Exploring the mechanisms and consequences of soybean aphid (Hemiptera: Aphididae) resistance to pyrethroids

by

Ivair Valmorbida

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Program of Study Committee:
Matthew E. O'Neal, Major Professor
Erin W. Hodgson, Major Professor
Brad S. Coates
David Onstad
Joel R. Coats

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

I am dedicating this dissertation to my wife, Tanise Coppetti, and our son, Theodoro C. Valmorbida. Tanise and I moved to Iowa in 2018, and in September 2021, Theodoro was born. Tanise has been endlessly supportive through my educational journey at Iowa State University. Most importantly, she has been such a strong, dedicated and attentive mother. I would never been able to accomplish what I have so far without her support and encouragement.

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ABSTRACT

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), was first detected in the United States in 2000 and has been actively managed by farmers with foliar insecticide applications. Bifenthrin and lambda-cyhalothrin are insecticides commonly used to control soybean aphid outbreaks. However, control failures were observed and laboratory bioassays confirmed that soybean aphid has evolved resistance to pyrethroids. This research investigated the susceptibility of virulent and avirulent aphids to lambda-cyhalothrin and the mechanisms and fitness costs related with pyrethroid-resistant soybean aphids. Leaf dip bioassays were performed to assess the susceptibility of laboratory and field-collected populations to insecticides. Also, molecular markers were developed to assess the frequency of resistant alleles before and after a foliar insecticide application. Toxicity bioassays revealed that virulent aphids had a higher LC_{50} compared with virulent aphids, and exposure to the LC_{25} of lambda-cyhalothrin can trigger hormesis in soybean aphids. These bioassays also confirmed that field-collected aphids have evolved resistance to pyrethroids and sequencing of the voltage-gated sodium channel (*vgsc*) genes identified non-synonymous mutations associated with resistant aphids. Additionally, molecular markers revealed a high frequency of aphids carrying at least one mutation in the *vgsc*, and foliar insecticide application significantly increased resistant allele frequency among survivors. Experiments using isofemale lines with varying levels of pyrethroid resistance did not find evidence of fitness cost. Field-evolved soybean aphid resistance to pyrethroids is concerning and will require the development and implementation of integrated pest management (IPM) and insect resistance management (IRM) plans to mitigate the spread of resistant aphids and delay resistance evolution to other management strategies.

CHAPTER 1. GENERAL INTRODUCTION

Soybean aphid

Distribution and bio-ecology

Native to eastern Asia, soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), is currently widespread in Asia and North America, and is present in Europe, and Oceania (Ragsdale et al. 2011, EPPO 2020). In North America, soybean aphid was first reported in Wisconsin in 2000 (Alleman et a. 2002, Ragsdale et al. 2011), and in less than a decade it spread to 30 U. S. states and three Canadian provinces (Ragsdale et al. 2011), becoming a predominant pest of soybeans, *Glycine max* (L.) Merrill, throughout the North Central USA (Tilmon et al. 2011).

Wingless soybean aphids are yellow-green with dark eyes, have an oval body shape, and are approximately 0.15 mm long. Adults have a pair of often pale basally darkening distally siphunculi (Voegtlin et al. 2004a). The nymphs are light yellowish green, smaller compared to adults and have four instars (Zhang 1988). Under favorable conditions of light and temperature (laboratory), soybean aphid can double population size in 1.5 to 2 days (McCornack and Ragsdale 2004). However, under field conditions it takes approximately 6-7 days (Ragsdale et al. 2007). During the summer on soybean, soybean aphid reproduces asexually and has about 15 overlapping generations (Tilmon et al. 2011).

Soybean aphid has a heteroecious, holocyclic lifecycle that utilizes a primary host plant for overwintering and a secondary host plant during the summer, and undergoes sexual and asexual reproduction (Ragsdale et al. 2011). The asexual reproduction occurs during the spring on buckthorn and summer on soybeans, and both wingless and winged forms are found. When photoperiod and temperature decrease, winged females (gynoparae) migrate from soybean to

Rhamnus species, where they feed and give birth to oviparae females. Males (androparae) also emigrate from soybean fields in the fall and mate with oviparae female on buckthorn where overwintering eggs are deposited (Ragsdale et al. 2004). In the U. S., common buckthorn, *R. cathartica* L., is the principal overwintering host for soybean aphid, however, other native *Rhamaceae* species can also serve as potential hosts (Voegtlin et al. 2004). The rapid spread and establishment of soybean aphid in North America was facilitated by the presence of its overwintering host, *R. cathartica* (Ragsdale et al. 2004).

Soybean aphid has piercing-sucking mouthparts and feeds on soybean leaves, stems, and pods (Tilmon et al. 2011). This species can also vector soybean mosaic virus and alfalfa mosaic virus to soybean plants (Hill et al. 2001). While feeding on soybeans, soybean aphid excretes sugar-rich droplets, commonly called honeydew, which can favor black sooty mold fungus growth (Tilmon et al. 2011). Soybean aphid injury can also interfere with soybean photosynthetic capacity (Macedo et al. 2003).

Soybean aphid feeding on soybean may reduce pods per plant, seed per pod, individual seed weight and consequently seed yield (Beckendorf et al. 2008, Pierson et al. 2010). In addition, aphid infestations on soybeans may interfere with soybean cyst nematode, *Heterodera glycines*, infection (McCarville et al. 2014). Growers are encouraged to scout fields and monitor soybean aphid populations. Insecticides are recommended to prevent yield loss when populations reach the economic threshold of 250 aphids per plant and 80% of plants are infested (Ragsdale et al. 2007, Koch et al. 2016). Soybean aphid outbreaks can lead to yield losses up to 40% when control methods are not adopted (Ragsdale et al. 2007). In Iowa, specifically, soybean aphid outbreaks are observed in 50% of the years in northern counties (Dean et al. 2020a).

Soybean aphid biotypes

Downie (2010) defined biotype as "*populations that are able to reproduce and survive on cultivars developed for resistance to this insect.*" To date four soybean aphid biotypes have been identified in North America, where virulent aphids are able to overcome different combinations of soybeans resistance to *Aphis glycines*. Soybean aphid biotype 1 is known to be avirulent to any soybean variety containing *Rag* genes (Cooper et al. 2015). In 2005, in fields planted with soybean cultivars containing *Rag1* gene, soybean aphids were found surviving on those plants and termed as biotype 2 (Kim et al. 2008). Soybean aphid biotype 3 was first identified in Indiana and is virulent to plants with *Rag2* resistance gene (Hill et al. 2010). In addition, soybean aphid biotype 4 was found surviving on plants with *Rag 1+2* genes in Wisconsin (Alt and Ryan-Mahmutagic et al. 2013). Alt et al. (2018) studied the geographic distribution of soybean aphid biotypes over three years in North America and found that biotypes have a widespread distribution.

Soybean aphid management

The introduction of soybean aphid changed the way farmers managed insects in soybean in the U. S. Prior to 2000, farmers rarely used insecticide in soybean (Costamagna and Landis 2006, Ragsdale et al. 2011). Currently, soybean aphid management is a priority for farmers in North America (Hurley and Mitchell 2017). After soybean aphid arrival, insecticide use in soybean fields increased (Hodgson et al. 2012, Coupe and Capel 2016). There has been an estimate of 130-fold increase in the use of insecticides on soybean fields in the North Central region (Ragsdale et al. 2011), leading to an increase of production costs of US$16-$33 per ha (Ragsdale et al. 2007).

Management approaches to suppress soybean aphid populations include biological control (Fox et al. 2004, Costamagna and Landis 2006, Ragsdale et al. 2011), host plant resistance (Ragsdale et al. 2011, Hesler et al. 2013, Dean et al. 2020), and chemical control (Myers et al. 2005, Magalhães et al. 2009, Hodgson et al. 2012). However, biological control and host plant resistance have limitations suppressing soybean aphid populations (Hesler et al. 2013, Gardiner et al. 2015), and management of soybean aphid outbreaks still relies on insecticides (Johnson et al. 2009, Chandrasena et al. 2011). Prophylactic measures to suppress soybean aphids, such as seed treatments using neonicotinoids, are also available. Nevertheless, the occurrence of this pest in soybean may not align with the control period provided by seed treatments and are unlikely to suppress soybean aphid populations (Krupke et al. 2017).

Synthetic pyrethroids and organophosphates are commonly used to manage soybean aphids (Johnson et al. 2009, Hodgson et al. 2012). These foliar broad-spectrum insecticides provided effective suppression (Chandrasena et al. 2011); however, short generation time and high growth rate of soybean aphid (Ragsdale et al. 2004) may result in population regrowth when a single-timed insecticide spraying is used (Myers et al. 2005). In addition, the prolonged use of the same insecticide or insecticides with the same mode(s) of action can lead to the evolution of insecticide resistance in aphids (Pedigo and Rice 2009, Chandrasena et al. 2011, Hanson et al. 2017).

Pyrethroids

Physical and chemical properties

Pyrethroids are neurotoxic insecticides and synthetic analogues of the naturally-occurring pyrethrins (Davies et al. 2007, Yu 2014). These pyrethrins were originally found in dried flowers of *Chrysanthemum cinerariaefolium* (Davies et al. 2007, Yu 2014). Large-scale production of

pyrethrins from *C. cinerariaefolium* began in the mid-19th century; however, its use in farming was limited because of high production costs and low photostability (Davies et al. 2007). Structural modifications of natural pyrethrins gave rise to synthetic pyrethroids, photostable compounds with low mammalian toxicity, limited soil persistence, and high insecticidal toxicity (Elliot et al. 1978, Casida and Quistad 1998, Davies et al. 2007, Yu 2014). In addition, synthetic pyrethroids are lipophilic chemicals with low volatility and high octanol-water partition coefficients (Laskowski 2002).

Synthetic pyrethroids are classified into two groups according to their effects in insects (Yu 2014). The type I pyrethroids (e.g., permethrin and bifenthrin) do not have a cyano moiety at the α-position and are characterized by symptoms such as hyperactivity, incoordination in response to a single stimulus and finally paralysis. Type II compounds (e.g., deltamethrin, cypermethrin, fenvalerate, and cyhalothrin) have an α-cyano moiety, and cause a pronounced convulsive phase, with membrane depolarization and suppression of the action potential (Gammon et al. 1981).

Mode of action of pyrethroids

Pyrethroids affect the central nervous system of insects (Soderlund and Bloomquist 1989), and target the voltage-gated sodium channels (*vgsc*), transmembrane domain proteins that play an important role in neuronal signaling (Zlotkin 1999, Silver et al. 2014, ffrench-Constant et al. 2016). Initially, pyrethroids cause nerve cell stimulation and production of repetitive discharges that lead to paralysis and death of the insects (Davies et al. 2007). Only a small amount of pyrethroid is necessary to modify the sodium channels, which result in the channel being open because the insecticides prevent channel closing, retaining the ability of the sodium channel to conduct at least small amounts of Na^+. In insects, this causes a state of

hyperexcitability, producing a sublethal effect known as '*knockdown'* (Davies et al. 2007).

Insecticide resistance

Insecticide resistance overview

In the late 1950s, the World Health Organization (WHO) defined insecticide resistance as "*the development of an ability of a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species*" (WHO 1957). Despite of the importance of the early conceptual definition of insecticide resistance, it did not clearly recognize the genetic basis, and did not take in consideration the role of phenotypic adaptations and epigenetics in the development of insecticide resistance (Guedes 2017). Later, Crow (1960) proposed that "*resistance marks a genetic change in response to selection.*" Sawicki (1987) used the previous definition by Crow (1960) and redefined it as "*Resistance marks a genetic change in response to selection by toxicants that may impair control in the field*." The Insecticide Resistance Action Committee (IRAC) defines resistance as "*a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species*" (IRAC 2022).

An insect population that can overcome effects of insecticides compounds through physiological conditions are called tolerant populations. Tolerance is not related to genetic variations or pressure selection and differs from resistance because resistance is a result of genetic alterations and selection pressure (Yu 2014). In many insect species, when an organism is resistance to a determined insecticidal compound it also has resistance to other insecticides. This is called "*cross resistance*" and occurs when a single mechanism that confers resistance to

an insecticide is also involved with the resistance of another insecticide, even if the population has not been previously exposed to the compound (Yu 2014, IRAC 2022).

Chemical insecticides are an effective tool in managing insect pests in the agroecosystems. However, several insect pest species have evolved resistance to one or more compounds (Perry et al. 2011, Pavlidi et al. 2018), posing a severe threat to agricultural production as well as human health (Knight and Norton 1989, Soderlund and Knipple 2003). The introduction of synthetic insecticides in the 1940s, presenting high efficacy of control led to an over-reliance on these chemicals, and consequently an increase of insecticide resistance reports. The number of resistance cases continue to increase, and over 500 cases have been reported (Sparks and Nauen 2015, Sparks et al. 2021). A variety of genetic, biochemical, biological and ecological, and operational factors have influence on the rates of insecticide resistant evolution and the persistence of resistant individuals in agroecosystems (Georghiou and Taylor 1977, Brattsten et al. 1986, Georghiou and Taylor 1986).

Insecticide resistance mechanisms

The first report of insecticide resistance was in 1914 (Melander 1914), and since then insects have evolved resistance through different mechanisms (Sparks et al. 2021). Insects have evolved resistance to insecticides through different mechanisms. These mechanisms are known as target site insensitivity, enhanced metabolism or detoxification (metabolic resistance), increased efflux, reduced penetration through the cuticle, and behavioral resistance (Casida and Quistad 1998, Scott 1990, ffrench-Constant 2013, Yu 2014, Feyereisen et al. 2015).

Target site resistance refers to point mutations on the sequences of insecticide target genes, reducing the binding or affinity of the insecticide to the target proteins (Dong 2007, Yu 2014). These amino acid changes are linked to insecticide resistance and were detected in genes

encoding for the *vgsc*, acetylcholinesterase, nicotinic acetylcholine, gamma-aminobutyric acid (GABA) receptors and ryanodine receptors (Hollingworth and Dong 2008, Yu 2014, Liu 2015).

Enhanced metabolism or detoxification by enzymes is one of most common mechanism of resistance to chemical compounds in insects (Scott 1999, Li et al. 2007, Pavlid et al. 2018, Nauen et al. 2022). Cytochrome P450s monooxygenases, carboxyl/cholinesterases (CCEs), glutathione S-transferases (GSTs), and UPD-glycosyltransferases are enzyme families that have been associated with insecticide resistance, and are involved in phase I and phase II reactions (Yu 2014). The ATP binding cassete proteins (ABC transporters) are also involved in resistance evolution and play important role in phase III reactions (Dermauw et al. 2014, Merzendorfer 2014). These detoxification enzymes and ABC transporters metabolize and eliminate chemical compounds before they reach the target site , respectively (McKenzie 19996, Scott 1999, Hemingway 2000, Dermauw et al. 2014, Merzendorfer 2014, Liu et al. 2015, Nauen et al. 2022).

Thickening and/or modification of the cuticle as a mechanism of resistance to insecticides is a well-recognized phenomenon in many insect species (Balabanidou et al. 2018). Modification of the content of lipids, proteins, and hydrocarbons may increase cuticle thickness, which has been linked to reduced penetration of insecticides, thus contributing to a decrease in their effectiveness (Balabanidou et al. 2018). Behavioral resistance refers to the ability of resistant insects to detect or recognize pesticides and stop feeding or leaving the treated area (IRAC 2022). It is also defined as any avoidance behavior that increases the chances of an insect or its offspring to survive a management tactic (Pittendrigh et al. 2014). For example, insects posing a behavioral mechanism of resistance respond to lower concentrations of insecticides than susceptible insects, indicating the existence of receptors that better detect the chemical

compound (Sparks et al. 1989).

Soybean aphid resistance to insecticides

Chandrasena et al. (2011) evaluated the susceptibility of soybean aphids to pyrethroids, organophosphates, and neonicotinoids and observed no alteration in the susceptibility of soybean aphids to these compounds. Ribeiro et al. 2018 evaluated the susceptibility of soybean aphid populations collected from 2012 to 2014 and found populations with low to moderate resistance levels to thiamethoxam. In China, field-collected soybean aphid populations exhibited resistance (Resistance Ratio ~ 6) to organophosphates compared to a susceptible population (Wang et al. 2012). More recently, resistance to pyrethroids has been detected in the United States. Field-collected soybean aphid populations demonstrated a reduction in mortality compared to a susceptible population (laboratory) (Hanson et al. 2017, Menger et al. 2020).

Although field-evolved resistance occurred in soybean aphid populations in the North Central U. S., the mechanism(s) involved in the evolution of resistance are not yet fully understood. In China, a laboratory-selected population exhibiting resistance to λ-cyhalothrin (40 generations, resistance ratio 76.67) presented cross-resistance to cypermethrin, esfenvalerate, and bifenthrin (pyrethroids), chlorpyrifos (organophosphate), and methomyl (carbamate) (Xi et al. 2015). Synergistic bioassays suggested cytochrome-P450-dependent monooxygenases and esterases (metabolic resistance) were associated with resistance to λ-cyhalothrin (Xi et al. 2015). A comparative proteome-wide analysis using another laboratory-selected population resistant to λ-cyhalothrin (43.42-fold resistance ratio) identified cytoskeleton-related proteins and energy metabolism proteins associated with resistance evolution in soybean aphids (Bi et al. 2016). More recently, field-collected pyrethroid-resistant aphids presented diverse patterns of

overexpressed detoxification enzyme genes (Paula et al. 2020) and four nonsynonymous mutations in the *vgsc* genes (Paula et al. 2021).

Mechanisms of resistance in aphids

Although only a few aphid species have evolved insecticide resistance among the thousands known, some species in Aphididae are notorious for damaging crops. Intense selection pressure due to insecticides to control aphid pests has selected resistant individuals (Foster et al. 2017). *Myzus persicae* (477 reported cases), *Aphis gossypii* (283), *Phorodon humuli* (72*), Brevicoryne brassicae* (22), *Rhopalosiphum padi* (20) and *Sitobion avenae* (19) are aphid species with most cases of insecticide resistance reported (APRD 2021).

In aphids, detoxification enzymes are associated with resistance to organophosphates, carbamates, and pyrethroids (Foster et al. 2017). The overproduction of carboxylases (E4 and/or FE4) has been related to the resistance of *M. persicae* to organophosphates and pyrethroids (Li et al. 2016) and to organophosphate in *A. gossypii* (Cao et al. 2008). Enhanced expression of a cytochrome P450-dependend monooxygenase gene (CYP6CY3) has been linked as a mechanism of resistance to neonicotinoids (imidacloprid) in *M. persicae* (Bass et al. 2013, Little et al. 2017). Overexpression of P450 monooxygenase CYP380C6 and a bioassay suppressing CYP380C6 revealed that this enzyme is involved with resistance to spirotetramat in *A. gossypii* (Pan et al. 2018).

