. Variant Calling Pipeline using GATK4. Available at: https://gencore.bio.nyu.edu/variant-calling-pipeline-gatk4/.

Khambay, B.P.S., and P.J. Jewess. **2005**. Pyrethroids. Compr. Mol. Insect Sci. 1–29.

Khan, S., M.N. Uddin, M. Rizwan, W. Khan, M. Farooq, A.S. Shah, F. Subhan, F. Aziz, K.U. Rahman, A. Khan, S. Ali, and Muhammad. **2020**. Mechanism of insecticide resistance in insects/pests. Polish J. Environ. Stud. 29: 2023–2030.

Kilpatrick, S. 2000. Education and training: Impacts on farm management practice. J. Agric. Educ. Ext. 7: 105–116.

Kim, J.S., and M. Zhen. **2009**. Neuronal Polarity. Neurol. Clin. 7: 1–181.

Kliot, A., and M. Ghanim. **2012**. Fitness costs associated with insecticide resistance. Pest Manag. Sci. 68: 1431–1437.

Koch, R., E. Hodgson, J. Knodel, and A. Varenhorst. **2019**. Management of Insecticide-resistance Soybean Aphids. J. Integr. Pest Manag. 9: 23.

Koch, R., E. Hodgson, J. Knodel, and A. Varenhorst. **2022**. Management of Insecticide-resistant Soybean Aphids. North Cent. Soybean Res. Progr. 1878: 1–4.

Koch, R.L., E.W. Hodgson, J.J. Knodel, A.J. Varenhorst, and B.D. Potter. **2018**. Management of insecticide-resistant soybean aphids in the upper Midwest of the United States. J. Integr. Pest Manag. 9: 23.

Koch, R.L., P.M. Porter, M.M. Harbur, M.D. Abrahamson, K.A.G. Wyckhuys,

D.W. Ragsdale, K. Buckman, Z. Sezen, and G.E. Heimpel. **2012**. Response of soybean insects to an autumn-seeded rye cover crop. Environ. Entomol. 41: 750–760.

Koch, R.L., B.D. Potter, P.A. Glogoza, E.W. Hodgson, C.H. Krupke, J.F. Tooker, C.D. DiFonzo, A.P. Michel, K.J. Tilmon, T.J. Prochaska, J.J. Knodel, R.J. Wright, T.E. Hunt, B. Jensen, A.J. Varenhorst, B.P. McCornack, K.A. Estes, and J.L. Spencer. **2016**. Biology and Economics of Recommendations for Insecticide-Based Management of Soybean Aphid. Plant Heal. Prog. 17: 265–269.

Koch, R.L., O. da Silva Queiroz, R.C. Aita, E.W. Hodgson, B.D. Potter, T. Nyoike, and C.D. Ellers-Kirk. **2020**. Efficacy of afidopyropen against soybean aphid (Hemiptera: Aphididae) and toxicity to natural enemies. Pest Manag. Sci. 76: 375–383.

Koramutla, M.K., R. Aminedi, and R. Bhattacharya. **2016**. Comprehensive evaluation of candidate reference genes for qRT-PCR studies of gene expression in mustard aphid, *Lipaphis erysimi* (Kalt). Sci. Rep. 6: 25883.

Kozera, B., and M. Rapacz. **2013**. Reference genes in real-time PCR. J. Appl. Genet. 54: 391–406.

Krupke, C.H., A.M. Alford, E.M. Cullen, E.W. Hodgson, J.J. Knodel, B. McCornack, B.D. Potter, M.I. Spigler, K. Tilmon, and K. Welch. **2017**. Assessing the value and pest management window provided by neonicotinoid seed treatments for management of soybean aphid (*Aphis glycines* Matsumura) in the Upper Midwestern United States. Pest Manag. Sci. 73: 2184–2193.

Lane, D.E., T.J. Walker, and D.G. Grantham. **2023**. IPM Adoption and Impacts in the United States. J. Integr. Pest Manag. 14.

Leach, A.B., C.A. Hoepting, and B.A. Nault. **2019**. Grower adoption of insecticide resistance management practices increase with extension-based program. Pest Manag. Sci. 75: 515–526.

Leal, M.F., D.C. Astur, P. Debieux, G.G. Arliani, C.E.S. Franciozi, L.C. Loyola, C.V. Andreoli, M.C. Smith, A. De Castro Pochini, B. Ejnisman, and M. Cohen. **2015**. Identification of suitable reference genes for investigating gene expression in anterior cruciate ligament injury by using reverse transcription-quantitative PCR. PLoS One. 10: 1–18.

Lee, S., and L. McCann. **2019**. Adoption of Cover Crops by U.S. Soybean Producers. J. Agric. Appl. Econ. 51: 527–544.

Van Leeuwen, T., W. Dermauw, K. Mavridis, and J. Vontas. **2020**. Significance and interpretation of molecular diagnostics for insecticide resistance management of agricultural pests. Curr. Opin. Insect Sci. 39: 69–76.

Li, H., and R. Durbin. **2009**. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 25: 1754–1760.

Li, Y., Z. Xu, L. Shi, G. Shen, and L. He. **2016**. Insecticide resistance monitoring and metabolic mechanism study of the green peach aphid, Myzus persicae (Sulzer) (Hemiptera: Aphididae), in Chongqing, China. Pestic. Biochem. Physiol. 132: 21–28.

Liu, N., M. Li, Y. Gong, F. Liu, and T. Li. **2015**. Cytochrome p450s –their expression, regulation, and role in insecticide resistance. Pestic. Biochem. Physiol. 120: 77–81.

Lozano, R.E., D.P. Paula, D.A. Andow, and R.L. Koch. **2022**. Validation of Reference Genes Across Populations of *Aphis glycines* (Hemiptera: Aphididae) for RT-qPCR Analysis of Gene Expression Related to Pyrethroid

Detoxification. J. Entomol. Sci. 57: 213–239.

Lü, F., K. Fu, Q. Li, W. Guo, and T. Ahmat. **2015**. Identification of carboxylesterase genes and their expression profiles in the Colorado potato beetle *Leptinotarsa decemlineata* treated with fipronil and cyhalothrin. Pestic. Biochem. Physiol. 122: 86–5.

Lü, J., C. Yang, Y. Zhang, and H. Pan. **2018**. Selection of reference genes for the normalization of RT-qPCR data in gene expression studies in insects: a systematic review. Front. Physiol. 9: 1560.

Lu, K.-H., J.Y. Bradfield, and L.L. Keeley. **1996**. Age and Starvation Effects on Hypertrehalosemic Gene Expression of Cytochrome P4504Cl in the Cockroach , Blaberus discoidalis. J. Insect Physiol. 42: 925–930.

Lu, Y., M. Yuan, X. Gao, T. Kang, S. Zhan, H. Wan, and J. Li. **2013**. Identification and validation of reference genes for gene expression analysis using quantitative PCR in *Spodoptera litura* (Lepidoptera: Noctuidae). PLoS One. 8: e68059.