Amino acid mutations in the target site of insecticides also are known to confer insecticide resistance to several insecticides. The *kdr* (knockdown resistance) comprises of non-synonymous mutation located in the *vgsc* genes, the target site of pyrethroids. A substitution at position 1014 (L1014F, leucine to phenylamine) confer pyrethroid resistance in several aphid species (Martinez-Torrez et al. 1997, Bass et al. 2014, Foster et al. 2017). This mutation confers

resistance to pyrethroids in *M. persicae* (Charaabi et al. 2016, Tang et al. 2017, Mingeto et al. 2021, Singh et al. 2021), *S. avenae* (Foster et al. 2014), and *A. gossypii* (Marshal et al. 2012). The *kdr* 918 (methionine for threonine at position 918) is associated with resistance in *M. persicae* (Martinez-Torres et al. 1997, Eleftherianos et al. 2008, Bass et al. 2014, Mingeto et al. 2021, Singh et al. 2021), and *A. gossypii* (Marshal et al. 2012). A M918L (methionine to leucine) confers resistance to pyrethroids in *R. padi* (Wang et al. 2020, Wang et al. 2021), *A. gossypii* (Carleto et al. 2009, Chen et al. 2017a), and *M. persicae* (Panini et al. 2014, Mingeot et al. 2021, Singh et al. 2021). In addition, pyrethroid-resistant *M. persicae* had a L932F (leucine to phenylamine) (Fontaine et al. 2011) and M918I (methionine to isoleucine) mutations in the *vgsc* (Singh et al. 2021). These mutations can occur alone (e.g. L1014F) or in combination (e.g. L1014F+M918L) and can confer varying levels of resistance to pyrethroids (Criniti et al. 2008, Eleftherianos et al. 2008).

Target site mutation in the gene encoding the enzyme acetylcholinesterase (AChE) and in the nicotinic acetylcholine receptor (nAChR) were associated with insecticide resistance in aphids. A point mutation in Ace1 gene (S431F), a serine-to-phenylalanine substitution, known as modified AChE (MACE), confers resistance to carbamates in *Macrosiphum euphorbiae* (Raboudi et al. 2012), carbamates and organophosphates in *A. gossypii* (Andrews et al. 2004, Benting et al. 2004, Li and Han 2004) and *M. persicae* (Nabeshima et al. 2003, Fontaine et al. 2011, Umina et al. 2014, Charaabi et al. 2016). The mutations F139L (phenylalanine to leucine) in Ace2 and A302S (alanine to serine) in Ace1 were also associated with resistance to carbamates in *A. gossypii* (Li et al. 2004). The nAChR mutation R81T (arginine to threonine), has been linked to neonicotinoid resistance in *A. gossypii* (Koo et al. 2014, Toda et al. 2017) and *M. persicae* (Bass et al. 2011, Slater et al. 2012, Panini et al. 2014, Mezei et al. 2021). A A302S

in the GABA receptor is known to confer resistance to cyclodienes in *M. persicae* (Anthony et al. 1998).

Behavioral resistance may also account for insecticide resistance in aphids. In a strain of *M. persicae* resistant to neonicotinoids, aphids demonstrated behavioral avoidance and enhanced dispersal compared with the susceptible line. In addition, the resistant strain had the increased ability to locate untreated areas, which may account for the elevated resistance to neonicotinoids (Fray et al. 2014).

Insecticide resistance and fitness costs

Although insecticide resistance evolution confers a selective advantage, fitness costs in resistant individuals can impact resistance evolution rates and fixation of the resistant allele in a population (Roush and McKenzie 1987, Kliot and Ghanin 2012, ffrench-Constant and Bass 2017, Hawkins et al. 2018, Freeman et al. 2021). This assumes that in an environment free of insecticide, the resistant individuals perform poorly compared with the susceptible ones (Kliot and Ghanin 2012, Freeman et al. 2021). Several biological and demographic parameters are used to measure fitness costs associated with resistance evolution. These include development times, reproductive performance, body size, survival, and susceptibility to natural enemies (Roush and McKenzie 1987, Foster et al. 2011, Kliot and Ghanin 2012, ffrench-Constant and Bass 2017, Freeman et al. 2021).

Among aphids, fitness costs associated with insecticide resistance were evaluated in several species. Reduced reproductive capacity was observed in insecticide-resistant *M. persicae* (Foster et al. 2003), *S. avenae* (Jackson et al. 2020), *A. gossypii* (Ullah et al. 2021) and *R. padi* (Wang et al. 2021). Insecticide-resistant aphids also presented increased vulnerability to natural enemies (Foster et al. 1999, Jackson et al. 2020), and reduced overwintering survivorship (Foster

et al. 1996). Evidence of lack of fitness cost in insecticide-resistant aphids was also observed (Fenton et al. 2010, Castañeda et al. 2011, Erdos et al. 2021, Walsh et al. 2021).

Synergist insecticides

Synergists are compounds that can inhibit detoxification enzymes, and when used in association with insecticides, they can increase insecticide efficacy against resistant insects (Metcalf et al. 1967, Raffa and Priester 1985). In addition to helping overcome insecticide resistance, synergist compounds can provide more efficient control of insects when in a mixture with insecticides and increase the spectrum of action of an insecticide (Metcalf et al. 1967). Because of their properties in inhibiting detoxification enzymes, synergists are an important tool to study potential resistance mechanisms (Scott 1990, Snoeck et al. 2017). Supposing an insect has evolved resistance to a compound and it is suspected to be related to metabolic resistance, the activity of detoxification enzymes can be measured by calculating the synergistic ratio (SR). The synergistic ratio is the LD_{50} (lethal dose) of insecticide alone divided by the LD_{50} of insecticide when mixed with a synergistic (Metcalf et al. 1967, Raffa and Priester 1985).

Piperonyl butoxide (PBO), an inhibitor of P450s, is a well-known and commonly used synergistic insecticide (Scott 1990, Yu 2014, Snoeck et al. 2017). This synergist binds to the active site of P450s, forming a pseudo-irreversible inhibitor complex between the electrophilic carbene and the ferrous iron of the P450, resulting in inhibition of P450 enzymes (Snoeck et al. 2017). The S,S,S-tributyl phosphorotrithioate (DEF) and triphenyl phosphate (TPP) are esterase inhibitors (Scott 1990, Wu et al. 2007, Yu 2014, Snoeck et al. 2017), and the diethyl maleate (DEM), a glutathione S-transferase inhibitor, are also used to investigate metabolic resistance (B-Bernard and Philogène 1993, Wu et al. 2007, Yu 2014).

Sublethal effects of insecticides

Hormesis is characterized as a biphasic relationship between low and high doses of a stressor resulting from insecticidal exposure (Kending et al. 2010, Guedes et al. 2022). Sublethal effects of insecticide exposure can vary among insect species and compounds, which can have a range of beneficial or adverse effects. Sublethal exposure can enhance mutation rates, favoring polygenic and multifactorial resistance (Gressel 2011). Insecticide-induced hormesis and induction/cross-induction of detoxification enzymes have also been observed (Guedes et al. 2017, Guedes et al. 2022). In addition, low insecticidal exposure can lead to induced hormesis and may be involved with pest resurgences (Guedes and Cutler 2013).

Hormesis effects have been reported in soybean aphids when exposed to sublethal concentrations of beta-cypermethrin and imidacloprid. Exposure to sublethal concentrations of beta-cypermethrin affected life-history traits. Specifically, soybean aphids exposed to some of these concentrations had reduced adult longevity and shorter oviposition period. However, when the aphids were exposed to 5 ug/L of beta-cypermethrin, net reproduction rate, intrinsic rate of increase, and finite rate of increase were significantly higher than those not exposed to the insecticide (Qu et al. 2017). Exposure to sublethal concentrations of imidacloprid also affected life-history parameters. Soybean aphids exposed to 0.20 mg/L of imidacloprid had slower juvenile development, reduced adult longevity, shorter reproductive period, and fecundity. However, hormesis effects were observed when aphids were exposed to lower concentrations. Net reproduction rate was higher in aphids exposed to those lower sublethal concentrations of imidacloprid than those in the control treatment (Qu et al. 2015).

Hormesis was also observed in several other aphid species. Direct and transgenerational effects of sublethal concentration (LC_{25}) of pirimicarb (carbamate) in *R. padi* and *S. avenae* were

observed, and it varied between the species. The parent generation of *R. padi* decreased in fecundity, juvenile development, reproductive period, and adult longevity was higher in the F_1 generation exposed to pirmicarb than in the control treatment (Xiao et al. 2015). Conversely, *S. avenae* exposed to LC_{25} of pirimicarb only reduced the pre-reproductive period in the F_1 generation (Xiao et al. 2015). Transgenerational hormesis effects of exposure to nitenpyram (neonicotinoid) were reported for the cotton aphid, *A. gossypii*. Demographic parameters in the F_1 generation of aphids exposed to sublethal concentrations of nitenpyram were higher than in the control treatment, which may result in pest resurgence in Chinese cotton fields (Wang et al. 2017). *Rhopalosiphum padi* exposed to LC_{10}, LC_{20} and LC_{25} of imidacloprid prolonged development, pre-oviposition period and adult longevity, and aphids exposed to LC_{20} demonstrated a longer oviposition period. However, imidacloprid concentrations significantly decreased pre-adult survival rate compared to control aphids (Li et al. 2018).

Plant defenses and insecticide resistance

Insect resistance to plant allelochemicals and synthetic insecticides pose a challenge to insect pest management (Li et al. 2007). Although there are specificities in each interaction, the mechanisms used by insects to overcome plant compounds and insecticides are similar and suggested to pre-adapt pest species to new insecticides (Alyokhin et al. 2017). This pre-adaptation to xenobiotics compounds is also suggested to predispose the insects to the development of insecticide resistance (Silva et al. 2012, Dermauw et al. 2018).

Silva et al. (2012) evaluated the ability of *M. persicae* carrying or not insecticide resistance mutations to reproduce on favorable (pepper) and unfavorable hosts (radish). The authors found that the insecticide resistance genotypes had a higher intrinsic rate of increase on radish than the susceptible genotypes. In addition, insecticide-susceptible aphids up-regulated

some genes to tolerate plant defense compounds in the same way they do when exposed to insecticides, suggesting that plant defenses might serve as pre-adaptation of *M. persicae* to insecticides (Silva et al. 2012). Differences in the susceptibility to insecticides when varying host plants were also observed for *Bemisia tabaci* (Xie et al. 2011), *Helicoverpa armigera* (Tao et al. 2012, Chen et al 2017b), *Spodoptera exigua* (Hafeez et al. 2018) and *Bradysia odoriphaga* (Zhu et al. 2017).

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CHAPTER 2. SOYBEAN APHID (HEMIPTERA: APHIDIDAE) RESPONSE TO LAMBA-CYAHLOTHRIN VARIES WITH ITS VIRULENCE STATUS TO APHID-RESISTANT SOYBEAN

Ivair Valmorbida[a], Dionei S. Muraro[b], Erin W. Hodgson[a], and Matthew E. O'Neal[a]

[a] Department of Entomology, Iowa State University, Ames, IA 50011, United States.

[b] Department of Entomology and Acarology, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo (USP), São Paulo, Brazil.

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Abstract

Soybean aphid, *Aphis glycines,* is an invasive insect in North America, considered one of the most important pests of soybean. Their management relies heavily on foliar insecticides, but there is growing effort to expand these tools to include aphid-resistant varieties. We explored if the LC_{50} and LC_{25} of lambda-cyhalothrin varied between virulent (Resistance to *Aphis glycines* (*Rag*) soybeans) and avirulent (susceptible to *Rag*-genes soybeans) populations of soybean aphid with a leaf-dip bioassay. We also investigated the response to the LC_{25} of lambda-cyhalothrin on adults (F0) and their progeny (F1) for both avirulent and virulent soybean aphid. The LC_{50} of the virulent aphid population was significantly higher compared with the LC_{50} of the avirulent population. The LC_{25} significantly reduced fecundity of the F0 generation of avirulent soybean aphid, but no significant effect was observed for virulent aphids. In addition, the LC_{25} significantly shortened the adult pre-oviposition period (APOP) and lengthened total pre-oviposition period (TPOP) of avirulent aphids, while the mean generation time (*T*) was significantly increased. For the virulent aphid, sublethal exposure significantly lengthened

development time of first and third instars, TPOP, and adult longevity. In addition, all demographic parameters of virulent soybean aphid were significantly affected when they were exposed to the LC_{25} of lambda-cyhalothrin. Our results demonstrate lambda-cyhalothrin is less toxic to virulent aphids and exposure to the LC_{25} can trigger hormesis which may have implications for the long-term management of this pest with this insecticide as well as with aphid-resistant varieties of soybean.

Keywords: Hormesis; pyrethroid; life table analysis; resistance; IPM

Introduction

Insect pests can be exposed to a plethora of chemicals, including defensive chemicals within the host plant[1] and insecticides used for their management.[2,3] Mechanisms used by insects to overcome plant defenses and chemical insecticides may be shared, which in turn may affect the susceptibility of insects to insecticides.[4,5,6] Indeed, decreased susceptibility to insecticides has been observed in several pest species due to the effect of plant allelochemicals.[6,7,8] Conversely, host plant resistance has also been reported to interfere with susceptibility of pest species to insecticides.[9,10,11]

Insects can be exposed to sublethal concentrations of insecticides in several situations within agro-ecosystems. Insecticide degradation by rainfall, temperature, and sunlight can lead to a reduction in concentration after initial application.[3] In addition, defective spraying equipment, drift, and missaplication may also affect the final concentration of the insecticide that the target insect experiences.[12] Furthermore, if the pest colonizes a field after an insecticide is applied, there is a potential for sublethal exposure as the active ingredient degrades. A sublethal concentration of an insecticide can induce hormesis in insects, a biphasic-response phenomenon

where a low-dose of an insecticide may have a stimulatory effect on population parameters, while a high-dose leads to inhibition.[2,13, 14,15] These sublethal effects can favor pest resurgence[2,15] and the development of insecticide resistance.[16]

Soybean aphid, *Aphis glycines* Matsumura (Hemiptera: Aphididae), was first detected in the United States in 2000,[17,18] and rapidly spread across the major soybean production areas of the North Central United States.[18] This species has a complex life cycle [18] and uses soybean as a secondary host during the summer resulting in as many as 15 generations.[19] Soybean aphid feeds on phloem while residing on soybean leaves and stems,[19] reducing pods per plant, seed per pod, individual seed weight, and consequently, seed yield.[20,21] If these populations are left unmanaged, soybean aphids can reduce yield by as much as a 40%.[22]

Evidence of hormesis in the form of stimulatory effects on life history traits resulting in increased longevity and fecundity have been observed in numerous arthropods species,[2,15] including the soybean aphid.[23,24] Soybean aphids were positively affected by sublethal exposure to imidacloprid (Group 4A)[23] and beta-cypermethrin (Group 3A).[24] Net reproduction rate was significantly higher in aphids exposed to sublethal concentrations of imidacloprid than those in the control treatment.[23] Similarly, net reproduction rate, intrinsic rate of increase, and finite rate of increase were significantly higher when soybean aphids were exposed to 0.005 μg mL^{-1} of beta-cypermethrin compared to unexposed aphids.[24] During the first 15 years of soybean aphid occurrence in North America (NA), the most common active ingredient used has been lambda-cyhalothrin (Group 3A).[18] To what extent lambda-cyhalothrin produces hormesis in soybean aphids found in NA is not known.

Despite passing through a genetic bottle-neck common for invasive species, there is evidence of genetic diversity with soybean aphid populations found in North America.[25,26,27] This

diversity has implications for management, in the form of phenotypic variation to genes confereing resitance to soybean aphid, i.e., *Rag*-genes.[27] Populations avirulent to all *Rag*-genes are referred to as biotype 1.[28] To date, several distinct virulent biotypes have been identified in North America[29] and found throughout a multi-state region of the US.[28] The most virulent biotype, biotype 4, is capable of surviving on soybeans with one or more *Rag*-genes.[30]

The role of *Rag*-genes against soybean aphids are not fully understood. Soybean isolines containing *Rag1* and *Rag2* genes confer resistance to soybean aphids primarily through antibiosis, although antixenosis may also play a role against soybean aphids.[31] The stress caused by the feeding of the aphid on a *Rag* plant induces a higher expression of genes related to the activation of response mechanisms common in resistant plants.[32] The identity of products from these genes and subsequent mechanisms has not been identified. Enzymatic systems in a virulent soybean aphid have been suggested to account for their capacity to develop on *Rag*-containing varieties of soybeans.[33] Although there is overlap between the mechanisms confering pesticide resistance and resistance to plant toxins,[34] especially for generalists herbivores, it is unclear how robust such overlap exists for specialists herbivores like the soybean aphid.

We used two distinct biotypes of soybean aphid to determine if virulence affected the aphids' susceptibility to lambda-cyhalothrin. Furthermore, we explored how both populations responded to the LC_{25} of lambda-cyhalothrin, and if this induced hormesis. We first determined if the LC_{50} for lambda-cyhalothrin varied between virulent and avirulent soybean aphids. Once we established this base-line susceptibility, we then tested if the biotypes responded differently to their respective LC_{25} for lambda-cyhalothrin. We predicted that a virulent biotype would be less susceptible to lambda-cyhalothrin and more likely to show evidence of hormesis than

avirulent biotypes. We looked for evidence for hormesis in adults (F0) exposed to lambda-cyhalothrin as well as their offspring (F1).

Materials and Methods

Insects, plants and insecticides

We used avirulent (biotype 1) and virulent (biotype 4) soybean aphids that came from colonies initially collected by colleagues at The Ohio State University. At Iowa State University, these colonies were maintained on their respective susceptible plants in growth chambers (25 ± 2°C, 50% RH and a photoperiod of 16:8 [L:D]) without exposure to insecticides. Avirulent soybean aphids (i.e., biotype 1) were reared on the soybean genotype LD14-8007, which does not contain *Rag*-genes. Virulent soybean aphids (i.e., biotype 4) were reared on soybean genotype LD14-8001 expressing both *Rag1* and *Rag2* genes (written as *Rag1+2* throughout). For the sublethal exposure bioassays, avirulent and virulent aphids were reared and tested on their respective plant genotypes that they were kept on while in these colonies. Technical grade of lambda-cyhalothrin (active ingredient 97.7%) was obtained from Control Solutions Inc. (Pasadena, USA).

Concentration-mortality response for virulent and avirulent soybean aphids

A leaf-dip bioassay[35] was used to assess the susceptibility of avirulent and virulent soybean aphids to a technical formulation of lambda-cyhalothrin. A stock solution of lambda-cyhalothrin was prepared in analytical acetone and diluted into seven to eight concentrations with distilled water containing 0.05% (v/v) Triton X-100 (Alfa Aesar, Tewksbury, USA). A control treatment contained distilled water, 0.05% (v/v) Triton X-100, and 0.01% of acetone, equal to the concentration of acetone in the treatment with the highest concentration of lambda-cyhalothrin.