Ma, K.S., F. Li, P.Z. Liang, X.W. Chen, Y. Liu, and X.W. Gao. **2016**. Identification and validation of reference genes for the normalization of gene expression data in qRT-PCR analysis in *aphis gossypii* (Hemiptera: Aphididae). J. Insect Sci. 16.

MacKenzie, T.D.B., I. Arju, R. Poirier, and M. Singh. **2018**. A genetic survey of pyrethroid insecticide resistance in aphids in New Brunswick, Canada, with particular emphasis on aphids as vectors of potato virusy. J. Econ. Entomol. 111: 1361–1368.

Mamidala, P., S.P. Rajarapu, S.C. Jones, and O. Mittapalli. **2011**. Identification and validation of reference genes for quantitative real-time polymerase chain reaction in *Cimex lectularius*. J. Med. Entomol. 48: 947–951.

Marshall, K.L., C. Moran, Y. Chen, and G.A. Herron. **2012**. Detection of kdr pyrethroid resistance in the cotton aphid, Aphis gossypii (Hemiptera: Aphididae), using a PCR-RFLP assay. J. Pestic. Sci. 37: 169–172.

Mayo, O. 2008. A Century of Hardy – Weinberg Equilibrium. 11: 249–256.

McCarville, M.T., and M.E. O'Neal. **2013**. Soybean Aphid (Aphididae : Hemiptera) Population Growth as Affected by Host Plant Resistance and an Insecticidal Seed Treatment. J. Econ. Entomol. 1302–1309.

Mccornack, B.P., M.A. Carrillo, R.C. Venette, and D.W. Ragsdale. **2005**. Physiological constraints on the overwintering potential of the soybean aphid (Homoptera: Aphididae). Environ. Entomol. 34: 235–240.

McCornack, B.P., and D.W. Ragsdale. **2006**. Efficacy of Thiamethoxam to Suppress Soybean Aphid Populations in Minnesota Soybean. Crop Manag. 5: 1–8.

McIntosh, C.H., J. Baird, E. Zinser, D.J. Woods, E.M. Campbell, and A.S. Bowman. **2016**. Reference gene selection and RNA preservation protocol in the cat flea, *Ctenocephalides felis*, for gene expression studies. Parasitology. 143: 1532–1542.

Melander, A.L. 1914. Can insects become resistant to sprays. J. Econ. Entomol. 7: 167.

Menger, J., P. Beauzay, A. Chirumamilla, C. Dierks, J. Gavloski, P. Glogoza, K. Hamilton, E.W. Hodgson, J.J. Knodel, I. V MacRae, D.T. Pezzini, B.D. Potter, A.J. Varenhorst, and R.L. Koch. **2020**. Implementation of a diagnostic-concentration bioassay for detection of susceptibility to pyrethroids in soybean aphid (Hemiptera: Aphididae). J. Econ. Entomol. 113: 932–939.

Menger, J.P., A. V. Ribeiro, B.D. Potter, and R.L. Koch. **2022a**. Changepoint analysis of lambda-cyhalothrin efficacy against soybean aphid (*Aphis glycines* Matsumura): Identifying practical resistance from field efficacy trials. Pest Manag. Sci. 78: 3638–3643.

Menger, J.P., A. V Ribeiro, B.D. Potter, I. Valmorbida, E.W. Hodgson, J.J. Knodel, R.L. Koch, and F. Musser. **2022b**. Lack of evidence for fitness costs in soybean aphid (Hemiptera: Aphididae) withresistance to pyrethroid insecticides in the upper Midwest region of the United States. J. Econ. Entomol. 115: 1191–1202.

Milani, R. 1954. Comportamento mendeliano della resistenza alla azione abbattante del DDT: correlazione abbattimento e mortalia in *Musca domestica* L. Riv. Parasitol. 15: 513–542.

Minnesota Invasive Terrestrial Plants and Pests Center (MITPPC). 2016. Minnesota's top 124 terrestrial invasive plants and pests: priorities for research. https://conservancy.umn.edu/bitstream/handle/11299/254604/MITPPC_top_124_research_priorities.pdf?sequence=1&isAllowed=y. Accessed: 2023-07-25

Nauen, R., C. Bass, R. Feyereisen, and J. Vontas. **2022**. The role of cytochrome P450s in insect toxicology and resistance. Annu. Rev. Entomol. 67: 105–124.

Nelson, D.R. 2018. Cytochrome P450 diversity in the tree of life. BBA - Proteins Proteomics. 1866: 141–154.

Nicholls, C., H. Li, and J.P. Liu. **2012**. GAPDH: A common enzyme with uncommon functions. Clin. Exp. Pharmacol. Physiol. 39: 674–679.

Niwa, R., and Y.S. Niwa. **2014**. Enzymes for ecdysteroid biosynthesis: Their

biological functions in insects and beyond. Biosci. Biotechnol. Biochem. 78: 1283–1292.

Oakeshott, J.G., C. Claudianos, P.M. Campbell, R.D. Newcomb, and R.J. Russell. **2005**. Biochemical Genetics and Genomics of Insect Esterases. Compr. Mol. Insect Sci. 5–6: 309–381.

Olson, K., T. Badibanga, and C. DiFonzo. **2008**. Farmers ' Awareness and Use of IPM for Soybean Aphid. Dep. Appl. Econ. Univ. Minnesota. 31.

Ormsby T., E. Napoleon, R. Burke, C. Groessl. 2009. Getting to Know ArcGIS Desktop. Updated for ArcGIS 9.3. Basics of ArcView, ArcEditor, ArcInfo. ESRI Press

Paim, R.M., M.H. Pereira, R. Di Ponzio, J.O. Rodrigues, A.A. Guarneri, N.F. Gontijo, and R.N. Araújo. **2012**. Validation of reference genes for expression analysis in the salivary gland and the intestine of *Rhodnius prolixus* (Hemiptera, Reduviidae) under different experimental conditions by quantitative real-time PCR. BMC Res. Notes. 5: 128.

Panini, M., G.C. Manicardi, G.D. Moores, and E. Mazzoni. **2016**. An overview of the main pathways of metabolic resistance in insects. Invertebr. Surviv. J. 13: 326–335.

Paula, D.P., R.E. Lozano, J.P. Menger, D.A. Andow, and R.L. Koch. **2021**. Identification of point mutations related to pyrethroid resistance in voltage-gated sodium channel genes in *Aphis glycines*. Entomol. Gen. 41: 243–255.

Paula, D.P., J. Menger, D.A. Andow, and R.L. Koch. **2020**. Diverse patterns of constitutive and inducible overexpression of detoxifying enzyme genes among resistant *Aphis glycines* populations. Pestic. Biochem. Physiol. 164: 100–114.

Pearson, K. 1901. On lines and planes of closest fit to systems of points in space. Philos. Mag. 2: 559–572.

Penn, H.J., J. Penn, M. Cunningham-Minnick, and W. Hu. **2021**. Kentucky Soybean Farmers' Supportiveness of Two Integrated Pest and Pollinator Management Tactics. J. Integr. Pest Manag. 12.

Perrin, B.J., and J.M. Ervasti. **2010**. The actin gene family: function follows isoform. Cytoskeleton. 67: 630–634.

Peterson, R.K.D., L.G. Higley, and L.P. Pedigo. **2018**. Whatever Happened to IPM. Am. Entomol. 64: 146–150.

Pfaffl, M.W., A. Tichopad, C. Prgomet, and T. Neuvians. **2004**. Determination of most stable housekeeping genes, differentially regulated target genes and sample integrity : BestKeeper. Biotechnol. Lett. 26: 509–515.

Phillips, J.R., J.B. Graves, and R.G. Luttrell. **1989**. Insecticide resistance management: Relationship to integrated pest management. Pestic. Sci. 27: 459–464.

Pinto, J., A. Lynd, N. Elissa, M.J. Donnelly, C. Costa, G. Gentile, A. Caccone, and V.E. Do Rosário. **2006**. Co-occurrence of East and West African kdr mutations suggests high levels of resistance to pyrethroid insecticides in *Anopheles gambiae* from Libreville, Gabon. Med. Vet. Entomol. 20: 27–32.

Platt, N., R.M. Kwiatkowska, H. Irving, A. Diabaté, R. Dabire, and C.S. Wondji. **2015**. Target-site resistance mutations (kdr and RDL), but not metabolic resistance, negatively impact male mating competiveness in the malaria vector *Anopheles gambiae*. Heredity (Edinb). 115: 243–252.

Plocik, A.M., and C. Guthrie. **2012**. Diverse forms of RPS9 splicing are part of

an evolving autoregulatory circuit. PLoS Genet. 8: e1002620.

Ponton, F., M.P. Chapuis, M. Pernice, G.A. Sword, and S.J. Simpson. **2011**. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. J. Insect Physiol. 57: 840–850.

Poplin, R., M.A. Ruano-Rubio, Valentin DePristo, T.J. Fennell, M. O Carneiro, G.A. Van der Auwera, D.E. Kling, L.D. Gauthier, A. Levy-Moonshine, D. Roazen, K. Shakir, T. Joel, C. Sheila, W. Chris, L. Monkol, G. Stacey, J.D. Mark, N. Ben, G.M. Daniel, and B. Eric. **2018**. Scaling accurate genetic variant discovery to tens of thousands of samples. BioRxIV. 1–22.

Quartagno, M., and J.R. Carpenter. **2019**. Multiple imputation for discrete data : evaluation of the joint latent normal model. Biometrical J. 61: 1003–1019.

Radonić, A., S. Thulke, I.M. Mackay, O. Landt, W. Siegert, and A. Nitsche. **2004**. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313: 856–862.

Raghavendra, K., P.S. Velamuri, V. Verma, N. Elamathi, T.K. Barik, R.M. Bhatt, and A.P. Dash. **2017**. Temporo-spatial distribution of insecticide-resistance in indian malaria vectors in the last quarter-century: Need for regular resistance monitoring and management. J. Vector Borne Dis. 54: 111–130.

Ragsdale, D.W., D.A. Landis, J. Brodeur, G.E. Heimpel, and N. Desneux. **2011**. Ecology and management of the soybean aphid in North America. Annu. Rev. Entomol. 56: 375–399.

Ragsdale, D.W., B.P. McCornack, R.C. Venette, B.D. Potter, I. V. Macrae, E.W. Hodgson, M.E. O'Neal, K.D. Johnson, R.J. O'Neil, C.D. Difonzo,

T.E. Hunt, P.A. Glogoza, and E.M. Cullen. **2007**. Economic Threshold for Soybean Aphid (Hemiptera: Aphididae). J. Econ. Entomol. 100: 1258–1267.

Ragsdale, D.W., D.J. Voegtlin, and R.J. O'Neil. **2004**. Soybean aphid biology in North America. Ann. Entomol. Soc. Am. 97: 204–208.

Ranson, H., and J. Hemingway. **2005**. Glutathione Transferases. Compr. Mol. Insect Sci. 5–6: 383–402.

Rasmussen, K.K., and J. Kollmann. **2008**. Low genetic diversity in small peripheral populations of a rare European tree (Sorbus torminalis) dominated by clonal reproduction. Conserv. Genet. 9: 1533–1539.

Rewitz, K.F., R. Rybczynski, J.T. Warren, and L.I. Gilbert. **2006**. The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. Biochem. Soc. Trans. 34: 1256–1260.

Rödiger, S., C. Liebsch, C. Schmidt, W. Lehmann, U. Resch-Genger, U. Schedler, and P. Schierack. **2014**. Nucleic acid detection based on the use of microbeads: A review. Microchim. Acta. 181: 1151–1168.

Rodrigues, T.B., C. Khajuria, H. Wang, N. Matz, D.C. Cardoso, F.H. Valicente, X. Zhou, and B. Siegfried. **2014**. Validation of reference housekeeping genes for gene expression studies in Western corn rootworm (*Diabrotica virgifera virgifera*). PLoS One. 9: e109825.

Roush, R.T., and J.A. McKenzie. **1987**. Ecological genetics of insecticide and acaricide resistance. Annu. Rev. Entomol. Vol. 32. 361–380.

Roush, R.T., and G.L. Miller. **1986**. Considerations for Design of Insecticide Resistance Monitoring Programs. J. Econ. Entomol. 79: 293–298.

Ruijter, J.M., C. Ramakers, W.M.H. Hoogaars, Y. Karlen, O. Bakker, M.J.B. van den hoff, and A.F.M. Moorman. **2009**. Amplification efficiency: linking

baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37: e45.

Saha, D. 2016. Biochemical Insecticide Resistance in Tea Pests. Insectic. Resist. chapter 18: 347–390.

Saingamsook, J., A. Saeung, J. Yanola, N. Lumjuan, C. Walton, and P. Somboon. **2017**. A multiplex PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid-resistant Aedes aegypti. Parasites and Vectors. 10: 1–9.

Sawicki, R.M. 1978. Unusual response of DDT-resistant houseflies to carbinol analogues of DDT. Nature. 275: 443–444.

Scharf, M.E., S. Parimi, L.J. Meinke, L.D. Chandler, and B.D. Siegfried. **2001**. Expression and induction of three family 4 cytochrome P450 (CYP4) genes identified from insecticide-resistant and susceptible western corn rootworms, *Diabrotica virgifera virgifera*. Insect Mol. Biol. 10: 139–146.

Scharlaken, B., D.C. de Graaf, K. Goossens, M. Brunain, L.J. Peelman, and F.J. Jacobs. **2008**. Reference gene selection for insect expression studies using quantitative real-time PCR: the head of the honeybee, *Apis mellifera* , after a bacterial challenge. J. Insect Sci. 8: 33.

Schmidt, N.P., M.E. O'Neal, P.F. Anderson, D. Lagos, D. Voegtlin, W. Bailey, P. Caragea, E. Cullen, C. Difonzo, K. Elliott, C. Gratton, D. Johnson, C.H. Krupke, B. McCornack, R. O'Neil, D.W. Ragsdale, K.J. Tilmon, and J. Whitworth. **2012**. Spatial distribution of a*phis glycines* (Hemiptera: Aphididae): A summary of the suction trap network. J. Econ. Entomol. 105: 259–271.