Soybean seeds were planted in plastic pots filled with a soil mixture (Sungro Horticulture Products, SS#1-F1P) and kept in a greenhouse (25 ± 5°C and a photoperiod of 16:8 [L:D]). Plants were watered three times per week, and after emergence, fertilized weekly with a water-soluble formulation (Peters Excel Multi-Purpose Fertilizer, 21-5-20 NPK). Disks (3.8-cm diameter) from first and second trifoliate leaves were cut with a hole punch (Fiskars, Helsinki, Finland) when plants reached the mid-vegetative stage (V4).[36] Leaf disks were manually submerged with gentle agitation in a treatment solution for 10 s and then allowed to air dry, abaxial side-up on a paper towel. Subsequently, leaf disks were placed with their abaxial surface downward onto 29.6 ml plastic souffle cups (Choice Paper Company, New York, USA) containing 1% w/v agar (Bacto™ Agar, Becton, Dickinson and Company, Franklin Lakes, USA) prior to congealing. Each cup was filled with approximately 20 ml of agar, leaving 10 ml to the top of the cups. A drop of distilled water was added to the agar bed to increase leaf disk adherence.

We selected apterous, mixed age adult aphids from our colonies and transferred them to the bottom of Petri dishes containing filter paper moistened with distilled water. We randomly selected twenty, uninjured aphids from these Petri dishes, transferring them onto a leaf disk. Each cup was sealed with a close-fitting, ventilated lid. Cups were stored in a growth chamber (25 ± 2°C, 70% RH and 16:8 [L:D]). Assessment of mortality was performed after 24 and 48h and data from 48h post treatment was used to estimate the LC_{50}. Aphids unable to right themselves within 10 s after they were turned on their back were considered dead.[35,37] Each cup contained 20 aphids and was considered an experimental unit, and each concentration of lambda-cyhalothrin and the control was replicated three times.

Effects of LC_{25} of lambda-cyhalothrin on F0 generation

The leaf-dip bioassay was used to determine the effects of lambda-cyhalothrin on both virulent and avirulent soybean aphids. We used data collected in the previous section to estimate the LC_{25} of lambda-cyhalothrin for each biotype. To determine the response of soybean aphids to this concentration, the same control treatment was used as described above, with adult aphids exposed to their respective LC_{25} of lambda-cyhalothrin prepared in acetone and diluted in distilled water containing 0.05% (v/v) Triton X-100. Aphid mortality was assessed at 48 h after exposure, and individual surviving aphids were gently transferred to an untreated leaflet kept in a Petri dish within a growth chamber (25 ± 2°C, 50% RH and 16:8 [L:D]). Each Petri dish contained a moistened circular filter paper at the bottom and a string of Parafilm (Fisher Scientific, Ottawa, Canada) was used to seal the Petri dishes preventing escape of aphids. Nymphs were recorded and removed daily until the death of the adult aphid. The soybean leaflet was replaced every 7 d, and filter paper was moistened when necessary. Each aphid was considered an experimental unit, and 100 adults were used for each treatment and biotype combination, for a total of 400 adult aphids.

Effects of LC_{25} of lambda-cyhalothrin on F1 generation

We used the same experimental protocol as described above to estimate the effect on the F1 generation of adult aphids exposed to the LC_{25} of lambda-cyhalothrin. The same control was used as described above, with adult aphids of virulent and avirulent biotypes exposed to their respective LC_{25} of lambda-cyhalothrin prepared in acetone and diluted in distilled water containing 0.05% (v/v) Triton X-100. Twenty-four hours after the F0 generation was exposed to a treatment, the F1 nymphs were removed and only the adults (F0 generation) remained on the leaf disks. At 48 h post-treatment of the F0 generation, the 24 h old nymphs (F1) were

transferred to untreated soybean leaflets and maintained individually in a Petri dish as described for the parental generation (F0). For the avirulent aphid, 100 nymphs were used for the control treatment and 61 for the LC_{25} treatment. For the virulent aphid, 100 nymphs were used for each treatment. The following parameters were assessed daily during the lifespan of the F1 generation: development time, number of surviving aphids at each life stage, nymphs per aphid, and longevity of adults. Exuviae were removed once detected and morphological characteristics of nymphs[38,39] were used to assess growth stage.

Data analysis

Concentration-mortality data was analyzed using a three-parameter log-logistic function of the 'drc' package in R[40] to estimate slope, LC_{50} and LC_{25} of lambda-cyhalothrin, and whether the LC_{50} of lambda-cyhalothrin differed between avirulent and virulent aphid populations. Individual aphid development time, survival rate, longevity, and daily fecundity of virulent and avirulent soybean aphids exposed to the LC_{25} of lambda-cyhalothrin and control treatments were analyzed following the age-stage, two-sex life table theory,[41,42] using TWOSEX-MSChart program.[43] Parameters such as age-stage specific survival rate (s_{xj}), probability a newly emerged nymph would survive to age x and stage j, (x is age in days and j is the stage), age-specific survival rate (l_x), age-specific fecundity (m_x), intrinsic rate of increase (r), net reproductive rate (R_0), finite rate of increase (λ), and mean generation time (T) were calculated according to Chi and Liu[41] and Chi[42]. Means and standard error of population parameters in the life table were estimated using a bootstrap procedure,[44] with 100,000 replicates. Differences between control and treated aphids within life table parameters were analyzed using a paired bootstrap test at 5% significant level using TWOSEX-MSChart program.[43]

Results

LC_{50} for virulent and avirulent soybean aphids

Susceptibility to lambda-cyhalothrin varied significantly between the two soybean aphid biotypes (Table 1). Lambda-cyhalothrin was less toxic to the virulent biotype than the avirulent biotype. Based on these data, we estimated an LC_{25} of 0.25 μg mL^{-1} and 0.53 μg mL^{-1} for avirulent and virulent biotypes, respectively. The corrected mortality for adult aphids exposed to the LC_{25} was 23.70% and 25.02% for avirulent and virulent aphids, respectively. Our estimate of the LC_{50} (0.40 ± 0.17 μg mL^{-1}) for the avirulent soybean aphid is similar to the LC_{50} (0.32–0.44 μg mL^{-1}) reported by Hanson[37] using leaf-dip bioassays to evaluate the susceptibility of biotype 1 to lambda-cyhalothrin. This comparison suggests that our estimate is within the range of what others have reported for avirulent soybean aphid populations.

Effects of LC_{25} of lambda-cyhalothrin on F0 generation

Regardless of virulence status, we did not observe a significant effect of lambda-cyhalothrin applied at the LC_{25} on the longevity of adults when compared to their respective controls. However, the LC_{25} of lambda-cyhalothrin had a variable effect on fecundity based on the virulence status of soybean aphid. The fecundity of avirulent aphids exposed to their LC_{25} was significant lower when compared with the control treatment ($t = 3.045$; d.f. = 198; $P = 0.002$), while the LC_{25} did not affect fecundity of virulent aphids ($t = 0.1502$; d.f. = 198; $P = 0.8808$) (Table 2).

Effects of LC_{25} of lambda-cyhalothrin on F1 generation of avirulent soybean aphid

Exposure to the LC_{25} of lambda-cyhalothrin had limited effects on biological and demographic parameters of avirulent soybean aphid when compared to the control treatment (Table 3). The developmental duration of 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} instars (N1 through N4), oviposition period, adult longevity and fecundity were not significantly affected by the exposure

to the LC_{25} of lambda-cyhalothrin. Conversely, the LC_{25} of lambda-cyhalothrin significantly shortened adult pre-oviposition period (APOP) and lengthened total pre-oviposition period (TPOP). There were no significant differences in the net reproductive rate (*Ro*), finite rate of increase (λ), intrinsic rate of increase (*r*) and gross reproduction rate (GRR) of avirulent soybean aphids exposed to LC_{25} when compared with the control treatment. However, the mean generation time (*T*) significantly increased when avirulent aphids were exposed to the LC_{25} of lambda-cyhalothrin.

Variability in developmental rates of individual avirulent soybean aphids, and overlap among stages were observed between those exposed to the LC_{25} and control treatments (Fig. 1). Nymphal development was delayed, as the peak of the fourth instar occurred at 5 d in the control treatment and 6 d in the LC_{25} treatment (Fig. 1). The maximum survival time was decreased in the LC_{25} treatment and a higher l_x was observed in the control group from age 2 to 12 d (Fig. 2). After 12 d, the l_x decreased, and a higher l_x was observed in the LC_{25} treatment from age 13 to 32 d. The age-specific maternity ($l_x m_x$) highest peaks occurred earlier in the control treatment (age 12) compared to the LC_{25} treatment (age 15) (Fig. 2). However, the fecundity peaks were higher for the LC_{25} treatment compared with control treatment (4 and 5.1 aphids/day, respectively). The age-stage life expectancy (e_{xj}) demonstrates the time that an individual of age *x* and stage *y* is expected to live. In general, the life expectancy decreased as age increased, and estimates of life expectancy were similar for both control and treated aphids. The age-stage reproductive values (v_{xj}) peaked earlier for the control treatment (12.12 at day 9) and was lower than the peak for aphids receiving the LC_{25} treatment (14.04 at day 10).

Effects of LC_{25} of lambda-cyhalothrin on F1 generation of virulent soybean aphid

Exposure to an LC_{25} of lambda-cyhalothrin significantly affected more biological and demographic parameters of the nymphs of virulent aphids than the avirulent aphids (Table 3). Development time of the first and third instars, TPOP, and adult longevity were significantly longer for virulent aphids exposed to lambda-cyhalothrin as compared to the control treatment. Net reproductive rate, mean generation time, and GRR were significantly lower for the treatment control. On the contrary, exposure to the LC_{25} significantly reduced finite rate of increase (λ) and intrinsic rate of increase (*r*). Overall, the effect of the LC_{25} on virulent aphids was more pronounced than in the avirulent aphid. For example, all the demographic parameters of the virulent aphid were significantly affected by the exposure to the LC_{25}, while for the avirulent aphid, only the mean generation time (*T*) was significantly affected.

Overlapping between stages showing variable developmental rates among individuals were observed in the control and LC_{25} treatments for the virulent soybean aphid (Fig. 1). Exposure to the LC_{25} delayed developmental time of the virulent aphid (Fig. 1). The virulent aphid exposed to the control treatment had a higher age-specific survival rate (l_x) at the beginning (age 3 and 4 d), which then decreased and was lower than that observed for aphids exposed to the LC_{25}, from ages six to 27 d (Fig 2). The LC_{25} did not affect the maximal survival time of the virulent aphids. The age-specific maternity ($l_x m_x$) peak occurred later for virulent aphids exposed to the LC_{25} compared to the control treatment; however, the number of aphids per day was higher in the LC_{25} treatment (Fig. 2). The age-stage life expectancy (e_{xj}) and the age-stage reproductive values (v_{xj}) for the virulent aphid followed a similar pattern as observed for the avirulent soybean aphid. The age-stage reproductive peak occurred later and was higher for the LC_{25} treatment when compared with control treatment.

Discussion

We observed differences between the two biotypes when exposed to two different concentrations of lambda-cyhalothrin, one representing a concentration more consistent with a lethal dose (LC_{50}) and a lower concentration (LC_{25}) that could represent a sublethal dose. This latter dose produced an interesting difference in the response of aphids consistent with hormesis in the F1 generation, at least for the virulent biotype. Difference in response by the two biotypes to lambda-cyhalothrin extended to effects on longevity and fecundity of the F0 generation when exposed to the LC_{25}. There were no significant differences on longevity and fecundity of virulent aphids exposed to the LC_{25} of lambda-cyhalothrin, whereas the fecundity of avirulent aphids were significantly reduced when exposed to the LC_{25} of lambda-cyhalothrin.

Based on life table analysis of the F1 generation, exposure to the LC_{25} of lambda-cyhalothrin had a stimulatory effect on several parameters for the virulent biotype but not the avirulent biotype. The F1 generation produced from virulent aphids exposed to the LC_{25} of lambda-cyhalothrin had greater adult longevity, longer oviposition period and produced more nymphs per female than those produced from a generation exposed to the control treatment. Furthermore, the LC_{25} exposure significantly increased net reproductive and gross reproductive rates of virulent soybean aphid compared to the untreated control. This is in contrast to the avirulent aphids, whose net reproductive and GRR rates were numerically higher; but did not significantly differ from the control treatment. All of these differences suggest a stimulatory effect from the exposure of this concentration of lambda-cyhalothrin is limited to the virulent biotype. These results suggest that this virulent biotype experiences hormesis when exposed to its LC_{25} of lambda-cyhalothrin.

Evidence of hormesis in the F1 generation of soybean aphid exposed to another pyrethroid insecticide has been revealed with lower concentrations. Soybean aphids exposed to the LC_{15} of beta-cypermethrin significantly decreased the intrinsic rate and finite rate of increase, while a lower concentration (nearly LC_5) increased these parameters.[24] The LC_5 of beta-cypermethrin significantly increased net reproduction rate, intrinsic rate of increase, and finite rate of increase for soybean aphids compared to the control treatment.[24] Although we did not evaluate the response of soybean aphids to concentration below the LC_{25} for lambda-cyhalothrin, it is likely concentrations below the LC_{25} for lambda-cyhalothrin could produce an even greater stimulatory effect, regardless of virulence status.

In summary, our results suggest that lambda-cyhalothrin was less toxic to the virulent biotype of the soybean aphid at varying concentrations than the avirulent biotype. This is the first evidence that virulence to *Rag*-genes affects the response to insecticides. Variation in susceptibility to insecticides has been demonstrated for sub-populations of other Hemipterans. For example, sub-populations of *Aphis gossypii* Glover,[45] *Bemisia tabaci* Gennadius,[10] and *Bactericera cockerelli* (Sulc)[9] that vary in their capacity to exploit different plant species, also had varying levels of insecticide susceptibility. Unlike these previous studies that include Hemipterans with a broad host-range, soybean aphid is specialist with a restricted host range (e.g., soybean as a summer host), and the sub-populations we used were identified based on their response to a genetic difference in their host plant (i.e., presence/absence of *Rag*-genes).

Although the mechanism for virulence to *Rag*-genes is not known, a role for effector proteins secreted by the aphid into the host plant[46,47] and detoxification enzymes within the aphid have been suggested.[33] Variation in the form and amount of these effector proteins injected into the plant by feeding aphids, as well as variation in detoxification enzymes may contribute to the

various biotype phenotypes observed in North America and Asia. For example, up-regulation of P450s, glutathione S-transferases (GSTs), carboxylesterases (COEs), and ABC transporters was observed when avirulent soybean aphids fed on soybean containing *Rag1*, suggesting a specific stress response to the xenobiotic compounds produced by *Rag1* soybean variety.[33] These mechanisms are similar to those used by insects against synthetic insecticides[34,48], and could explain the difference in susceptibility of avirulent and virulent soybean aphid to lambda-cyhalothrin, assuming the virulent aphid also presents similar mechanisms that allow them to survive on *Rag1+2* plants. For example, similar mechanisms against plant secondary compounds were observed for *Bradysia odoriphaga* larva reared on garlic and humus, leading to a higher tolerance to insecticides (e.g., Phoxim and clothianidin) compared with other host plants.[11] Furthermore, *Helicoverpa armigera* (Hübner) larvae fed on a gossypol-diet demonstrated higher tolerance to deltamethrin, associated with an increase in P450 activity within the midgut.[7] Activity of EST and P450 were also associated with development of resistance to deltamethrin in *Spodoptera exigua* (Hübner) fed gossypol.[8]

To what extent the response of the F0 generation of soybean aphid to lambda-cyhalothrin is typical for other insecticides is not clear. Similar experiments suggest effects on longevity and fecundity for the F0 generation varies by aphid species and insecticide combination. For example, the exposure of *Myzus persicae* (Sulzer) to the LC_{25} of flupyradifurone significantly reduced adult longevity and fecundity.[49] However, no differences in longevity and fecundity were observed for *A. gossypii* when exposed to theLC_{25} of flupyradifurone[50] and sulfoxaflor.[51] The longevity of *A. gossypii* was not affected by the LC_{10} and LC_{50} of nitenpyram, but fecundity was significantly reduced.[52]

Although we observed a difference in the LC_{50} between virulent and avirulent biotypes when exposed to lambda-cyhalothrin, this difference is likely not immediately important for management of the soybean aphid. However, the difference observed at a lower concentration (as defined by the LC_{25}) is important for soybean aphid management, and reinforces a rational application of insecticides within an IPM program.

Insecticide induced hormesis in agricultural pests can be a serious problem, because it can result in pest resurgence.[16] Sublethal exposure may also increase mutation frequencies, and if related to the target-site of insecticides, it might reduce the pest's susceptability to insecticides.[53] In addition, sublethal exposure to insecticides may directly increase the selection of resistant by stimulating the expression of advantageous phenotypes, and indirectly by providing conditions that may prime the insect pest to better tolerate stressful conditions (e.g., resistant host plants).[54] Interestingly, our data suggest that the virulent soybean aphid may have an advantage over avirulent aphid when exposed to low concentrations of lambda-cyhalothrin. This is disconcerting given the often prophylactic or calendar-based use of insecticides for managing the soybean aphid in North America.[55] This approach to insect pest a management may inadvertently favor the selection of virulent over avirulent soybean aphids within North America. Such selection pressure may limit the durability of aphid-resistant soybean varieties that are in development,[31,56] and this relatively new technology also may suffer the consequences of unnecessary insecticide applications in soybean fields.

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Tables and Figures

Table 2.1. Concentration-mortality response and corrected mortality of both virulent and avirulent adult *Aphis glycines* to lambda-cyhalothrin.

Population	Slope±SE[a]	LC_{50} (95% FL)[b]	LC_{25} (95% FL)	$\chi 2$ (d.f.)[c]	Mortality (%)[d]
Avirulent	2.20±0.654	0.40 (0.23 - 0.57) b	0.25 (0.07 - 0.41)	9.30 (6)	23.70
Virulent	2.77±0.480	0.78 (0.67 - 0.88) a	0.53 (0.40 - 0.64)	10.35 (5)	25.05

[a] SE = standard error.
[b] LC_{50} values designated by different letters within a column are significantly different from each other through non-overlap of 95% fiducial limits.
[c] Chi-square testing linearity of concentration-mortality responses.
[d] LC_{25} induced mortality; Henderson-Tilton correction.

Table 2.2. Longevity and fecundity of adult *Aphis glycines* treated with the LC_{25} of lambda- cyhalothrin and untreated control treatments for 48 h post exposure.

Population	Longevity (days) ± SE					Fecundity ± SE				
	Control	LC_{25}	df	t[a]	*P*	Control	LC_{25}	df	t[a]	*P*
Avirulent	10.06±0.49	8.82±0.41	198	1.913	0.057	20.49±1.23	15.43±1.10	198	3.045	0.002
Virulent	7.31±0.42	7.71±0.43	198	-0.651	0.515	18.06±1.23	17.8±1.20	198	0.150	0.880

[a] Student's *t* test for differences between LC_{25} and control treatments for each biotype.