Seagraves, M.P., and J.G. Lundgren. **2012**. Effects of neonicitinoid seed treatments on soybean aphid and its natural enemies. J. Pest Sci. (2004).

85: 125–132.

Shang, F., D.D. Wei, X.Z. Jiang, D. Wei, G.M. Shen, Y.C. Feng, T. Li, and J.J. Wang. **2015**. Reference gene validation for quantitative pcr under various biotic and abiotic stress conditions in *Toxoptera citricida* (Hemiptera, Aphidiae). J. Econ. Entomol. 108: 2040–2047.

Sharma, H.C. 2007. Host plant resistance to insects, modern approaches and limitations.

da Silva Queiroz, O., A.A. Hanson, and R.L. Koch. **2020**. Effects of aphid-resistant soybean on nontarget herbivores in Minnesota. Arthropod. Plant. Interact. 14: 79–87.

Shahbandeh, M. 2023a. Major soybean producing U.S. states from 2018 to 2022. Statista. https://www.statista.com/statistics/192076/top-10-soybean-producing-us-states/. Accessed: 2023-07-25.

Shahbandeh, M. 2023b. Leading soybean producing countries worldwide from 2012/13 to 2022/23. https://www.statista.com/statistics/263926/soybean-production-in-selected-countries-since-1980/. Accessed: 2023-07-25.

Silva, W.M., M. Berger, C. Bass, V.Q. Balbino, M.H.P. Amaral, M.R. Campos, and H.A.A. Siqueira. **2015**. Status of pyrethroid resistance and mechanisms in Brazilian populations of *Tuta absoluta*. Pestic. Biochem. Physiol. 122: 8–14.

Simon, J. Y. 2011. The toxicology and biochemistry of insecticides. CRC press.

Sinha, D.K., and C.M. Smith. **2014**. Selection of reference genes for expression analysis in *diuraphis noxia* (Hemiptera: Aphididae) fed on resistant and susceptible wheat plants. Sci. Rep. 4: 5059.

Smitha, P.K., K. Vishnupriyan, A.S. Kar, M. Anil Kumar, C. Bathula, K.N.

Chandrashekara, S.K. Dhar, and M. Das. **2019**. Genome wide search to identify reference genes candidates for gene expression analysis in *Gossypium hirsutum*. BMC Plant Biol. 19: 1–11.

Soderlund, D.M. 2005. Sodium Channels. Compr. Mol. Insect Sci. 5–6: 1–24.

Soderlund, D.M. 2010. Toxicology and Mode of Action of Pyrethroid Insecticides, Third Edit. ed, Hayes' Handb. Pestic. Toxicol. Elsevier Inc.

Soderlund, D.M. 2012. Molecular mechanisms of pyrethroid insecticide neurotoxicity: Recent advances. Arch. Toxicol. 86: 165–181.

Soderlund, D.M., and J.R. Bloomquist. **1989**. Neurotoxic actions of pyrethroid insecticides. Annu. Rev. Entomol. 34: 77–96.

Soderlund, D.M., and J.R. Bloomquist. **1990**. Pesticide Resistance in Arthropods.

Soderlund, D.M., and D.C. Knipple. **1999**. Knockdown resistance to DDT and pyrethroids in the house fly (Diptera: Muscidae): From genetic trait to molecular mechanism. Ann. Entomol. Soc. Am. 92: 909–915.

Song, F., S.M. Swinton, C. DiFonzo, M. O'Neal, David, and Ragsdale. **2006**. Profitability Analysis Of Soybean Aphid Control Treatments In Three Northcentral States. Michigan State Univ. 1–20.

Sonoda, S. 2010. Molecular analysis of pyrethroid resistance conferred by target insensitivity and increased metabolic detoxification in *Plutella xylostella*. Pest Manag. Sci. 66: 572–575.

Sparks, T.C., and R. Nauen. **2015**. IRAC : Mode of action classification and insecticide resistance management. Pestic. Biochem. Physiol. 121: 122–128.

Stern, C. 1943. The hardy-weinberg law. Science (80-.). 137–138.

Sun, H., B. Yang, Y. Zhang, and Z. Liu. **2017**. Metabolic resistance in *Nilaparvata lugens* to etofenprox, a non-ester pyrethroid insecticide. Pestic. Biochem. Physiol. 136: 23–28.

Sun, W., Y. Jin, L. He, W.-C. Lu, and M. Li. **2010**. Suitable reference gene selection for different strains and developmental stages of the carmine spider mite, *Tetranychus cinnabarinus*, using quantitative real-time PCR. J. Insect Sci. 10: 208.

Tabashnik, B.E., D. Mota-Sanchez, M.E. Whalon, R.M. Hollingworth, and Y. Carrière. **2014**. Defining terms for proactive management of resistance to Bt crops and pesticides. J. Econ. Entomol. 107: 496–507.

Thellin O., Zorzia W., Lakaye B., De Borman B., Coumans B., Hennen G., Grisar T., Igout A., and Heinena E. 1999. Housekeeping genes as internal standards: use and limits. J. Biotechnol. 75: 291–295.

Tilmon, K.J., E.W. Hodgson, M.E. O'Neal, and D.W. Ragsdale. **2011**. Biology of the soybean aphid, *Aphis glycines* (Hemiptera: Aphididae) in the United States. J. Integr. Pest Manag. 2.

Tilmon, K.J., A. Michel, and M.E. O'Neal. **2021**. Aphid resistance is the future for soybean production, and has been since 2004: efforts towards a wider use of host plant resistance in soybean. Curr. Opin. Insect Sci. 45: 53–58.

Tooker, J.F., M.R. Douglas, and C.H. Krupke. **2017**. Neonicotinoid Seed Treatments: Limitations and Compatibility with Integrated Pest Management. Agric. Environ. Lett. 2: 1–5.

Tossou, E., I. Djegbe, F. Zeukeng, and R. Akoton. **2019**. Inhibitory effect of PBO and DEF on detoxification mechanisms involved in deltamethrin

desistance in Helicoverpa armigera (Hübner) (Lepidoptera : Noctuidae) in Benin, West Africa. Eur. J. Sci. Res. 154: 385–398.

United States Environmental Protection Agency (EPA). 2022. Chlorpyrifos. https://www.epa.gov/ingredients-used-pesticide-products/chlorpyrifos. Accessed: 2023-05-03.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, and S.G. Rozen. **2012**. Primer3-new capabilities and interfaces. Nucleic Acids Res. 40: 1–12.

USDA National Agricultural Statistics Service. 2023a. Minnesota Ag News-2022 Soybean County estimates. https://www.nass.usda.gov/Statistics_by_State/Minnesota/Publications/County_Estimates/2023/MN-CtyEst-Soybeans-02-23.pdf. Accessed: 2023-05-03.

USDA National Agricultural Statistics Service. 2023b. NASS - Quick Stats Ad-hoc Query Tool. https://quickstats.nass.usda.gov/results/9F2BB18D-415E-348E-BD13-80E1798C8022. Accessed: 2023-19-07

USDA National Agricultural Statistics Service. 2023c. Statistics by subject results. https://www.nass.usda.gov/Statistics_by_Subject/result.php?64D20FB3-8DF5-35B9-A9AA-1A2FF7F7B232&sector=CROPS&group=FIELD%20CROPS&comm=SOYBEANS. Accessed: 2023-19-07

Valmorbida, I., B.S. Coates, E.W. Hodgson, M. Ryan, and M.E. O'Neal. **2022a**. Evidence of enhanced reproductive performance and lack-of-fitness costs among soybean aphids, *Aphis glycines* , with varying levels of pyrethroid resistance. Pest Manag. Sci. 0–2.

Valmorbida, I., J.D. Hohenstein, B.S. Coates, J.G. Bevilaqua, J. Menger,

E.W. Hodgson, R.L. Koch, and M.E.O. Neal. **2022b**. Association of voltage - gated sodium channel mutations with field - evolved pyrethroid resistant phenotypes in soybean aphid and genetic markers for their detection. Sci. Rep. 1–14.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and S. Frank. **2002**. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3: 1–12.

VanGuilder, H.D., K.E. Vrana, and W.M. Freeman. **2008**. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques. 44: 619–626.

Van den Berg, J., Greyvenstein, B., & du Plessis, H. 2022. Insect resistance management facing African smallholder farmers under climate change. Curr. Opin. Insect. Sci. 50: 100894.

Venette, R.C., and D.W. Ragsdale. **2004**. Assessing the invasion by soybean aphid (Homoptera: Aphididae): Where will it end? Ann. Entomol. Soc. Am. 97: 219–226.

Wang, C.L., N.I. Siang, G.S. Chang, and H.F. Chu. **1962**. Studies on the soybean aphid, *Aphis glycines* Matsumura. Acta Entomol. Sin. 11: 31–43.

Wang, R.Y., and S.A. Ghabrial. **2002**. Effect of aphid behavior on efficiency of transmission of Soybean mosaic virus by the soybean-colonizing aphid, *Aphis glycines*. Plant Dis. 86: 1260–1264.

Wenger, J.A., B.J. Cassone, F. Legeai, J.S. Johnston, R. Bansal, A.D. Yates, B.S. Coates, V.A.C. Pavinato, and A. Michel. **2017**. Whole genome sequence of the soybean aphid, *Aphis glycines*. Insect Biochem. Mol. Biol. 123: 102917.

Williamson, M.S., D. Martinez-Torres, C.A. Hick, and A.L. Devonshire. **1996**. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. Mol. Gen. Genet. 252: 51–60.

Wu, K., W. Liu, T. Mar, Y. Liu, Y. Wu, and X. Wang. **2014**. Sequencing and validation of reference genes to analyze endogenous gene expression and quantify yellow dwarf viruses using RT-qPCR in viruliferous *Rhopalosiphum padi*. PLoS One. 9: e97038.

Wu, Z., D. Schenk-Hamlin, W. Zhan, D.W. Ragsdale, and G.E. Heimpel. **2004**. The soybean aphid in China: A historical review. Ann. Entomol. Soc. Am. 97: 209–218.

Xi, J., Y. Pan, R. Bi, X. Gao, X. Chen, T. Peng, M. Zhang, H. Zhang, X. Hu, and Q. Shang. **2015**. Elevated expression of esterase and cytochrome P450 are related with lambda-cyhalothrin resistance and lead to cross resistance in *Aphis glycines* Matsumura. Pestic. Biochem. Physiol. 118: 77–81.

Xu, Q., L. Tain, L. Zhang4, and N. Liu. **2012**. Sodium Channel Genes and Their Differential Genotypes at the L-to-F Kdr Locus in the Mosquito *Culex quinquefasciatus*. Biochem Biophys Res Commun. 23: 1–7.

Yainna, S., N. Nègre, P.J. Silvie, T. Brévault, W.T. Tay, K. Gordon, E. Dalençon, T. Walsh, and K. Nam. **2021**. Geographic monitoring of insecticide resistance mutations in native and invasive populations of the fall armyworm. Insects. 12: 1–12.

Yan, S., F. Cui, and C. Qiao. **2009**. Structure, function and applications of carboxylesterases from insects for insecticide resistance. Protein Pept. Lett. 16: 1181–1188.

Yang, C., H. Pan, Y. Liu, and X. Zhou. **2014**. Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, *Acyrthosiphon pisum* (Harris) (hemiptera, aphidiae). PLoS One. 9: e110454.

Yang, C., H. Pan, Y. Liu, and X. Zhou. **2015**. Temperature and development impacts on housekeeping gene expression in cowpea aphid, *Aphis craccivora* (Hemiptera: Aphidiae). PLoS One. 10: e0130593.

Yang, Q., Z. Li, J. Cao, S. Zhang, H. Zhang, X. Wu, Q. Zhang, and X. Liu. **2014**. Selection and assessment of reference genes for quantitative PCR normalization in migratory locust *Locusta migratoria* (Orthoptera: Acrididae). PLoS One. 9: e98164.

Yang, Y., S. Chen, S. Wu, L. Yue, and Y. Wu. **2006**. Constitutive Overexpression of Multiple Cytochrome P450 Genes Associated with Pyrethroid Resistance in Helicoverpa armigera. J. Econ. Entomol. 99: 1784–1789.

Yu S.J. 2011. The toxicology and biochemistry of insecticides. CRC press Inc. Boca Raton, FL. p 358.

Zalucki, M.P., D. Adamson, and M.J. Furlong. **2009**. The future of IPM: Whither or wither?: Overview. Aust. J. Entomol. 48: 85–96.

Zhai, Y., Q. Lin, X. Zhou, X. Zhang, T. Liu, and Y. Yu. **2014**. Identification and validation of reference genes for quantitative real-time PCR in *Drosophila suzukii*. PLoS One. 9: e106800.

Zhou, G., T. Somasundaram, E. Blanc, G. Parthasarathy, W.R. Ellington, and M.S. Chapman. **1998**. Transition state structure of arginine kinase: implications for catalysis of bimolecular reactions. Proc. Natl. Acad. Sci. U. S. A. 95: 8449–8454.

Zhou, W., S. Wang, L. Yang, Y. Sun, Q. Zhang, B. Li, B. Wang, L. Li, D. Wang, and Z. Wang. **2019**. Reference genes for qRT-PCR normalisation in different tissues, developmental stages, and stress conditions of *Hypericum perforatum*. PeerJ. 2019: 1–17.

Zhou, X., C. Yang, N. Liu, M. Li, Y. Tong, X. Zeng, and X. Qiu. **2019b**. Knockdown resistance (kdr) mutations within seventeen field populations of Aedes albopictus from Beijing China: First report of a novel V1016G mutation and evolutionary origins of kdr haplotypes. Parasites and Vectors. 12: 1–16.

Zimmer, C.T., A. Müller, U. Heimbach, and R. Nauen. **2014**. Target-site resistance to pyrethroid insecticides in German populations of the cabbage stem flea beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae). Pestic. Biochem. Physiol. 108: 1–7.