Table 2.3. Biological and demographic parameters of avirulent and virulent soybean aphid exposed to the LC_{25} of lambda-cyhalothrin.

Biological parameter	Avirulent		Virulent	
	Control	LC_{25}	Control	LC_{25}
N1 (days)	2.34±0.06a	2.67±0.07a	1.55±0.00b	2.08±0.00a
N2 (days)	1.53±0.06a	1.54±0.07a	1.38±0.04a	1.26±0.04a
N3 (days)	1.36±0.06a	1.50±0.06a	1.07±0.02b	1.21±0.04a
N4 (days)	1.20±0.04a	1.24±0.06a	1.21±0.04a	1.19±0.04a
APOP	1.12±0.06a	0.98±0.03b	0.52±0.00a	0.45±0.00a
TPOP	7.55±0.11b	7.94±0.13a	5.75±0.07b	6.20±0.05a
Oviposition period (days)	10.73±0.37a	11.82±0.42a	11.28±0.30b	12.76±0.25a
Adult longevity (days)	18.13±0.78a	19.62±0.85a	14.66±0.53b	16.19±0.37a
Fecundity (no. nymphs per female)	34.42±1.31a	38.42±1.56a	45.43±1.45b	51.51±1.00a
Demographic parameter				
Net reproductive rate (Ro)	29.84±1.64a	31.49±2.27a	42.25±1.78b	47.91±1.61a
Finite rate of increase (λ, d^{-1})	1.32±0.00a	1.30±0.00a	1.44±0.00a	1.41±0.00b
Intrinsic rate of increase (r, d^{-1})	0.27±0.00a	0.26±0.00a	0.36±0.00a	0.34±0.00b
Mean generation time (T, days)	12.16±0.16b	12.94±0.16a	10.16±0.06b	11.07±0.07a
GRR	39.79±1.12a	41.26±0.99a	51.03±0.95b	54.46±0.87a

Mean ± standard error (SE) were estimated using 100,000 bootstrap replications. Different letters within the same row for avirulent and virulent soybean aphid, indicates significant differences between the control and LC_{25} group at $P < 0.05$ level, with a paired bootstrap test. APOP: Adult pre-oviposition period; TPOP: Total pre-oviposition period; GRR: Gross reproductive rate.

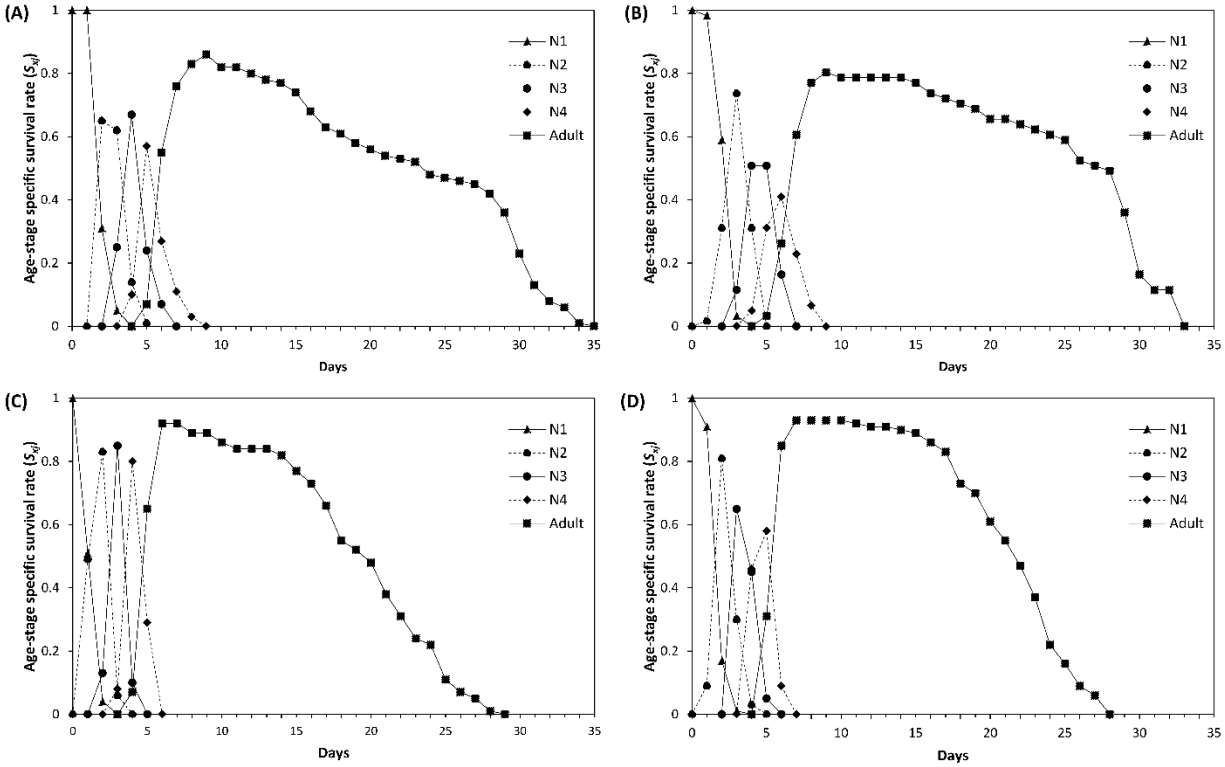

Figure 2.1. Age-stage specific survival rate (S_{xj}) of *Aphis glycines* exposed to control and LC_{25} of lambda-cyhalothrin. (A) avirulent control, (B) avirulent LC_{25}, (C) virulent control, (D) virulent LC_{25}.

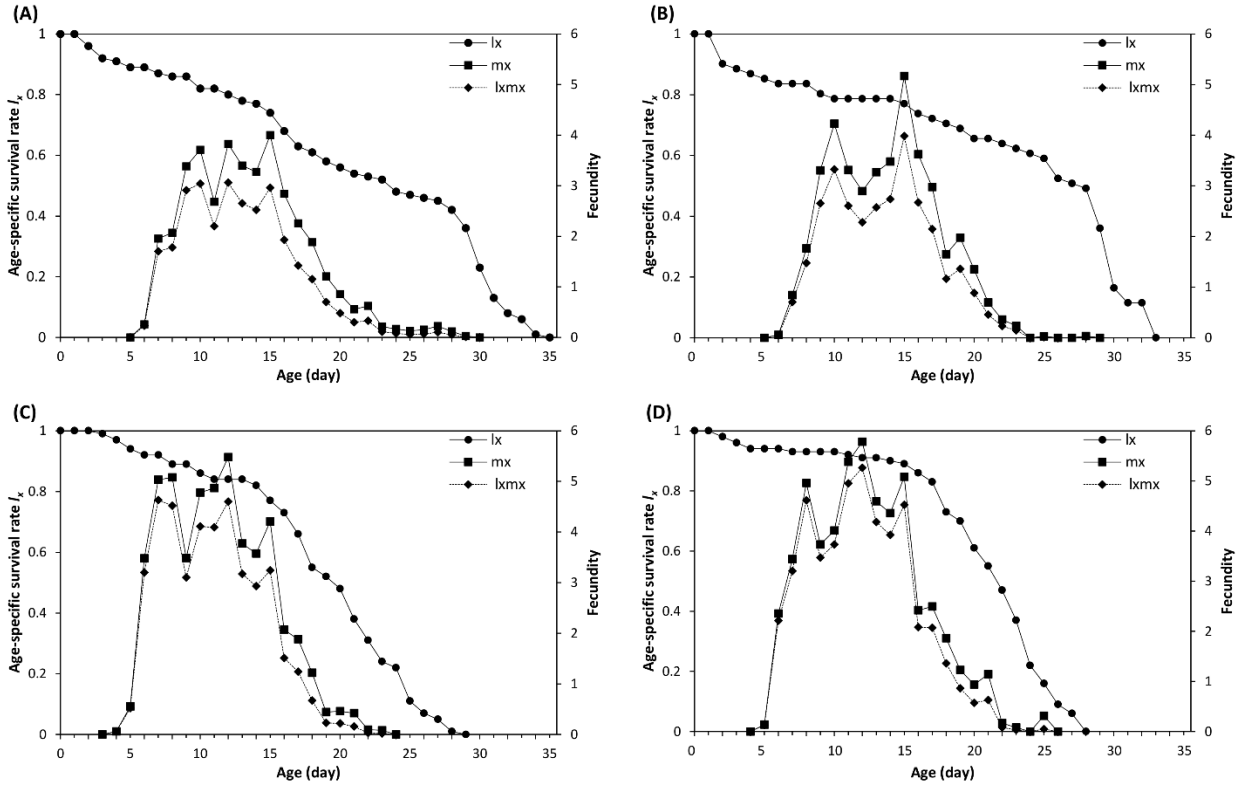

Figure 2.2. Age-specific survival rates (l_x), age-specific fecundity (m_x) and net maternity ($l_x m_x$) of *Aphis glycines* exposed to control and LC_{25} of lambda-cyhalothrin. (A) avirulent control, (B) avirulent LC_{25}, (C) virulent Control, (D) virulent LC_{25}.

CHAPTER 3. ASSOCIATION OF VOLTAGE-GATED SODIUM CHANNEL MUTATIONS WITH FIELD-EVOLVED PYRETHROID-RESISTANT PHENOTYPES IN SOYBEAN APHID AND GENETIC MARKERS FOR THEIR DETECTION

Ivair Valmorbida[a#], Jessica D. Hohenstein[a#], Brad S. Coates[b#], Júlia G. Bevilaqua[c], James Menger[d], Erin W. Hodgson[a], Robert L. Koch[d], and Matthew E. O'Neal[a]

[a] Department of Entomology, Iowa State University, Ames, IA, USA

[b] United States Department of Agriculture, Agricultural Research Service, Corn Insects & Crop Genetics Research Unit, Ames, IA, USA

[c] Department of Crop Protection, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

[d] Department of Entomology, University of Minnesota, Saint Paul, MN, USA

# contributed equally to the completion of this work.

Corresponding author: Matthew E. O'Neal, oneal@iastate.edu

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Abstract

The frequent use of insecticides to manage soybean aphids, *Aphis glycines* (Hemiptera: Aphididae), in the United States has contributed to field-evolved resistance. Pyrethroid-resistant aphids have nonsynonymous mutations in the voltage-gated sodium channel (*vgsc*). We identified a leucine to phenylalanine mutation at position 1014 (L1014F) and a methionine to isoleucine mutation at position (M918I) of the *A. glycines vgsc*, both suspected of conferring knockdown resistance (*kdr*) to lambda-cyhalothrin. We developed molecular markers to identify these mutations in insecticide-resistant aphids. We determined that *A. glycines* which survived exposure to a diagnostic concentration of lambda-cyhalothrin and bifenthrin via glass-vial bioassays had these mutations, and showed significant changes in the resistance allele frequency

(RAF) between samples collected before and after field application of lambda-cyhalothrin. Thus, a strong association was revealed between aphids with L1014F and M918I *vgsc* mutations and survival following exposure to pyrethroids. Specifically, the highest survival was observed for aphids with the *kdr* (L1014F) and heterozygote *super-kdr* (L1014F + M918I) genotypes following laboratory bioassays and in-field application of lambda-cyhalothrin. These genetic markers could be used as a diagnostic tool for detecting insecticide-resistant *A. glycines* and monitoring the geographic distribution of pyrethroid resistance. We discuss how generating these types of data could improve our efforts to mitigate the effects of pyrethroid resistance on crop production.

Keywords: insecticide resistance, soybean, Insect Resistance Management

Introduction

The prevalence of insecticide resistance among arthropods continues to increase globally[1,2], and can dramatically reduce the ability of farmers to control damage and manage the spread of insect-vectored diseases[3-5]. Additionally, increased production costs can be incurred when resistance evolves to less expensive active ingredients, necessitating a transition to more-expensive alternative chemistries[2,5]. Furthermore, higher insecticide application rates used to control more resistant insect populations have greater detrimental effects on the environment[6,7]. This scenario threatens the sustainability of crop production practices and global food security.

Pyrethroids function as neurotoxins through the strong binding and maintenance of an open state for the voltage-gated sodium channel (*vgsc*) protein[8]. Nucleotide mutations leading to non-synonymous changes to amino acids in or flanking the target site, alone or in combination, are associated with resistance to pyrethroids[9-12]. Knockdown resistance (*kdr*) genotypes with a leucine to phenylalanine amino acid substitution at positions orthologous to 1014 (L1014F) in

the *vgsc* protein of house fly, *Musca domestica*[13], are reported to confer low to moderate levels of pyrethroid resistance[12,14]. Additionally, this L1014F *kdr* mutation in combination with a methionine to threonine amino acid substitution at *vgsc* protein position 918 (M918T) is causal of the *super-kdr* phenotype in *M. domestica* that confers increased levels of resistance[15,16].

Pyrethroid resistance in field populations of several aphid species including *Aphis glycines* is associated with non-synonymous mutations in the *vgsc*[17-23]. Despite these associations, pyrethroid resistance is reported to be multimodal[24]. In aphids, the up-regulation of detoxification genes can also contribute to field-evolved pyrethroid resistance[25-27]. Understanding the mechanism(s) by which resistance develops among pests can lead to increased capacity to evaluate the efficacy of insect resistance management (IRM) programs and track the spread of resistant phenotypes[28], leading to improved locally appropriate control recommendations.

Aphis glycines was first observed in the United States in 2000 when populations were discovered on soybean, *Glycine max*, in Wisconsin[29]. By 2003, *A. glycines* was established throughout a 12-state region, including all 99 counties of Iowa. Foliar-applied insecticides are primarily used to prevent yield losses caused by *A. glycines* throughout the northcentral United States[30]. Failures of foliar-applied pyrethroids to control field outbreaks have been reported since 2015 across several northcentral states[31]. Subsequent laboratory bioassays confirmed a decreased susceptibility to pyrethroids (bifenthrin and lambda-cyhalothrin) among populations collected from soybean fields[31,32].

The mechanism(s) of pyrethroid resistance in *A. glycines* is not fully understood, but the up-regulation of detoxification genes, cytochrome P450-dependent monooxygenases and esterases, were detected among resistant populations in the United States[33] and China[34]. In

addition, *kdr* and *super-kdr* mutations were characterized in a field survey of 24 *A. glycines* collected in the northcentral United States[23]. We tested the hypothesis that *kdr* and *super-kdr* mutations have a role in conferring resistance to pyrethroids in *A. glycines* through the application of relatively high throughput molecular screening technique. We confirmed the presence of *kdr* and *super-kdr* genotypes, and observed a significant increase of *kdr* alleles in field populations following the application of lambda-cyhalothrin. Our results revealed an association between these survivors and molecular markers for the *kdr* mutation, in particular a *super-kdr* (L1014F + M918I) genotype. The development and application of single-locus genetic markers as demonstrated in this study is not commonly undertaken for crop pests, and we discuss their potential use as diagnostic tools for predicting the occurrence of resistant phenotypes in field populations.

Materials and methods

Aphid populations

Laboratory colonies were established for susceptible and pyrethroid-resistant *A. glycines*, and used to explore the occurrence and phenotype of *vgsc* mutations. For this, separate susceptible laboratory colonies of biotype 1 (SBA-ISU-B1) and biotype 3 (SBA-ISU-B3) were reared in a Percival growth chamber (Percival Scientific, Perry, Iowa, USA) on *G. max* cultivars LD14-8007 and LD14-8002, respectively, as described previously[35]. The biotypes of these colonies are based on their response to *G. max* containing *Rag* (Resistance to *A. glycines*) genes; Biotype 1 is avirulent on any *Rag* cultivar while Biotype 3 is virulent on *Rag2 G. max*. Both SBA-ISU-B1 and –B3 colonies were maintained at Iowa State University for ≥ 6 years, and never exposed to insecticides. Additionally, putatively resistant *A. glycines* populations were initiated from survivors collected after exposure to field-applied rates of lambda-cyhalothrin

(Warrior II, Syngenta Crop Protection, Greensboro, NC) in Minnesota during 2017 (SBA-MN1-2017 and SBA-MN2-2017), and Iowa in 2017 (SBA-Sutherland-2017) and 2018 (SBA-Nashua-2018; Table 1). These populations were reared in separate growth chambers on *G. max* cultivar (NK S24-K2; Syngenta) without further exposure to insecticide. Insecticidal treatment free *G. max* seeds were sown into plastic pots filled with a soil mixture (Sungro Horticulture Products, SS#1-F1P) in plastic pots, and kept in a greenhouse at 25 ± 5°C and a 16:8 [L:D]. Plants were watered three times per week and after emergence they were fertilized weekly with a water-soluble formulation (Peters Excel Multi-Purpose Fertilizer, 21-5-20 NPK). Aphid-free *G. max* at V2-V3 growth stage[36] were added to population-specific lines weekly, whereon *A. glycines* propagated by parthenogenetic (clonal) reproduction.

Estimates of pyrethroid survivorship among field-derived aphids

We assessed the susceptibility to lambda-cyhalothrin of *A. glycines* populations (Table 1) by comparing estimated lethal concentrations required to cause 50% mortality (LC_{50}) following a leaf-dip bioassay[37]. A stock solution of technical grade lambda-cyhalothrin (97.7% purity, Control Solutions Inc., Pasadena, USA) was prepared in analytical acetone, and diluted into 7-8 treatment concentrations (0.0008-60 μg ml^{-1}) with distilled water containing 0.05% (v/v) Triton X-100 (Alfa Aesar, Tewksbury, USA). The final concentration of acetone in any treatment was ≤ 0.5% (v/v). The control treatment contained distilled water, 0.05% (v/v) Triton X-100, and ≤ 0.5% (v/v) acetone. The *G. max* cultivar NK S24-K2 (Syngenta) was grown in a greenhouse at 25 ± 5 °C and a 16:8 [L:D] as described above. Leaflets from first and second trifoliate were excised from V3-V4 *G. max*[36], cut into disks (3.8-cm diameter) with a hole punch (Fiskars, Helsinki, Finland). Each disk was manually submerged in a solution at each treatment concentration for 10 s with gentle agitation, and then air dried on a paper towel at room

temperature with the abaxial leaf side up. Subsequently, leaf disks were placed with their abaxial side up into 29.6 ml plastic souffle cups (Choice Paper Company, New York, USA) containing 1% w/v agar (Bacto™ Agar, Becton, Dickinson and Company, Franklin Lakes, USA) prior to congealing. Each cup was filled with approximately 20 ml of agar, leaving 10 ml to the top of the cups. A drop of distilled water was added to the agar bed to increase leaf disk adherence as described previously[31,37].

Apterous, mixed-aged adult *A. glycines* from SBA-ISU-B1, SBA-ISU-B3, and field-derived populations (Table 1) were collected from leaves of laboratory-grown soybean plants and transferred to Petri plates containing a filter paper moistened with distilled water. Twenty uninjured aphids from each population were randomly selected and transferred separately onto each leaf disk. A cup was considered an experimental unit, and each treatment had three independent replications with 20 aphids each. Cups were sealed with a close-fitting, ventilated lid and stored in a growth chamber at 25 ± 2 °C, 70% RH and 16:8 L:D. Mortality was assessed 48h post-treatment. Aphids unable to right themselves within 10 s once turned on their back were considered dead[31,37]. Slope, LC_{50} and 95% confidence interval (CI) were estimated for each population using a three-parameter log-logistic function of the 'drc' package in R[38]. LC_{50} values were considered different when there was no overlap of the 95% CI. A resistance ratio (RR) was calculated by dividing individual LC_{50} estimates for field collected populations or SBA-ISU-B3 by the LC_{50} of SBA-ISU-B1.

All the *G. max* plants used in the bioassays were grown from commercially available seeds. The experiments complied with relevant institutional, national, and international guidelines and legislation.

Synergist and cross-resistance bioassays

We used the most resistant (SBA-MN1-2017) and susceptible (SBA-ISU-B1) populations from the previously described leaf-dip bioassay to explore for evidence of metabolic resistance. The effects of the cytochrome P450 monooxygenase inhibitor, piperonyl butoxide (PBO; 91.2% purity, EcoSMART Technologies, Inc., Roswell, GA), the carboxylesterase inhibitor, triphenyl phosphate (TPP) (> 99% purity, Sigma-Aldrich, St. Louis, MO, USA), and the esterase inhibitor, *S,S,S*-tributyl phosphorotrithioate (DEF) (96% purity, Crescent Chemical Co., Inc, Islandia, NY, USA), were evaluated. First, leaf-dip bioassays were performed at six concentrations of each synergist to determine the highest concentration which resulted in ≤ 10% mortality in SBA-ISU-B1. Leaf-dip assays were performed as described above, except leaf discs were treated with a constant rate of PBO, TPP, or DEF (100 μg ml^{-1}) and a range of lambda-cyhalothrin (0.0008-60 μg ml^{-1}). The synergist alone served as the treatment control. Mortality was measured after 48 h. A three-parameter log-logistic function of the 'drc' package in R[38] was used to estimate the slope, LC_{50} and fiduciary 95% CI, and synergist ratio (SR; LC_{50} estimate lambda-cyhalothrin alone ÷ LC_{50} estimate lambda-cyhalothrin with PBO, TPP, or DEM) calculated for all dose-responses.

To assess patterns of cross-resistance to other insecticides, concentration-response leaf-dip bioassays were performed using SBA-MN1-2017 (pyrethroid-resistant) and SBA-ISU-B1 (susceptible) populations. We used a pyrethroid (bifenthrin, 98% purity, Chem Service, West Chester, PA), a pyridinecarboxamide (flonicamid, 99.5% purity, Sigma-Aldrich, St. Louis, MO, USA), a butenolide (flupyradifurone, 99.8% purity, Sigma-Aldrich, St. Louis, MO, USA), a sulfoximine (sulfoxaflor, analytical standard, Down Agrosciences, Indianapolis, IN, USA), and a tetramic acid derivative (spirotetramat, 99.6% purity, Sigma-Aldrich, St. Louis, MO, USA). All

were conducted using leaf-dip bioassays as described above, except mortality was assessed at post-exposure periods of 48 h for bifenthrin, flupyradifurone and sulfoxaflor, 72 h for spirotetramat, and 96 h for flonicamid. Slope, LC_{50} and fiduciary 95% CI were estimated as described above, and resistance ratio (RR; LC_{50} estimate for SBA-MN1-2017 ÷ LC_{50} values for SBA-ISU-B1).

cDNA sequencing of voltage-gated sodium channel alleles

The molecular basis for differing levels of pyrethroid resistance among the *A. glycines* laboratory populations (Table 1) estimated from between leaf-dip bioassays (above) was investigated next. This involved a candidate gene approach to predict the association of any nucleotide differences (mutations) in the full-length *A. glycines vgsc* transcript sequence in the pyrethroid-resistant populations. Since *vgsc* genes are not well annotated in the current *A. glycines* genome assembly, the 2105 amino acid conceptional translation (AAB47604.1) from the house fly, *Musca domestica, para*-type *vgsc* gene[39] (accession U38813.1) was used as the query to search protein models from the official gene set (OGS) v 6.0 (Ag_bt1_v6.0) of the *A. glycines* biotype 1 genome [40]. We searched with the BLASTp algorithm at a web interface maintained at AphidBase (https://bipaa.genouest.org/sp/aphis_glycines/blast/)[41], and filtered results for "hits" with *E*-values ≤ $1.0e^{-100}$. Genome scaffold positions of gene models were retrieved from the OGS6.0_20180125.gff3 file. BLAST output was used to define the targets for our subsequent confirmatory sequencing and generation of evidence for gene annotation.

To generate evidence for gene annotation, putative *vgsc* transcripts (cDNAs) corresponding to *A. glycines* Ag_bt1_v6.0 gene models AG6007485, AG6007488, and AG6007489 were sequenced from susceptible and field-derived resistant populations (Table 1). Specifically, oligonucleotide primer pairs AG6007485-F and -R, and AG6007489-F: (5'-ATG

AGT GTG TAT AGT AGT GAG GAA CTC C-3') and AG6007488-R (5'-TTA GAC ATC GGC GAG TCT TGA G-3') were designed using Primer3[42] (start ATG codons and reverse complements of stop codons TAA and TAG are double underlined). Total RNA was extracted in duplicate from a pool of 2-3 mixed age apterous *A. glycines* from each population using the RNeasy® Plus Micro Kit (Qiagen Hilden, Germany) according to the manufacturer's protocol. Genomic DNA contamination was removed using TURBO DNA-free™ kit (Ambion®, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's directions. First strand cDNA was synthesized from 30 to 200 ng DNase-treated total RNA in iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, California, USA) reactions with an extension of 30-45 min at 46 °C. RT-PCR reactions included 18.625 µl deionized H_20, 10.0 µl 5X GoTaq polymerase buffer (Promega, Madison, WI, USA), 3.75 µl 25.0 mM MgCl, 0.3125 µl 25 mM dNTPs, 1.0 µl of each forward and reverse primer pair (10 µM), 0.3125 µl of 5U μl^{-1} Go*Taq* DNA polymerase (Promega), and 15.0 µl of 10 ng μl^{-1} first strand cDNA. Amplification was performed by a touchdown procedure: initial denaturation at 96°C for 2.5 min, then seven cycles of 96°C for 30 s, 66°C for 30s (decreasing 2°C each cycle), 72°C for 8 min, then 40 cycles of 96°C for 30 s, 54°C for 30 s, 72°C for 8 min with a final extension at 72°C for 10 min on a Tetrad2 thermocycler (BioRad). PCR products (10.0 µl each) were separated by 1% agarose gel electrophoresis, and residual primers dephosphorylated and degraded in the remaining PCR volume as described earlier[43]. Treated PCR products were diluted 1:8 with deionized H_2O and submitted to the Iowa State University DNA Facility (Ames, IA, USA) for a bidirectional primer walking by Sanger sequencing on an ABI3700 (Applied Biosystems, Forest City, CA, USA) using internal oligos.

Trimmed high quality Sanger reads were assembled into individual cDNAs using CAP3[44], and conceptual translations were predicted for each using TransDecoder v5.5.0 (https://github.com/TransDecoder/TransDecoder/releases). A multiple translated protein sequence alignment between *M. domestica* (AAB47604.1), the *A. glycines* gene model AG6007485-RA, and our cDNAs was generated using the Clustal Omega algorithm[45] (default parameters) with the web-based tool located at https://www.ebi.ac.uk/Tools/msa/clustalo/. Structural annotations consisting of four domains (DI-DIV) each with six transmembrane region (TMR) segments (S1-S6) from the *M. domestica vgsc* protein[16] were used to identify orthologous positions in aligned *A. glycines* proteins. Multiple sequence alignments were similarly generated between the conceptual translations from *A. glycines* gene models and cDNAs, and orthologs in GenBank accessions from aphids (NCBI Taxonomy ID 27482). These included recently published translations for *A. glycines* (QTJ01838.1- QTJ01843.1)[23].

Comparisons among assembled *A. glycines vgsc* cDNAs were then used to predict variation with and between resistant and susceptible populations. An intraspecific multiple nucleotide sequence alignment among *de novo* assembled cDNAs and corresponding *A. glycines* gene models was performed using the Clustal Omega algorithm[45] as described above. Alignments were overlaid with conceptual translations, structural annotations, and nucleotide variation within individual contigs predicted as co-occurring electropherogram peaks in constituent Sanger trace data using Pearl[46].

Prediction and validation of *kdr* and *super-kdr* mutations

Given that the putative *A. glycines vgsc* mutations are fixed differently between resistant and susceptible populations based on our preceding comparisons of cDNA sequencing, we subsequently 1) used direct Sanger sequencing of genomic DNA amplicons to verify these

mutations within these regions and detect variation in a larger number of individual aphids from each of the laboratory populations (increased sample size compared to that for cDNA), and 2) developed and validated genetic makers to detect mutations in individual aphids. For the first goal we used predicted mutations in DIIS4-6 based on our cDNA data, and mutations in DIIIS6-DIVS1, and DIVS4-S6 previously reported to be associated with pyrethroid resistance in mosquitoes[47], and mutations in DIIS4-S6 described in *A. glycines*[23]. Primers to amplify these regions were designed from SBAphidCtg1013 sequence data of the *A glycines* Ag_Bt_V6.0 genome assembly[40] using Primer3[42]. Individual aphids (4-8) were sampled from 1) four lines collected from fields with suspected pyrethroid resistance and susceptible laboratory populations (Table 1) and 2) two laboratory populations previously shown to be susceptible to insecticides[23,48] .

Genomic DNA was extracted from all aphids individually using QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI) as described by the manufacturer, except volume adjusted to 50.0 µL per sample. DNA quantities were estimated on a DeNovix DS-11 (DeNovix Inc, Wilmington, DE, USA), and samples diluted to 10ng μl^{-1} with deionized water. PCR reaction setup and touchdown amplification reactions were performed as described above, except reaction volumes scaled to 10 µl and included primers for DIIS4-6, DIIIS6-DIVS1, or DIVS4-S6. Thermocycler extension times were reduced to 1 min. A 5.0 µl aliquot of each PCR reaction product was separated by 2% agarose gel electrophoresis. Residual primers digested and dephosphorylated in remaining product volumes, then diluted 1:8 or 1:10 and submitted for Sanger sequencing with corresponding forward or reverse primers as described above. Electropherogram data were aligned against genomic scaffold SBAphidCtg1013, trimmed, and variant nucleotide positions predicted at a Phred quality cutoff score of 20 using novoSNP[49].

Resulting trimmed sequences were aligned and annotated as described above and accessioned in the NCBI non-redundant nucleotide database.

The putative *A. glycines* L1014F *kdr* mutation was detected by a PCR-restriction enzyme fragment length polymorphism (RFLP) assay. Primers AG*kdr*-F and -R primers were designed to amplify a 439 bp fragment in DII S6 (positions 2847 to 3286 of AG6007485-RA) with a single *Bst*EII restriction enzyme recognition site (GGTNAA[C/A]; variant nucleotides causing L1014F mutation in brackets) using Primer3[42], and synthesized at Integrated DNA Technologies (IDT; Coralville, IA, USA). Individual reactions consisted of 3.75 µl deionized H_20, 2.0 µl 5X GoTaq polymerase buffer (Promega, Madison, WI), 0.75 µl 25.0 mM $MgCl_2$, 0.0625 µl 25 mM dNTPs, 0.1875 µl of each forward and reverse primer at 10 µM, 0.0625 µl of 5U μl^{-1} Go*Taq* DNA polymerase (Promega), and 3.0 µl of 10 ng μl^{-1} gDNA. All gDNA templates used for *kdr* validation were from the same samples used in Sanger sequencing (above). Amplification of the locus used a touchdown procedure[43]. The entire volume of each PCR product (10.0 µl) was digested by addition of 8.9 µl deionized H_20, 1.0 µl 10X Buffer 3.1 (New England Biolabs, Ipswich, MA), and 0.1 µl *Bst*EII (0.1 U; New England Biolabs). Digestion reactions were incubated overnight at 60°C, then separated by 2% agarose gel electrophoresis. Samples were genotyped based on of two fragments (154 and 285 bp) among homozygotes for susceptible alleles, an undigested 439 bp fragment for homozygous resistant individuals, and heterozygotes defined by co-occurrence of resistant and susceptible alleles (154 bp, 285 bp, and 439 bp).

A ligase chain reaction (LCR) based marker assay was developed to detect the *A. glycines* M918I mutation. For this, a 151 bp region of the *vgsc* gene encompassing the M918I locus (positions 2725 to 2876 of AG6007485-RA) was PCR amplified with primers AG*skdr*-F and -R. The same reaction and thermocycler parameters and samples were used as for the *kdr*

amplicon (above). PCR reaction products were diluted 1:20 using deionized H_20, and used as template in subsequent LCR reactions.

LCR assays consisted of three separate oligonucleotide probes. The upstream allele-specific wildtype susceptible P1-918_Met_G and mutant P1-918_Ile_A probes. The 5'-phosphorylated P2-918_Phos probe annealing downstream and immediately adjacent to P1 probes. Individual 10.0 µl LCR reactions included 1.0 µl of 10X *Taq* DNA Ligase Reaction Buffer (New England Biolabs), 0.4 µl of *Taq* DNA Ligase (40U μl^{-1}; New England Biolabs), 1.0 µl of each P1-918_Met_G, P1-918_Ile_A, and P2-Phos probes at 0.2 µM, and 5.0 µl of 1:20 diluted AG*skdr*-F and -R amplified PCR product as template. Ligation reactions were incubated at 94ºC for 2 min, followed by 2 cycles of 94ºC for 20 s and 75ºC for 10 min, and then held at 15ºC.

LCR reaction were diluted 1:20 with deionized H_2O, and 2.0 µl used in PCR reaction and amplification conditions identical to those above except for use of M13_5p17nt-F and M13_5p18nt-R primers. Genotypes were determined according to predicted sizes from amplified probes specific for wildtype P1-918_Met_G (141 bp) and mutant P1-918_Ile_A alleles (165 bp) following 3% agarose gel electrophoresis.

In-field association of pyrethroid resistant genotype to phenotype

We conducted two experiments to determine the relationship between markers for the *vgsc* mutations and *A. glycines* survival when exposed to pyrethroids. We used field collected aphids for both experiments. In the first experiment, we used a previously determined diagnostic concentration of lambda-cyhalothrin and bifenthrin developed for glass-vial bioassays to assign aphids to survivor (putative resistant) and moribund (susceptible) groups[32]. This diagnostic concentration is an accepted tool that can be used by field entomologists for making management

decisions. Aphids tested within the bioassays were subsequently genotyped with our L1014F *Bst*EII PCR-RFLP and M918I LCR genetic markers. We tested the hypothesis that survivors of the glass-vial bioassay would have a higher frequency of mutations in the *vgsc* genes.

Individual *A. glycines* were collected from three locations (Darwin, Sutherland, and Kanawha) with a history of frequent pyrethroid use to manage *A. glycines* and one (Boone) with lack of this history. Aphids were collected during August 2019 and had not been treated with foliar insecticides. In 2020, aphids were similarly collected at two locations (Sutherland and Kanawha) in late July and early August. Infested *G. max* leaflets were collected randomly from approximately 40 plants at each location, and transported to the laboratory. Leaflets were transferred to Petri plates containing a moistened filter paper, sealed with Parafilm and incubated in a growth room at 25 ± 2 °C, 50% RH and 16:8 [L:D]. Aphids were taken from leaflets for use in glass-vial bioassays within one week after collection. The bioassays were based on a previously published methodology[31,32] using 20-ml glass-vials coated with technical grade of bifenthrin (0.0215 µg A.I./ 0.5 ml/vial) and lambda-cyhalothrin (0.2521 µg A.I./0.5 ml/vial), along with control treatment (acetone). Briefly, ten healthy apterous mixed-age adult aphids were transferred to the bottom of each treated glass-vial, capped, and incubated upright at room temperature. Mortality was assessed 4 h post-infestation[31,32]. Surviving and moribund (dead) aphids were collected, placed individually into 1.5 ml microcentrifuge tubes, and stored at -20°C. DNA was extracted and genotypes were determined for survivor and moribund aphids using L1014F *Bst*EII PCR-RFLP and M918I LCR assays as described above. The association between genotypes and corresponding phenotype, surviving (resistant) *vs*. moribund (susceptible) following bioassay, was performed for each field collection site using Fisher's exact tests implemented in R version 3.5.1[50].

In a second experiment, changes in the frequency of genotypes before and after an application of lambda-cyhalothrin (Warrior II, Syngenta Crop Protection, Greensboro, NC; full rate of 0.14 l ha^{-1}) were assessed at three locations in 2019 and at two locations in 2020 (Table 5). For this, a "pre-application" sample was taken < 7 days prior to the foliar application of lambda-cyhalothrin, and a "post-application" sample taken 2-3 days after an application. L1014F *Bst*EII PCR-RFLP and M918I LCR assays were performed as described above on individual aphids from pre- and post-application samples. Differences in genotypic frequencies between pre- and post-application groups were analyzed for each field using Fisher's exact tests implemented in R version 3.5.1[50]. A binomial generalized linear model (GLM) with a logit link function implemented in base R[50] was used to evaluate changes in resistance allele frequency (RAF) between groups. The model included time (pre- and post-application) and location as predictor variables, and RAF for 1014F *kdr* and 918I compared to the wild type susceptible (S) alleles 1014L and 918M, respectively, as explanatory variables. Estimates of allele frequencies, confidence intervals, contrasts, and odds ratios (OR) were computed using the R package 'emmeans'[51].

Results

Estimates of pyrethroid survivorship among field-derived aphids

Initial leaf-dip bioassay results revealed that populations collected from fields with a history of reduced pyrethroid efficacy had significantly higher estimated LC_{50} for lambda-cyhalothrin compared to susceptible controls. Specifically, the LC_{50} estimates for susceptible SBA-ISU-B1 (0.38 ± 0.09 μg ml^{-1}) and SBA-ISU-B3 (0.43 ± 0.07 μg ml^{-1}) were significantly lower than the LC_{50} estimated for all field-collected aphids (range 1.51 ± 0.32 to 18.33 ± 4.41 μg

ml^{-1}; Table 1). The corresponding RR of the field populations derived from the LC_{50} of the SBA-ISU-B1 ranged from 3.94 to 48.23.