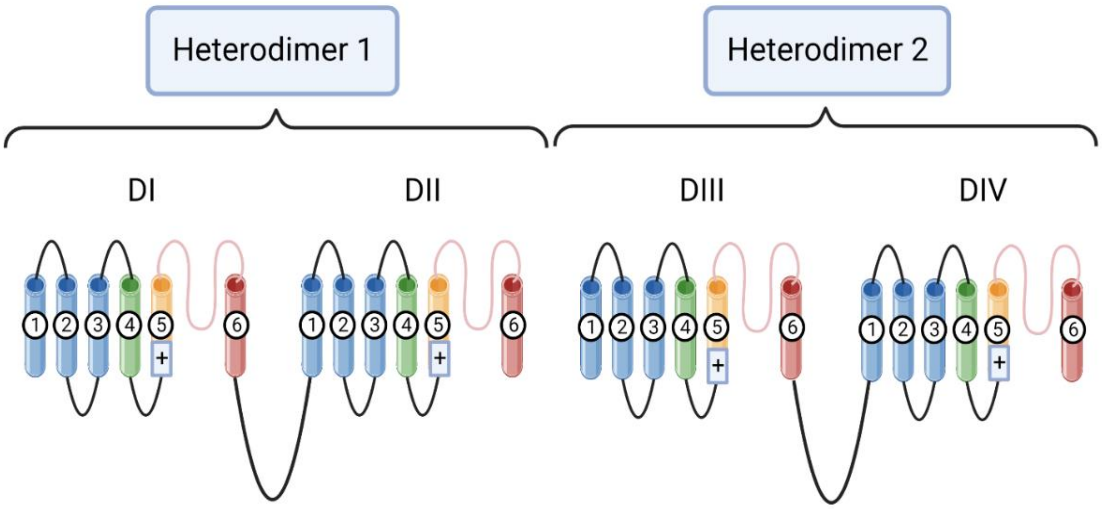
Appendices

Appendix A**.** Supplementary information for Chapter I. Hardy-Weinberg Equilibrium (HWE) genotype frequencies and test statistics for each VGSC-II variant identified in *Aphis glycines* from 32 field populations sampled in Minnesota-US from 2019 to 2021. Heterozygous, homozygous reference allele genotypes (Homozygous 1), homozygous alternate allele genotypes (Homozygous 2), and Chi-square test (ChiSq).

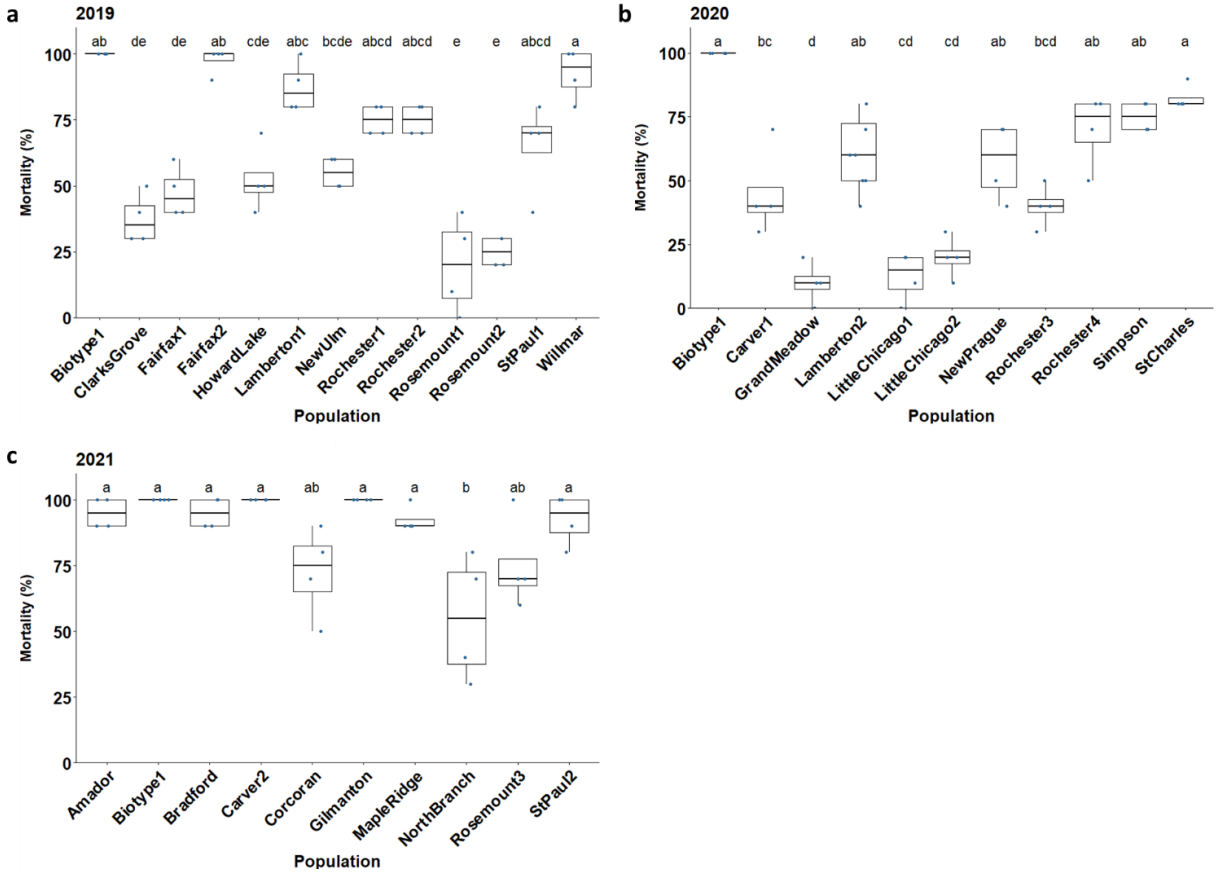
Musca domestica Amino Acid Position*	Observed frequency (Homozygous 1/ Heterozygous / Homozygous 2)	Expected frequency (Homozygous 1/ Heterozygous / Homozygous 2)	ChiSq	*p*-value	*p*-value Heterozygous Deficit	*p*- value Heterozygous Excess
905	469/1/0	469.00/1.00/0.00	5.33E-04	1.00E+00	1.00E+00	1.00E+00
906	469/1/0	469.00/1.00/0.00	5.33E-04	1.00E+00	1.00E+00	1.00E+00
908	468/2/0	468.00/2.00/0.00	2.14E-03	1.00E+00	1.00E+00	9.99E-01
913	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
918	463/5/0	463.01/4.97/0.01	1.35E-02	1.00E+00	1.00E+00	9.89E-01
918	342/113/11	340.78/115.45/9.78	2.09E-01	5.79E-01	3.73E-01	7.54E-01
922	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
925	362/93/10	358.87/99.27/6.87	1.85E+00	1.89E-01	1.23E-01	9.40E-01
925	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
934	467/2/0	467.00/2.00/0.00	2.14E-03	1.00E+00	1.00E+00	9.99E-01
939	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
942	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00