Synergist and cross-resistance bioassays

Bioassays that included a pyrethroid with a detoxification enzyme inhibitor (i.e. synergists) did not significantly affect the estimated LC_{50} for SBA-MN1-2017 based on the non-overlapping 95% confidence intervals (CI). The synergist ratio for PBO, TPP and DEF was estimated at 1.27, 1.28 and 1.30, and 1.31, 1.26, and 1.31 for SBA-MN1-2017 and SBA-ISU-B1, respectively (Table S1). The exposure of pyrethroid-resistant and susceptible populations to other insecticides revealed limited variation, indicating no cross-resistant to insecticides with different mode of action (MoA; Table 2). SBA-MN1-2017 was 33.90-fold more resistant to bifenthrin compared to the susceptible control. There were no significant differences in estimated LC_{50} between SBA-MN1-2017 and SBA-ISU-B1 for the other insecticides.

cDNA sequencing voltage-gated sodium channel alleles

Results of BLAST searches and evidence from our full-length cDNA sequencing defined two *A. glycines vgsc* heterodimers that together comprise the complete coding sequence that was lacking from the current gene models. A search of proteins from the Ag_bt1_v6 OGS with the translated *M. domestica vgsc* protein sequence, AAB47604.1, identified three putative hits; the 1174 aa AG6007485-PA, 649 aa AG6007488-PA, and 359 aa AG6007489-PA (*E*-values $\leq 2.0e^{-103}$, identities $\geq 52.7\%$). Parent transcripts AG6007485-RA, AG6007488-RA, and AG6007489-RA were 3525, 1950, and 1080 bp, respectively, and were encoded on contig SBAphidCtg1013 Ag_bt1_v6 (Fig. 1A). These results demonstrated that the *A. glycines* gene model is fragmented. Our annotation data came from full-length cDNA amplification products which provided evidence for two distinct *A. glycines vgsc* transcripts. Transcript sizes did not vary within or

between susceptible and resistant individuals; an ~3,500 bp product from AG6007485-RA and an ~3,100 bp cDNA produced from primers annealing to the C-terminal CDS of AG6007488-RA and N-terminal CDS of AG6077489-RA (results not shown). AG6007485-RA and combined AG6007488-RA and AG6077489-RA gene models were referred to a heterodimer H1 (*vgsc-h1*) and H2 (*vgsc-h2*), respectively, following convention for heterodimeric *vgsc* among aphids. Assembly of 2749 to 3453 bp cDNA sequences (contigs) for AG6077485-RA from twelve individuals resulted in six with a putative full-length 1150 aa translated open reading frame (ORF) (GenBank accessions MW759883.1–MW759893.1). The 3453 bp consensus *vgsc-h1* cDNA showed ≥ 99.8% nucleotide similarity to the 3525 bp gene model AG6007485-RA, and 3489, 3453, and 3588 bp isoforms, X1, X2, and X3, respectively, previously predicted in accession MT379843.1. Intraspecific splice variation involved 33 bp (11 aa) of exon 2 and the initial 39 bp of exon 16 in AG6007485-RA compared to all cDNAs in this study, and inclusion of a single valine (GTA) in MT379843.1 isoform X2.

Translated *A. glycines vgsc-h1* transcript variants showed ≥ 68.48% identity when aligned to *M. domestica* AAB47605.1 wherein residues putatively orthologous DI S1-S6 and DII S1-S6 were identified in *A. glycines* and in other aphid species. This orthology was also used to define location of putative variation among *A. glycines*. Five putative nucleotide variant sites (e.g. single nucleotide polymorphisms, SNPs) were predicted among *A. glycines vgsc-h1* cDNAs, two that putatively cause amino acid changes in DII S1-S6; a C to T transition mutation at AG6007485-RA position 3070 (1st codon position of residue 1014) leads to a nonsynonymous leucine to phenylalanine change (L1014F), and a G to A transition at 2784 was predicted to cause a methionine to isoleucine change at amino acid position 918 (M918I) in AG6007485-PA (Fig. 1B). Corresponding electropherograms showed co-occurring C and T signals (pyrimidine;

Y) at position 3070, and G and A (purine; R) at AG6007485-RA position 2784. Electropherograms with one or both of these co-occurring signals (Fig. 1C) were only observed in *A. glycines* resistant to the pyrethroid lambda-cyhalothrin. No other amino acid changing mutations were detected.

The 13 independently assembled *A. glycines vgsc-h2* cDNAs were between 2,682 to 2,877 bp (MW759894.1 – MW759904.1), wherein six encoded a complete 958 aa ORF and all encoded DIII S1-6 and DIV S1-6 (named isoform *vgsc-h2* X5). Assemblies provided evidence for a merger of gene models AG6007488-RA and AG6077489-RA (Fig. 1A), predicted to encode DIII S1-6 and DIV S1-6. Our cDNAs encoded a protein within the 813 to 960 aa range and showed ≥ 92.9% identity to other aphid *vgsc* proteins. N-terminal residues in previously defined isoforms X1 and X2 predicted in QTJ01841.1 and QTJ01842.1 (n = 36), respectively, or isoform X3 in QTJ01843.1 (n = 9) were not encoded in our isoform X4 nor shared with any aphid orthologs. Additionally, isoforms X1 and X3 encode a 41 aa insertion that putatively interrupted DIIIS4. A multiple sequence alignment among *A. glycines vgsc-h2* transcripts identified 13 variant sites, 10 of which were in 3rd codon positions and not predicted to cause amino acid changes. Of the three nonsynonymous changes, those orthologous to *M. domestica* positions 1219, 1424, and 1430 caused putative glycine to serine, leucine to valine, and valine to glycine changes, respectively. The G1219S mutation was in a non-conserved linker region between DIIS6 and DIIIS1. V1424G and L1430V mutations are both in the DIII S5 α-helix, of which the former is only predicted in two resistant aphids to involve residues with short chain aliphatic side changes. The L1430V mutation was predicted in resistant as well as susceptible aphids.

Prediction and validation of *kdr* and *super-kdr* mutations

Results of comparisons made among Sanger reads generated from short genomic DNA amplicons from a larger sample set provided secondary confirmation of cDNA-based predictions. Specifically, comparison of aligned Sanger sequence from DIIS4-S6 (range 1323 to 949 bp; GenBank accessions MW846869-MN846958), DIIIS6-DIVS1 (414 bp; MW847052-MW847146), and DIVS4-S6 (536 bp; MW846959-MW847051) predicted four, one, and two nucleotide substitutions, respectively. Of these seven variable sites, five were predicted in 3rd codon positions and to be synonymous (non-amino acid changing). Two of four variants among *vgsc-h1* DIIS4-S6 fragments were in introns, and two putatively causing amino acid changes when comparing among susceptible SBA-ISU-B1 -B3, and -B4 and field collected resistant SBA-MN1-2017, SBA-MN2-2017, SBA-Sutherland-2017, and SBA-Nashua-2018 populations (Table 1; Table 3). Specifically, a putative G to A transition in the extracellular loop region between DII S4 and S5 at position 87 of the 1323 bp consensus alignment was predicted to cause a methionine to isoleucine mutation at aa position 918. The M918I locus was predicted to be homozygous for the wildtype G allele (single electropherogram peak) among all 21 susceptible and 21 resistant aphids, but heterozygous with co-occurring G and A nucleotides (Fig. 1C) for 17 aphids from the resistant lines. No A nucleotides were predicted among susceptible aphids. This DIIS4-S6 fragment also showed a C to T transition (Table 3), leading to a putative L1014F *kdr* mutation in the *A. glycines vgsc-h1* DII S6 transmembrane region (Fig. 1B). All aphids from susceptible lines were homozygous for the C allele. In contrast, 33 and 5 aphids from the resistant lines were heterozygotes (co-occurring C and T peaks) and homozygous for the T allele, respectively (Fig. 1C).

Furthermore, our results validated two single locus genetic markers that detect nonsynonymous (amino acid changing) mutations in the *A. glycines vgsc-h1* DII S1-S6. Preliminary validation of the L1014F *kdr* mutation by a *Bst*EII PCR-RFLP assay resulted in digestion reaction fragments of 154bp and 285bp across all individuals from the SBA-ISU-B1 population (n = 16), and corresponded to homozygotes for the C nucleotide that retain the *Bst*EII recognition sequence (5'-GGTNAC**C**-3') and fixed for the leucine amino acid (Fig. 1B). In contrast, all 16 aphids from the SBA-MN1-2017 population were heterozygotes showing three gel fragments in *Bst*EII PCR-RFLP assays; 154, 285, and 439 bp. These corresponded to an overlap in 154 and 285 bp fragments indicative of the C nucleotide alleles and the non-digested 439bp fragment derived from alleles with a T nucleotide that removes the *Bst*EII recognition site (5'-GGTNACT-3') and encoded a phenylalanine (F) amino acid.

The LCR assay resulted in a single 141 bp amplified fragment derived from ligation of P1-918_Met_Gand P2-918_Phos probes among all susceptible SBA-ISU-B1 individuals, and corresponded to predicted susceptible G allele homozygotes encoding a methionine at *A. glycines vgsc-h1* position 918. This 141 bp fragment was also amplified along with a 165 bp fragment among all resistant SBA-MN1-2017 individuals, where the latter corresponded to predicted size of the P1-918_Ile_A P2-918_Phos probe ligation product. This co-amplification represented heterozygous genotypes, with alternate alleles encoding methionine (M) and isoleucine (I) amino acids at position 918. A single 165 bp LCR product was not generated among pyrethroid-resistant SBA-MN1-2017 individuals.

In-field association of pyrethroid resistant genotype to phenotype

In our first experiment, we observed significant changes in the frequency of *vgsc* mutations in *A. glycines* following exposure to a diagnostic concentration of bifenthrin and

lambda-cyhalothrin (n = 462 aphids tested across both insecticides and all locations; Table 4). For all aphids, the frequency of *kdr* mutations was greater in survivors than moribund aphids (Table 4). The *super-kdr* heterozygote genotype (1014 L/F:918 M/I) showed the greatest proportional increase (≥ 16.9%) among survivors across locations in both years, whereas wild type aphids (L/L:M/M) decreased ≥ 10.7%. We also observed variation by year and active ingredient, for example, genotype frequency changed significantly after bifenthrin was applied only in 2019. In 2020, the proportions of *A. glycines* with a mutation changed significantly between survivor and moribund aphids exposed to lambda-cyhalothrin (P-value = 0.024), but not bifenthrin (P-value = 0.722; Table 4). We did not observe a significant change in genotypic frequency between survivor and moribund aphids following bifenthrin exposure despite 94.4% of survivors being homozygous *kdr* (F/F:M/M) (Table 4).

In our second experiment, we observed significant changes in the RAF for aphids collected pre- and post-application of a pyrethroid in the field (Table 5). Based on a total of 575 pre-application and 378 post-application *A. glycines* collected and genotyped from fields in Iowa, we observed a significant difference in genotypic frequencies between pre- and post-application in 2019 and 2020 when pooled across locations (P-values ≤ 0.0397; Table S2). Significant changes were detected at Boone and Kanawha in 2019, and Sutherland in 2020. The heterozygote *kdr* genotype was the most prevalent among survivors after insecticide was sprayed across all locations in 2019 (55.1%) and homozygote *kdr* was correspondingly most prevalent in 2020 (Table S2). However, the *super-kdr* heterozygote genotype showed the greatest proportional increase across locations in 2019 (+17.2%) and 2020 (+29.7%). There were significant changes in the RAF for 1014 *kdr* and 918 loci between pre- and post- application in 2019 and 2020 (P-values ≤ 0.0174; Table 5). A significant increase in *kdr* allele frequency was

detected at two of three Iowa locations in 2019 (P-values ≤ 0.0240) and one of two locations in 2020 (P-value < 0.0001; Table 5). There was no significant change in the RAF for 1014 *kdr* at Kanawha in 2019 and Sutherland in 2020. This is likely due to a high occurrence of mutations before insecticide was applied as the RAF was high in the pre-application sample (≥ 57.5%) and nearly equal to post-application estimate (≥ 60.7%) at these locations. Combined across all locations, the odds of the 1014F *kdr* allele being found among *A. glycines* post-application was 1.73- and 1.56-times greater compared to pre-application in 2019 and 2020, respectively. Significant differences were also predicted in RAF of M918I between pre- and post-application aphids when pooled across all locations in 2019 and 2020 (P-values ≤ 0.0002; Table 5), but individually only at Kanawha in 2019 and Nashua in 2020. Odds ratio for 918I allele presence in post- compared to pre-application aphids was 2.15 and 3.63-times greater across locations in 2019 and 2020, respectively. The RAF for 918I (range 2.9 to 14.8) was lower compared to that of 1014F among pre-application sampled aphids (23.2 to 82.7), as well as among post-application samples (918I: 5.3 to 50.0; 1014F: 50.0 to 77.9).

Discussion

We identified populations of field-collected *A. glycines* with a resistant phenotype as determined by estimating the LC_{50} using a leaf-dip bioassay. Two non-synonymous mutations in *vgsc* genes that are known to confer the knockdown resistant (*kdr*) phenotype in other insects were found in pyrethroid-resistant *A. glycines*. This phenotype has reduced sensitivities to paralysis caused by pyrethroids and DDT[52,53], which are linked to mutations in the α subunit of the *vgsc* gene expressed in neurological tissues[12]. Insect *vgsc* genes encode four protein domains (DI–DIV), each containing six α-helical transmembrane segments (S1-S6)[54], where S5-S6 and their linker region form the sodium pore channel. In most insects, a single *vgsc* gene encodes all

functional domains, with the exception of species in the Aphididae that encode DI-DII and DIII-DIV in separate heterodimers referred to as H1 and H2, respectively[55,56]. Our cDNA evidence and prior sequence data[23] support the presence of *A. glycines vgsc-h1* and *-h2*, where the latter is a revision of two Ag_bt1_v6 gene models into a single 958 aa *A. glycines vgsc-h2* protein encoding DIII-DIV. Multiple *vgsc* isoforms may arise via extensive alternate transcript splicing[57] with up to six *vgsc* isoforms predicted for a given aphid species, and four isoforms for both *A. glycines vgsc-h1* and *-h2*.

The structure of *A. glycines vgsc-h1* and *-h2* heterodimers defined in this study partially differs with recent *A. glycines* transcript models[23], where the latter predicts splice variation and coding sequence that lacks homology and is not supported by comparative analyses to aphid orthologs. Specifically, N- and C-terminal coding regions of the *vgsc-h2* isoforms X1, X2 and X3 and *vgsc-h1* isoform X3, respectively, are not present among orthologs from other aphids, suggesting validation of these prior transcript models[23] may be warranted. There may be a range of diversity in *vgsc* isoforms among *A. glycines* but we did not observe differences in splice variation between pyrethroid-resistant and susceptible *A. glycines*. Regardless, the specific nonsynonymous mutations we identified within the *vgsc* genes appear to be at least associated with, if not responsible for, resistance to pyrethroids.

The substitution mutations, L1014F and M918I, are among 61 in the *vgsc* gene found to be associated with varying levels of pyrethroid resistance in other insect species[10,58]. Our sequence data from cDNA and genomic DNA fragments of *A. glycines vgsc-h2* show no variation at positions orthologous to some of these other mutations (i.e. 1524, 1528, 1538, or 1549 in DIIIS6, or 1752 or 1821 in DIVS5-S6)[16]. The M918T and L925M mutations previously detected in a survey of *A. glycines*[23] were not present in *vgsc-h1* sequences sampled in this study,

which suggests that multiple genotypes may lead to the general phenotype of pyrethroid resistance. The L1014F mutation in the *M. domestica* is associated with pyrethroid resistance, but higher resistance levels were observed when it co-occurs with the M918T mutation (e.g. *super-kdr* M918T + L1014F genotype)[15,16]. Functional studies demonstrate resistance is conferred by the *vgsc* 918T variant alone[59], but the greatest resistance has been observed for the 918T + 1014F *super-kdr* variant in the *Drosophila vgsc* (paralytic, *para*) protein[60]. Analogously, the M918I mutation is present in pyrethroid resistant tropical beg bug, *Cimex hemipterus*[61], but only the *super-kdr* genotype (L1014F + M918I) was identified in resistance populations from Hawaii[62] and China[63]. When considering each of these independent cases (*C. hemipterus*, *M. domestica*, and *Drosophila*), the combined evidence suggests an interdependence of mutations at 918 and 1014 positions which can produce high levels of resistance. A previous study suggested a similar phenomenon may occur with *A. glycines*. The M918I + L1014F genotype was described in five *A. glycines* from three Minnesota populations with a history of pyrethroid resistance[23]. Data reported herein suggest that the heterozygote *kdr* (L1014F) and *super-kdr* (L1014F + M918I) genotypes are associated with survival of *A. glycines* exposed to bifenthrin and lambda-cyhalothrin. We observed a significant increase in RAF for alleles encoding both 918I and 1014F, which was directly connected to more aphids with the *super-kdr* (918I + 1014F) genotype in field populations.

This study demonstrated the significant increase in survival among *A. glycines* with a homozygous *kdr* genotype, which partially agrees with evidence that the L1014F *kdr* mutation alone is associated with pyrethroid resistance in field-derived strains of *M. persicae*[17], *A. gossypii*[22], and *S. avenae*[21]. Furthermore, genotypes homozygous for 1014F are more resistance than the heterozygous genotypes[14,64]. This study revealed that field-collected resistant *A. glycines*

were mostly either *kdr* or *super-kdr* heterozygotes. Specifically, pyrethroid resistant phenotypes were likely conferred by the M918I + L1014F *super-kdr* genotype, but a smaller proportion were heterozygote and homozygote 1014F *kdr* genotypes. This pattern is consistent with phenotypic evidence derived from *M. domestica*[15,16] and assays that evaluated how different mutations in the *vgsc* affect pyrethroid efficacy[60].

There was a stronger association between our genetic markers and a resistant phenotype when aphids were screened using in-field application versus a diagnostic concentration via glass vials treated with insecticides (Table 5 and Table 4, respectively). These differences can be attributed to two sources of variation. The first is the concentration of insecticide that the aphids were exposed to in the glass vials compared to the field. The exposure and conditions in the vials are different compared with field conditions, given that aphids are unable to feed and are consistently exposed to the insecticide. Second, a limited number of individuals survived the glass vial assays due to likelihood that the diagnostic concentration used could have resulted in the death of a high number of resistant individuals[65,66]. This result from the bioassay may not accurately or consistently define field-resistant phenotypes. Therefore, a significant increase in the M918I + L1014F *super-kdr* genotype following a field application of pyrethroids is likely more relevant to field scenarios.

Pyrethroid resistance in *A. glycines* has been previously associated with an increase in the expression of detoxification enzymes, including cytochrome P450 monooxygenases[33,34]. When we combined synergists that inhibit detoxification enzymes to a pyrethroid, we did not observe a significant change in mortality of the SBA-MN1-2017 population (Table S1). While these results suggest that enhanced detoxification may not be involved in the pyrethroid resistance observed in SBA-MN1-2017, we did not confirm that the synergists inhibited enzyme activity. In addition,

PBO is known not to inhibit all P450 monooxygenases equally[67], which could further influence the ability to accurately evaluate the role of P450s in pyrethroid resistance. Future studies including companion measurements of enzyme activity and transcript expression likely will provide more definitive results.

The increased frequency of pyrethroid resistance among *A. glycines* populations in the northcentral United States[31,32] likely evolved due to strong selection pressure from prophylactic application of insecticides with a single mode of action[68]. This resistance is an economic and environmental concern, and arguably would benefit from the implementation of IRM strategies. Specifically, IRM prescribes the application of measures and tactics that delay or prevent the onset of insecticide resistance, or mitigate the effects resistance that has developed to one class of insecticides by maintaining susceptibly to alternative control measures that remain efficacious[69]. Monitoring changes in the frequency of resistance using genetic markers is feasible[70,71]. Due to the likely involvement of multiple *vgsc* mutations in *A. glycines* resistance, markers in addition to ours for M918I and L1014F may be required to account for *vgsc*-based resistance in all populations. Incorporation of such molecular-based diagnostic data into crop management decisions and pest management strategies is yet to be fully realized. Genetic markers are arguably better suited for detecting resistance because they do not require the use of living insects and may be more efficient in processing larger sample sizes compared to using diagnostic bioassays[72]. Additional research is necessary to determine the contribution of other mechanisms and traits that confer resistance in *A. glycines* (e.g. detoxification enzyme production) alone or in association with *kdr*, *super-kdr*, or other genotypes. Despite this partial knowledge regarding the mechanism(s) of resistance, the genetic markers developed in this study

are resources for estimating the frequency and tracking the spread of resistance in field populations of *A. glycines*.

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Author contributions

I.V., J.D.H. and B.S.C. contributed equally to this work. I.V., J.D.H. and J.G.B. performed laboratory methods. I.V., J.D.H and B.S.C. analyzed data and led manuscript writing. J.M and R.L.K. provided aphids and glass-vial materials. I.V., J.D.H., B.S.C., E.W.H. and

M.E.O. contributed critically to the study design. All the authors contributed to the manuscript drafts and gave approval for publication.

Competing interests

The authors declare no competing interests.

Tables and Figures

Table 3.1. Estimates of pyrethroid (lambda-cyhalothrin) resistance among *Aphis glycines* at Iowa (Nashua and Sutherland) and Minnesota (MN1 and MN2) locations from leaf-dip bioassays, compared to susceptible controls from biotype 1 (SBA-ISU-B1) and biotype 3 (SBA-ISU-B3) specific laboratory colonies.

Collection	Origin	*n*	LC_{50}[a]	95% CI[b]	Slope	χ^{2}[c]	df	RR[d]
SBA-ISU-B1	Laboratory	480	0.38d	0.29-0.46	2.51 ± 0.45	5.08	5	-
SBA-ISU-B3	Laboratory	540	0.43d	0.36-0.50	4.22 ± 0.98	7.27	6	1.16
SBA-MN1-2017	Field	540	18.33a	13.92-22.74	2.08 ± 0.404	6.53	6	48.23
SBA-MN2-2017	Field	480	14.66a	10.42-18.91	1.91 ± 0.412	9.96	5	38.57
SBA-Nashua-2018	Field	1080	6.19b	4.49-7.90	1.25 ± 0.13	18.00	6	16.28
SBA-Sutherland-2017	Field	540	1.51c	1.18-1.83	2.86 ± 0.48	10.60	6	3.94

[a]LC_{50} values designated by different letters within a column are significantly different from each other through non-overlap of 95% confidence intervals.
[b]CI, confidence interval.
[c]Chi-square testing linearity of concentration-mortality responses.
[d]Resistance ratio (RR): LC_{50} of individual test population divided by LC_{50} of susceptible SBA-ISU-B1.

Table 3.2. Levels of cross-resistance of *Aphis glycines* from field-collected SBA-MN1-2017 across different classes of insecticides; common name (IRAC classification).

Insecticide	Population	n[a]	LC_{50} (95% CI)	Slope± SE	χ^{2} (df)[b]	RR[c]
Bifenthrin	SBA-ISU-B1	540	0.66 (0.52 - 0.81)	2.28 ± 0.37	11.18 (6)	33.90
(pyrethroid)	SBA-MN1-2017	540	22.38 (16.18 - 28.59)	1.99 ± 0.40	8.13 (6)	
Flonicamid	SBA-ISU-B1	540	0.44 (0.22 - 0.65)	1.16 ± 0.29	4.49 (6)	0.97
(flonicamid)	SBA-MN1-2017	540	0.43 (0.25 - 0.61)	1.22 ± 0.25	6.99 (6)	
Flupyradifurone	SBA-ISU-B1	840	0.07 (0.04 - 0.11)	0.65± 0.06	15.84 (10)	2.28
(butenolides)	SBA-MN1-2017	840	0.16 (0.10 - 0.23)	0.83 ± 0.07	10.95 (10)	
Sulfoxaflor	SBA-ISU-B1	540	0.02 (0.01 - 0.03)	1.07± 0.14	9.53 (6)	2.00
(sulfoximines)	SBA-MN1-2017	540	0.04 (0.02-0.05)	1.07± 0.12	3.85 (6)	
Spirotetramat	SBA-ISU-B1	540	55.07 (31.58 - 78.57)	1.02±0.19	4.36 (6)	0.73
(tetramic acid)	SBA-MN1-2017	540	40.71 (25.40-56.01)	0.95±0.13	9.51 (6)	

[a] Number of aphids tested.
[b] Chi-square (degrees of freedom).
[c] Resistance ratio (RR): LC_{50} of resistant SBA-MN1-2017 divided by LC_{50} of susceptible SBA-ISU-B1.

Table 3.3. Nonsynonymous mutations associated with pyrethroid resistance identified in the voltage-gated sodium channel genes of *Aphis glycines* at positions 1014 (L1014F) and 918 (M918I) from laboratory and field-collected aphids. Genotypes predicted from Sanger sequences from individual aphids.

Collection	Origin	M918I	L1014F
SBA-ISU-B1	Laboratory	SS	SS
SBA-ISU-B3	Laboratory	SS	SS
SBA-ISU-B4	Laboratory	SS	SS
SBA-UIL-B1	Laboratory	SS	SS
SBA-MN1-2017	Field	SS and RS	RS and RR
SBA-MN2-2017	Field	SS and RS	RS and RR
SBA-Nashua-2018	Field	SS and RS	RS
SBA-Sutherland-2017	Field	SS and RS	RS

Table 3.4. Association of survival among randomly sampled *Aphis glycines* from field populations with different voltage-gated sodium channel (*vgsc*) amino acid (aa) changes (L1014F:M918I). Phenotypes defined as survivor and moribund following exposure to bifenthrin and lambada-cyhalothrin at LC_{99} levels in glass-vial bioassays. Counts given for diploid individuals with predicted amino acids (aa/aa) at each locus (1014:918). Numbers in parenthesis represent the percentage of each genotype. Significance of association between encoded aa and aphid survival determined using Fisher's exact tests.

Location	Phenotype	n	*A. glycines vgsc* genotype (L1014F:M918I) [a]									
			L/L:M/M	L/L:M/I	L/L:I/I	L/F:M/M	L/F:M/I	L/F:I/I	F/F:M/M	F/F:M/I	F/F:I/I	*P*
2019 - Bifenthrin												
Boone-IA	Moribund	32	2 (6.3)	10 (31.2)	0 (0.0)	19 (59.4)	1 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.048
	Survivor	8	1 (12.5)	2 (25.0)	0 (0.0)	5 (62.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Kanawha-IA	Moribund	29	0 (0.0)	0 (0.0)	0 (0.0)	28 (96.6)	0 (0.0)	0 (0.0)	1 (3.4)	0 (0.0)	0 (0.0)	0.083
	Survivor	11	0 (0.0)	0 (0.0)	0 (0.0)	8 (72.7)	1 (9.1)	0 (0.0)	2 (18.2)	0 (0.0)	0 (0.0)	
Sutherland-IA	Moribund	37	5 (13.5)	1 (2.7)	0 (0.0)	27 (73.0)	4 (10.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.444
	Survivor	2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Darwin-MN	Moribund	20	4 (20.0)	3 (15.0)	0 (0.0)	7 (35.0)	3 (15.0)	0 (0.0)	3 (15.0)	0 (0.0)	0 (0.0)	0.012
	Survivor	17	0 (0.0)	1 (5.9)	0 (0.0)	9 (52.9)	6 (35.3)	0 (0.0)	1 (5.9)	0 (0.0)	0 (0.0)	
All locations	Moribund	118	11 (9.3)	14 (11.9)	0 (0.0)	81 (68.6)	8 (6.8)	0 (0.0)	4 (3.4)	0 (0.0)	0 (0.0)	0.008
	Survivor	38	1 (2.6)	3 (7.9)	0 (0.0)	22 (57.9)	9 (23.7)	0 (0.0)	3 (7.9)	0 (0.0)	0 (0.0)	
2019 - Lambda-cyhalothrin												
Boone-IA	Moribund	40	12 (30.0)	3 (7.5)	0 (0.0)	23 (57.5)	2 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	N/A
	Survivor	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Kanawha-IA	Moribund	35	0 (0.0)	0 (0.0)	0 (0.0)	34 (97.1)	0 (0.0)	0 (0.0)	1 (2.9)	0 (0.0)	0 (0.0)	0.083
	Survivor	5	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	2 (40.0)	0 (0.0)	2 (40.0)	0 (0.0)	0 (0.0)	
Sutherland-IA	Moribund	15	5 (33.3)	1 (6.7)	0 (0.0)	5 (33.3)	4 (26.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.079
	Survivor	22	3 (13.6)	2 (9.1)	0 (0.0)	8 (36.4)	9 (40.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Darwin-MN	Moribund	20	4 (20.0)	6 (30.0)	0 (0.0)	8 (40.0)	1 (5.0)	0 (0.0)	1 (5.0)	0 (0.0)	0 (0.0)	0.040
	Survivor	20	1 (5.0)	5 (25.0)	0 (0.0)	5 (25.0)	9 (45.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
All locations	Moribund	110	21 (19.1)	10 (9.1)	0 (0.0)	70 (63.6)	7 (6.4)	0 (0.0)	2 (1.8)	0 (0.0)	0 (0.0)	0.008
	Survivor	47	4 (8.5)	7 (14.9)	0 (0.0)	14 (29.8)	20 (42.6)	0 (0.0)	2 (4.3)	0 (0.0)	0 (0.0)	
2020 - Bifenthrin												
Nashua-IA	Moribund	38	27 (71.0)	0 (0.0)	0 (0.0)	9 (23.7)	2 (5.3)	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)	N/A
	Survivor	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Sutherland-IA	Moribund	20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	20 (100)	0 (0.0)	0 (0.0)	0.222
	Survivor	18	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (94.4)	0 (0.0)	0 (0.0)	
All locations	Moribund	58	27 (46.6)	0 (0.0)	0 (0.0)	9 (15.5)	2 (3.4)	0 (0.0)	20 (34.5)	0 (0.0)	0 (0.0)	0.722
	Survivor	18	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	17 (94.4)	0 (0.0)	0 (0.0)	

Table 3.4. (Continued)

Location	Phenotype	n	*A. glycines vgsc* genotype (L1014F:M918I) [a]								
			L/L:M/M	L/L:M/I	L/L:I/I	L/F:M/M	L/F:M/I	L/F:I/I	F/F:M/M	F/F:M/I	F/F:I/I	*P*
2020 - Lambda-cyhalothrin												
Nashua-IA	Moribund	29	17 (58.6)	1 (3.4)	0 (0.0)	10 (34.5)	1 (3.4)	0 (0.0)	0 (0.00)	0 (0.0)	0 (0.0)	0.107
	Survivor	8	3 (37.5)	0 (0.0)	0 (0.0)	4 (50.0)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	
Sutherland-IA	Moribund	33	1 (3.0)	0 (0.0)	0 (0.0)	3 (9.1)	0 (0.0)	0 (0.0)	29 (87.9)	0 (0.0)	0 (0.0)	0.333
	Survivor	3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	0 (0.0)	
All locations	Moribund	62	18 (29.0)	1 (1.6)	0 (0.0)	13 (21.0)	1 (1.6)	0 (0.0)	29 (46.8)	0 (0.0)	0 (0.0)	0.024
	Survivor	11	3 (27.3)	0 (0.0)	0 (0.0)	4 (36.4)	0 (0.0)	0 (0.0)	4 (36.4)	0 (0.0)	0 (0.0)	

[a] Alleles at the *A. glycines* vgsc 1014 *kdr* locus encoding leucine (L; CTT codon) or phenylalanine (F; TTT codon) at amino acid position 1014: homozygous susceptible (L/L), heterozygote (L/F), or homozygous resistant (F/F); Alleles at encoding methionine (M; ATG codon) or isoleucine (I; ATA codon) at amino acid position 918: homozygous susceptible (M/M), heterozygote (M/I), or homozygous resistant (I/I).

Table 3.5. Association of genotypes defining *Aphis glycines* amino acids at voltage-gated sodium channel (*vgsc*) positions 1014 (L1014F; *kdr* mutation) and 918 (M918I) with of field applied rates of lambda-cyhalothrin. Genotypes shown for *A. glycines* collected pre- and post-application of lambda-cyhalothrin, and significance of corresponding changes in resistant allele frequency (RAF) at *A. glycines vgsc* positions 1014 (1014F allele) and 918 (918I allele) in 2019 and 2020 determined using a binomial GLM.

2019

Location	Application collection	n	Genotype (1014)[a]			RAF[b]	Model (95% CI)[c]	Odds Ratio	*P*-value	Genotype (918)[d]			RAF	Model (95% CI)	Odds Ratio	*P*-value
			L/L	L/F	F/F					M/M	M/I	I/I				
Boone-IA	Pre	120	51	69	0	28.8	28.7 (23.4-34.8)	2.94	<0.0001	92	28	0	11.7	11.7 (8.1-16.3)	1.55	0.1957
	Post	47	4	35	8	54.3	54.3 (44.1-64.0)			31	16	0	17.0	17.2 (10.7-26.0)		
Kanawha-IA	Pre	120	0	102	18	57.5	57.5 (51.2-63.6)	1.14	0.4960	113	7	0	2.9	2.9 (1.4-5.9)	6.12	<0.0001
	Post	103	1	79	23	60.7	60.7 (53.8-67.1)			71	32	0	15.5	15.5 (11.2-21.1)		
Sutherland-IA	Pre	115	25	90	0	39.1	39.1 (33.0-45.6)	1.56	0.0240	81	34	0	14.8	14.8 (10.7-19.9)	1.04	0.8908
	Post	95	0	95	0	50.0	50.0 (43.0-57.0)			66	29	0	15.3	15.2 (10.8-21.1)		
All locations	Pre	355	76	261	18	41.8	41.4 (37.7-45.1)	1.73	<0.0001	286	69	0	9.7	8.1 (6.1-10.7)	2.15	0.0002
	Post	245	5	209	31	55.3	55.0 (50.3-59.6)			168	77	0	15.7	15.9 (12.7-19.7)		

2020

Location	Application collection	n	Genotype			RAF	Model (95% CI)	Odds Ratio	*P*-value	Genotype			RAF	Model (95% CI)	Odds Ratio	*P*-value
			L/L	L/F	F/F					M/M	M/I	I/I				
Nashua-IA	Pre	110	61	47	2	23.2	23.2 (18.1-29.2)	3.34	<0.0001	98	12	0	5.5	5.4 (3.1-9.3)	17.33	<0.0001
	Post	38	0	38	0	50.0	50.0 (38.9-61.1)			0	38	0	50.0	50.0 (38.9-61.0)		
Sutherland-IA	Pre	110	14	10	86	82.7	82.7 (77.2-87.2)	0.736	0.2192	95	15	0	6.8	6.8 (4.1-11.0)	0.75	0.5128
	Post	95	1	40	54	77.9	77.9 (71.4-83.2)			85	10	0	5.3	5.2 (2.8-9.5)		
All locations	Pre	220	75	57	88	53.0	54.6 (48.7-60.3)	1.56	0.0174	193	27	0	6.1	6.1 (4.2-8.77)	3.63	<0.0001
	Post	133	1	78	54	69.9	65.2 (58.6-71.3)			85	48	0	18.0	19.1 (13.7-25.8)		

[a]L/L, wild type (susceptible); L/F, *kdr* heterozygote; F/F, *kdr* homozygous resistance allele
[b]RAF= Resistant allele frequency (((2 × RR+SR)/2n) *100)
[c]Binomial generalized linear model with a logit link function
[d]M/M wild type (susceptible); M/I heterozygote; I/I homozygous resistance allele

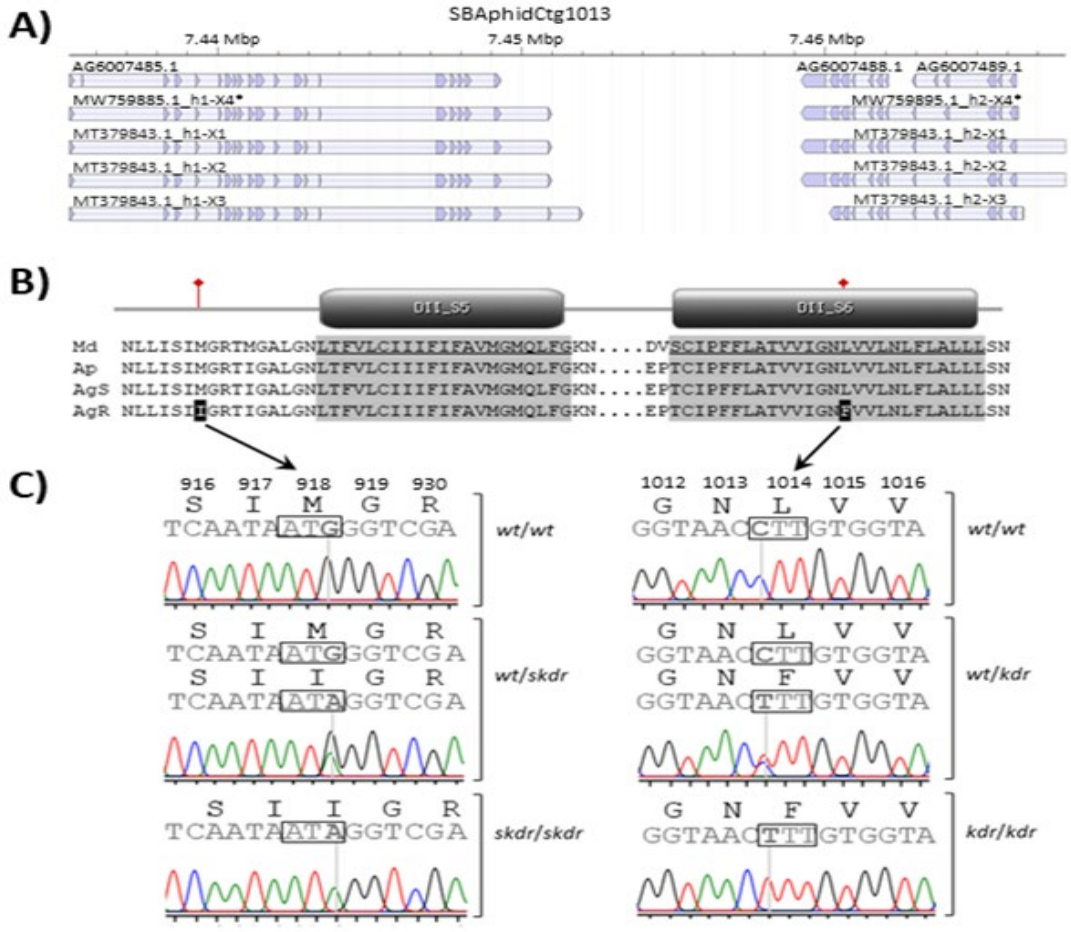

Figure 3.1. Structural variation in the *Aphis glycines* voltage gated sodium channel gene, *vgsc*, and validation of mutations associated with pyrethroid resistance within field populations.Genome organization of the *A. glycines* *vgsc* on SBAphidCtg1013 of the Ag_bt1_v6 assembly[40] with predicted gene models AG60074085.1, AG60074088.1 and AG60074089.1. The *vgsc* heterodimer (*vgsc-h*1 and *-h*2) isoforms X1, X2, and X3 were annotated based on previous evidence (GenBank accession MT379843.1)[23], and isoform X4 supported by sequence assemblies in this study (* representative full-length cDNA accession; MW759883.1 to MW759893.1). **B)** Partial alignment of conceptual protein translations for *vgsc* orthologs from *Musca domestica* (Md; GenBank accession AAB47604.1), *Acyrthosiphon pisum* (Ap; XP_008183365.1), and *A. glycines* pyrethroid resistant (AgR; MW75988.1 – MW759893.1) and susceptible (AgS; MW759883.1 and MW759884.1) alleles showing domain II (DII) segment 5 (S5) and 6 (S6), and positions of knockdown (*kdr*) and super-*kdr* (*skdr*) mutations. **C)** Representative electropherograms from Sanger sequencing with arrows showing substitution mutations predicted to cause amino acid variation at positions 918 (M918I) and 1014 (L1014F; *kdr*), where pyrethroid resistant *A. glycines* are homozygous (*kdr*/*kdr*) or heterozygous for the alleles encoding 1014F allele (*wt*/*kdr*). A portion of resistant *A. glycines* genotypes show *kdr* mutations in combination with the M918I mutation.