943	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
945	466/3/0	466.00/2.99/0.00	4.83E-03	1.00E+00	1.00E+00	9.97E-01
953	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
953	468/1/0	468.00/1.00/0.00	5.34E-04	1.00E+00	1.00E+00	1.00E+00
958	467/1/0	467.00/1.00/0.00	5.35E-04	1.00E+00	1.00E+00	1.00E+00
964	467/0/1	466.00/2.00/0.00	4.68E+02	1.07E-03	1.07E-03	1.00E+00
965	467/1/0	467.00/1.00/0.00	5.35E-04	1.00E+00	1.00E+00	1.00E+00
969	465/1/1	464.00/2.99/0.00	2.07E+02	3.22E-03	3.22E-03	1.00E+00
970	467/1/0	467.00/1.00/0.00	5.35E-04	1.00E+00	1.00E+00	1.00E+00
984	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
984	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
984	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
991	469/2/0	469.00/2.00/0.00	2.13E-03	1.00E+00	1.00E+00	9.99E-01
997	468/2/1	467.01/3.98/0.01	1.17E+02	6.37E-03	6.37E-03	1.00E+00
998	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
999	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
1004	468/2/1	467.01/3.98/0.01	1.17E+02	6.37E-03	6.37E-03	1.00E+00
1012	470/1/0	470.00/1.00/0.00	5.32E-04	1.00E+00	1.00E+00	1.00E+00
1014	214/223/33	225.43/200.15/44.43	6.13E+00	1.69E-02	9.95E-01	8.75E-03
1016	0/0/467	0.00/0.00/467.00	-nan	1.00E+00	1.00E+00	1.00E+00
1020	465/5/0	465.01/4.97/0.01	1.34E-02	1.00E+00	1.00E+00	9.89E-01

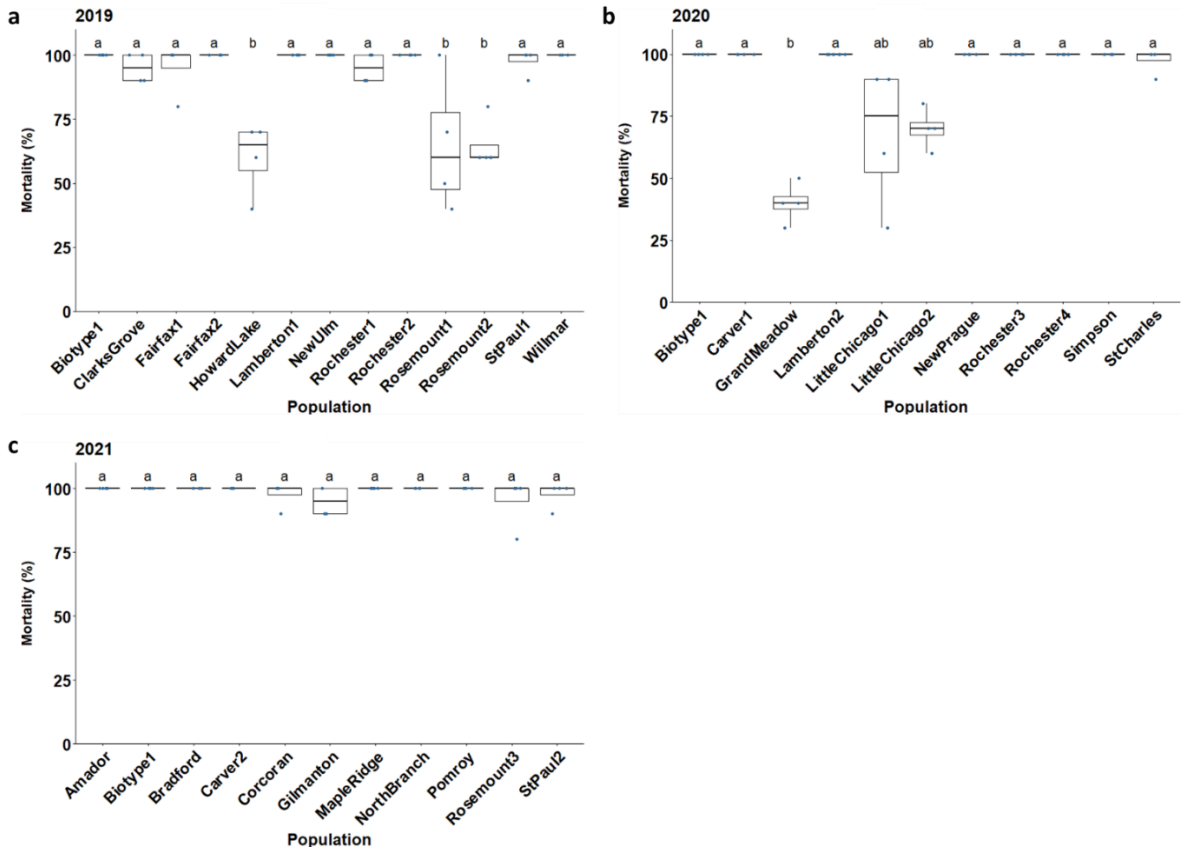
**Musca domestica* amino acid position used for *kdr* nomenclature (GenBank: U38813)
-nan = SNP is monomorphic (mono-allelic).



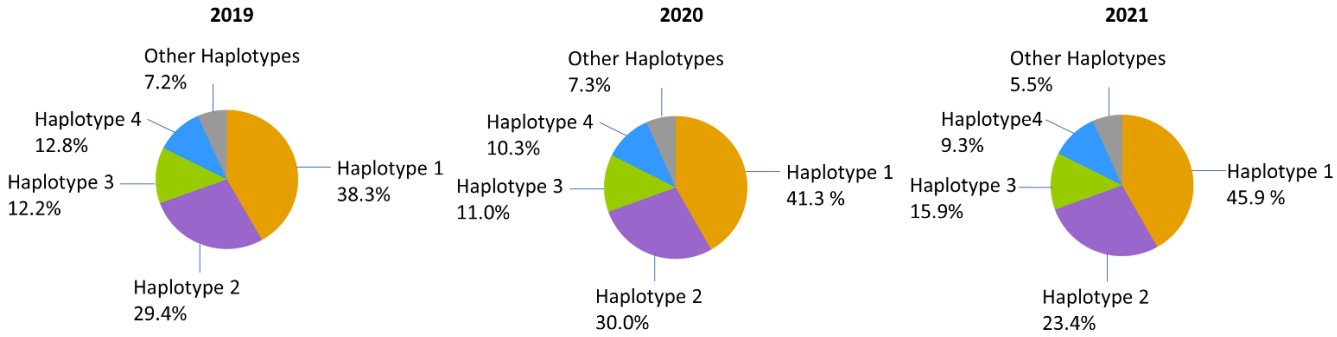
Appendix B. Supplementary information for Chapter I. Representation of the voltage gated sodium channel showing the four repeated domains (I–IV) and the six hydrophobic transmembrane segments. Adapted from Soderlund and Knipple (2003).



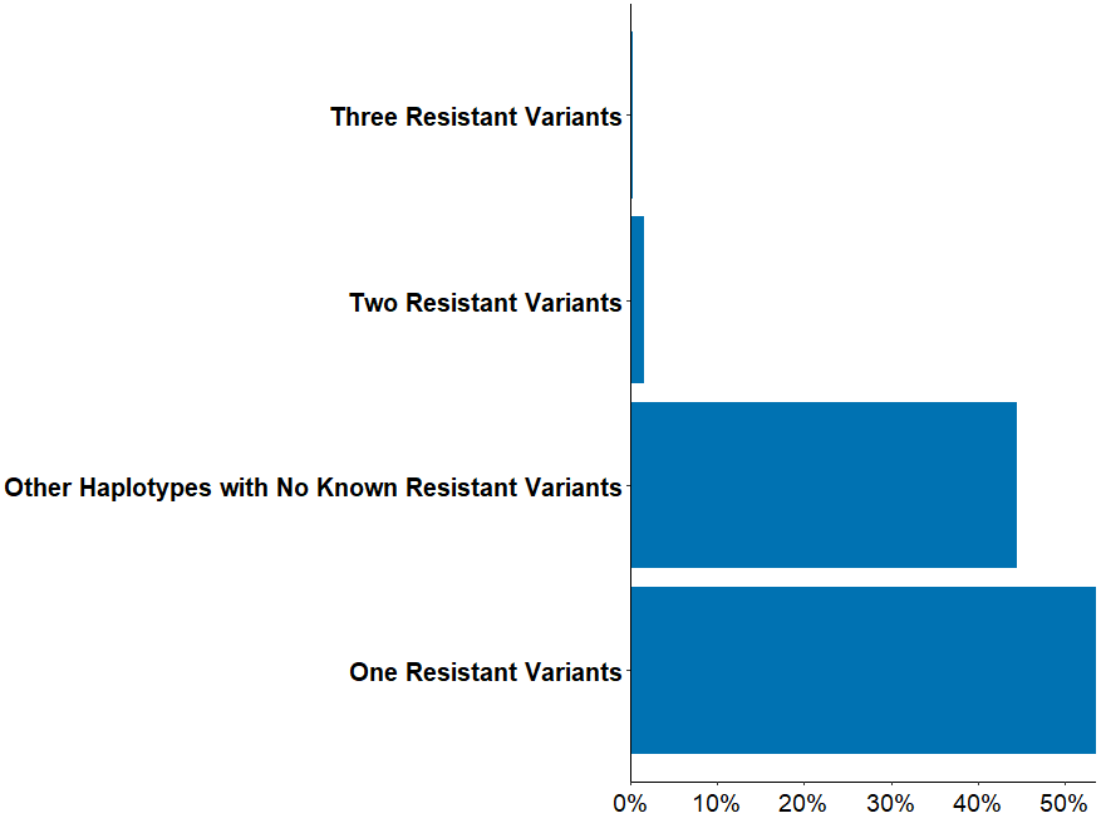
Appendix C. Supplementary information for Chapter I. Bifenthrin mortality of an *Aphis glycines* susceptible laboratory population (i.e., biotype 1) and multiple field-collected populations from Minnesota-US in 2019, 2020, or 2021. Different letters denote significant differences (P<0.05) among populations within years based on ANOVA and Tukey test.



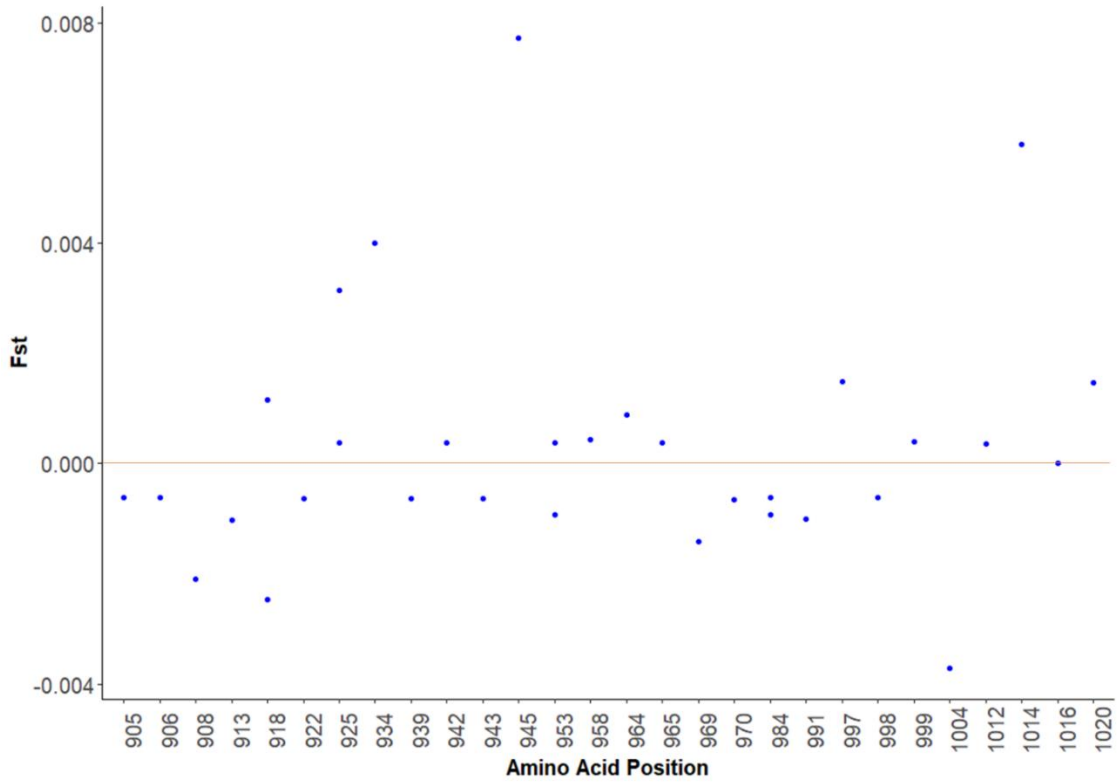
Appendix D. Supplementary information for Chapter I. Lambda cyhalothrin mortality of an *Aphis glycines* susceptible laboratory population (i.e., biotype 1) and multiple field-collected populations from Minnesota-US in 2019, 2020, or 2021. Different letters denote significant differences (P<0.05) among populations within years based on ANOVA and Tukey test.



Appendix E. Supplementary information for Chapter I. VGSC-II haplotype frequencies in the 32 field populations of *Aphis glycines* from Minnesota-US in 2019, 2020, or 2021.



Appendix F. Supplementary information for Chapter I. VGSC-II haplotype frequencies of known pyrethroid resistance variants in 32 field populations of *Aphis glycines* from Minnesota-US in 2019, 2020, or 2021.



Appendix G. Supplementary information for Chapter I. Comparison of the fixation index (F_{ST}) between VGSC-II amino acid positions where the 33 variants were identified in 32 field populations of *Aphis glycines* from Minnesota-US sampled from 2019 to 2021. A FST of 0 indicates no differentiation. Each data point in the graph represents the average of allele frequencies between years.