Appendix A. Chapter 3 supplemental information

Table S1. Effect of synergists on the toxicity of lambda-cyhalothrin on susceptible and resistant *Aphis glycines* in leaf-dip bioassays.

Treatment	Susceptible SBA-ISU-B1				Resistant SBA-MN1-2017				
	LC_{50}	95% CI	Slope	SR[a]	LC_{50}	95% CI	Slope	SR[a]	RR[b]
Lambda–cyhalothrin	0.38	0.29-0.46	2.51 ± 0.45	-	18.33	13.92-22.74	2.08 ± 0.40	-	48.23
Lambda–cyhalothrin + PBO[c]	0.29	0.23-0.35	1.97 ± 0.26	1.31	14.38	9.53-19.22	1.56 ± 0.31	1.27	49.58
Lambda–cyhalothrin + TPP[d]	0.30	0.19-0.41	1.37 ± 0.19	1.26	14.25	10.28-18.23	1.03 ± 0.11	1.28	47.50
Lambda–cyhalothrin + DEF[e]	0.29	0.19-0.39	1.56 ± 0.23	1.31	14.02	9.43-18.61	1.77 ± 0.39	1.30	48.34

[a] Synergistic ratio: LC_{50} of the insecticide alone divided by the LC_{50} of the insecticide + synergist.

[b] Resistance ratio (RR): LC_{50} of resistant SBA-MN1-2017 divided by LC_{50} of susceptible SBA-ISU-B1.

[c] Piperonyl butoxide: cytochrome P450 inhibitor

[d] Triphenyl phosphate: carboxylesterase inhibitor

[e] *S,S,S*-tributyl phosphorotrithioate: esterase inhibitor

Table S2. Frequencies of predicted amino acid changes among *Aphis glycines* collected pre- and post-application of a foliar pyrethroid insecticide spray. Reported as absolute number (proportion) of A. glycines individuals predicted to give rise to amino acid mutations L1014F and M918I based on BstEII PCR-RFLP and LCR assays, respectively. Fisher Exact test P-values showing significant differences between pre- and post-application genotypes are indicated with an asterisk (*). Data for pre- and post-application super-kdr heterozygote genotypes (L/F:M/I) across locations are highlighted grey in both 2019 and 2020.

2019											
Location	Collection time	Genotype (L1014F:M918I)									
		L/L:M/M	L/L:M/I	L/L:I/I	L/F:M/M	L/F:M/I	L/F:I/I	F/F:M/M	F/F:M/I	F/F:I/I	*P*-value
Boone-IA	Pre	33 (27.5)	18 (15)	0 (0.0)	59 (49.2)	10 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.0397*
	Post	1 (2.1)	3 (6.4)	0 (0.0)	22 (46.8)	13 (27.7)	0 (0.0)	8 (17.0)	0 (0.0)	0 (0.0)	
Kanawha-IA	Pre	0 (0.0)	0 (0.0)	0 (0.0)	95 (79.2)	7 (5.8)	0 (0.0)	18 (15.0)	0 (0.0)	0 (0.0)	0.0476*
	Post	1 (1.0)	0 (0.0)	0 (0.0)	47 (45.6)	32 (31.1)	0 (0.0)	23 (22.3)	0 (0.0)	0 (0.0)	
Sutherland-IA	Pre	20 (17.4)	5 (4.3)	0 (0.0)	61 (53.0)	29 (25.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.1667
	Post	0 (0.0)	0 (0.0)	0 (0.0)	66 (69.5)	29 (30.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
All locations	Pre	53 (14.9)	23 (6.5)	0 (0.0)	215 (60.6)	46 (13.0)	0 (0.0)	18 (5.1)	0 (0.0)	0 (0.0)	0.0079*
	Post	2 (0.8)	3 (1.2)	0 (0.0)	135 (55.1)	74 (30.2)	0 (0.0)	31 (12.7)	0 (0.0)	0 (0.0)	
2020											
Location	Collection time	Genotype (L1014F:M918I)									
		L/L:M/M	L/L:M/I	L/L:I/I	L/F:M/M	L/F:M/I	L/F:I/I	F/F:M/M	F/F:M/I	F/F:I/I	*P*-value
Nashua-IA	Pre	59 (53.6)	2 (1.8)	0 (0.0)	37 (33.6)	10 (9.1)	0 (0.0)	2 (1.8)	0 (0.0)	0 (0.0)	0.3333
	Post	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	38 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Sutherland-IA	Pre	3 (2.7)	11 (10.0)	0 (0.0)	6 (5.5)	4 (3.6)	0 (0.0)	86 (78.2)	0 (0.0)	0 (0.0)	0.0397*
	Post	1 (1.1)	0 (0.0)	0 (0.0)	30 (31.6)	10 (10.5)	0 (0.0)	54 (56.8)	0 (0.0)	0 (0.0)	
All locations	Pre	62 (28.2)	13 (5.9)	0 (0.0)	43 (19.5)	14 (6.4)	0 (0.0)	88 (40.0)	0 (0.0)	0 (0.0)	0.0397*
	Post	1 (0.8)	0 (0.0)	0 (0.0)	30 (22.6)	48 (36.1)	0 (0.0)	54 (40.6)	0 (0.0)	0 (0.0)	

CHAPTER 4. EVIDENCE OF ENHANCED REPRODUCTIVE PERFORMANCE AND LACK-OF-FITNESS COSTS AMONG SOYBEAN APHID, A*phis glycines*, WITH VARYING LEVELS OF PYRETHROID RESISTANCE

Ivair Valmorbida[1], Brad S. Coates[2], Erin W. Hodgson[1], Molly Ryan[3] and Matthew E. O'Neal[1]

[1] Department of Entomology, Iowa State University, Ames, IA, USA

[2] United States Department of Agriculture, Agricultural Research Service, Corn Insects & Crop Genetics Research, Ames, IA, USA

[3] Corteva Agriscience, Agriculture Division of DowDuPont, Dallas Center, IA, USA

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Abstract

Foliar application of insecticides is the main strategy to manage soybean aphid, *Aphis glycines* (Hemiptera: Aphididae), in the northcentral United States. Subpopulations of *A. glycines* have multiple non-synonymous mutations in the voltage-gated sodium channel (*vgsc*) genes that are associated with pyrethroid resistance. We explored if fitness costs are associated with phenotypes conferred by *vgsc* mutations using life table analyses. We predicted that there would be significant differences between pyrethroid susceptibility and field-collected, parthenogenetic isofemale clones with differing, non-synonymous mutations in *vgsc* genes. Estimated resistance ratios for the pyrethroid-resistant clones ranged from 3.1 to 37.58 and 5.6 to 53.91 for lambda-cyhalothrin and bifenthrin, respectively. Although life table analyses revealed some biological and demographic parameters were significantly different among the clonal lines, there was no association between levels of pyrethroid resistance and a decline in fitness. In contrast, one of the

most resistant clonal lines (SBA-MN1-2017) had a significantly higher finite rate of increase, intrinsic rate of increase, and greater overall fitness compared to the susceptible control and other pyrethroid-resistant clonal lines. Our life history analysis suggests that there are no negative pleotropic effects associated with the pyrethroid resistance in the clonal *A. glycines* lines used in this study. We discuss the potential impact of these results on efficacies of insecticide resistance management (IRM) and integrated pest management (IPM) plans directed at delaying the spread of pyrethroid-resistant *A. glycines*.

Keywords: lambda-cyhalothrin; bifenthrin; *vgsc* mutations; insecticide; IRM

Introduction

The use of pyrethroids are an integral part of global insect pest management strategies, where they account for 15% of the market share worldwide[1]. Pyrethroids bind to the voltage-gated sodium channel (*vgsc*) protein, which alters function of the pore channel, causing repetitive neurological impulses, and results in paralysis then death of the insect[2-5]. The frequent use and duration of exposure to this insecticide class has contributed to the widespread occurrence of resistance in populations of many insect pests[1,5,6]. In general, two mechanisms confer resistance to pyrethroids, increased activity or expression of cytochrome P450 monooxygenases (P450), glutathione transferase (GST), or esterase detoxification enzymes, or amino acid substitutions that alter the target sites domains of the *vgsc*[2,7-11].

Pyrethroid resistance in several insect species is associated with mutations that alter amino acid sequences in domain II (DII) α-helical segments 4-6 (S4-6), DIIIS6-DIVS1, and DIVS4-S6 regions of the *vgsc* gene[2-4,9]. Specifically, a knockdown resistance (*kdr*) mutation causing a leucine to phenylalanine substitution at amino acid position 1014 (L1014F) in DIIS4-6 of the house fly (*Musca domestica*) *vgsc* protein is associated with low to moderate levels of

pyrethroid resistance[4,12,13]. Increased pyrethroid resistance happens when the L1014F mutation co-occur with a methionine to threonine substitution at *vgsc* position 918 (M918T), producing a genotype in *M. domestica* referred to as super-*kdr* (L1014F + M918T) [4,14,15]. Super-*kdr* like L1014F + M918I genotypes are found among pyrethroid resistant *Cimex hemipterus*[16-18]. Pyrethroid resistance is associated with non-synonymous mutations in the *vgsc* of aphids[19-23]. For example, the L1014F, M918T and M918L and their allelic combinations are found among pyrethroid-resistant *Myzus persicae*[24-26] and *Aphis gossypii*[27,28]. Super-*kdr* genotypes (L1014F + M918I) were associated with the highest levels of pyrethroid resistance among field-collected *Aphis glycines*[29,30], similar to that reported for *M. persicae*[31]. The L925F (leucine to phenylalanine) mutation has been found in several aphid species[32] and the L925M + L1014F and M918L+L1014F genotypes are also associated with resistant *A. glycines* individuals[29].

Although mutations that reduce the toxic effects of pyrethroids confer a selective advantage to insects leading to their increased prevalence in field populations, pleiotropy is also observed (i.e., a fitness cost)[33]. Several factors impact the rate of increase and persistence of resistant phenotypes in insect populations, such as the pest management practices used, the initial frequency and selective advantage of resistance alleles, selection pressure, gene flow, and the strength and type of fitness costs[34-39]. Fitness costs are measured as reductions in vigor, survival, or reproductive capacity compared to susceptible counterparts in the absence of selection. Among aphids, fitness costs associated with insecticide resistance have been observed as reduced reproductive capacity[40-43], increased vulnerability to natural enemies[41,44], and reduced overwintering survivorship[45]. These disadvantages can reduce the rate at which resistant alleles approach fixation within a population. The impact of these disadvantages may be most important at the initial stages of resistance development when associated alleles are at a low frequency and

mostly present among heterozygotes[46]. Allelic dominance can also affect the degree fitness costs impact the effects of selection[47] and the persistence of resistance alleles when selection pressures are relaxed[48].

Soybean aphid*, A. glycines* (Hemiptera: Aphididae), is an invasive pest of soybean, (*Glycine max*), in the United States[49] that can reduce soybean yield by up to 40% when left unmanaged[50]. Foliar applications of pyrethroids are the primary strategy adopted by farmers to manage *A. glycines*[51,52], resulting in the increased prevalence of resistant phenotypes in field populations of the northcentral United States[30,53]. If pyrethroid resistance becomes more frequent, reaching 50% of a given field population, the capacity for these insecticides to prevent economic yield loss is predicted to decrease[52]. Efforts to develop an insecticide resistance management (IRM) plan to prevent this outcome would benefit from understanding the trade-offs *A. glycines* may experience in absence of the selective advantage conferred by pyrethroid resistance.

We conducted a series of experiments to estimate the degree to which fitness costs are related with lambda-cyhalothrin and bifenthrin resistance associated with different mutations in *vgsc* genes of isofemale lines (i.e. clones). Life table analyses were constructed and used to determine if any differences in fitness occurred among isofemale lines with one or two *vgsc* mutations (M918I, M918L, L925M, and L1014F) compared to a susceptible control. The occurrence and magnitude of fitness costs can contribute to our understanding of the future prevalence of different mutations conferring pyrethroid resistance in field populations, and IRM strategies.

Materials and Methods

Aphis glycines isofemale lines

Aphis glycines were collected from G. max plants from fields at Iowa State University (ISU) and University of Minnesota research farms, and one commercial farm (n = 5; Table 1). Aphids were collected either before an insecticide application (n = 2) or collected 2-3 days after a foliar application of Warrior II (lambda-cyhalothrin active ingredient; Syngenta Crop Protection, Greensboro, NC) at a full rate of 0.40 L ha^{-1} (n = 3). Live individuals from each location were transported to ISU and maintained on G. max plants (Syngenta S24-K2) without insecticide exposure in separate Percival growth chambers (Percival growth chamber (Percival Scientific, Perry, Iowa, USA) at 25 ± 2 ºC, 50% relative humidity (RH), and a photoperiod of 16:8 (L:D). Plants used to maintain the aphid colonies were grown in a greenhouse [25 ± 2 °C, 50 ± 10% relative humidity (RH), and 16:8 (L:D)] in 16-cm diameter plastic pots filled with a soil mixture (Sungro Horticulture Products, SS#1-F1P, Agawam, MA, USA), watered three times per week and fertilized weekly after emergence (Peters Excel Multi-Purpose Fertilizer, 21-5-20 NPK). V3-V4 growth stage plants[54] were added to the colonies weekly.

After at least 25 generations (all through asexual reproduction), a single clonal female was randomly selected from each colony and used to initiate an isofemale line, propagated in growth chambers as described above. Initial females of each isofemale line were propagated by parthenogenesis, whereby each consisted of clonal daughters that were used for further analyses. Naming of each A. glycines (soybean aphid, SBA) line indicated the location and year, and initiation from a single individual female (isofemale line, ISO). For example, SBA-Darwin-2019-ISO. For brevity, ISO was removed throughout the text as every line in this study were derived from single individuals.

Sequencing of voltage-gated sodium channel genes

Mutations in the *vgsc* genes previously associated with pyrethroid resistance in *A. glycines*[29,30] were detected in each isofemale line by direct Sanger sequencing. Specifically, fragments from the *A. glycines vgsc* DIIS4-6, DIIIS6-DIVS1, and DIVS4-S6 were amplified by polymerase chain reaction (PCR) and products sequenced as described previously[30]. In brief, PCR amplification of these three fragments was performed each isofemale line (Table 1), except SBA-MN1-2017 which had been previously characterized[30]. Genomic DNA was isolated separately from individual clones using QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI) according to manufacturer instructions, except that the per sample volume was adjusted to 50.0 µl. Each fragment was amplified from four independently extracted replicates of each isofemale line. PCR products were then purified and bidirectional Sanger sequence data generated on an ABI3700 (Applied Biosystems, Forest City, CA, USA) at the Iowa State University DNA Facility (Ames, IA). Inter- and intraspecific variation in individual Sanger reads was predicted using the application Tracy[55] by alignment to the genomic reference (gene model AG6007485.1 from the *A. glycines* genome assembly Ab_bt1_v6.0)[56], where co-occurrence of electropherogram peaks at a nucleotide position were detected using default parameters and defined as putative heterozygotes.

Detection of mutations through sequencing of *vgsc* genes

Direct Sanger sequencing of DIIS4-6, DIIIS6-DIVS1, and DIVS4-S6 regions of the *A. glycines vgsc* genes (GenBank accessions OL321811-321825) revealed a total of eight mutations within and between the four pyrethroid-resistant isofemale lines compared to the reference genome. Four of the eight mutations were nonsynonymous (amino acid changing). No mutations were predicted in sequence data for the pyrethroid susceptible line SBA-Boone-2019. There

were no differences among the four replicate reads from each line. Specifically, comparisons within and between resistant isofemale lines predicted four non-synonymous (amino acid changing) mutations in the DIIS4-S6 fragment electropherogram results: An A to T transversion at AG6007485-RA position 2782 causing the M918L mutation; a G to A transition at AG6007485-RA position 2784 causing the M918I mutation; a T to A transversion at AG6007485-RA position 2803 causing the L925M mutation; and a C to T transition at AG6007485-RA position 3070 resulting in a predicted L1014F knockdown resistance (*kdr*) mutation (Fig. 1). Analyses also predicted either single incidence (homozygosity) or co-occurrence of respective nucleotide signals (heterozygosity) for M918I, M918L, L925M and L1014F in electropherogram data from the pyrethroid-resistant clones (Fig. 1).

Considering these sequence results, genotypes for SBA-Nashua-2018 and SBA-Kanawha-2019 were heterozygous and homozygous, respectively, for the L1014F mutation. SBA-Darwin-2019 was heterozygous for M918L and L925M mutations, and SBA-MN1-2017 was heterozygous for both the M918I and L1014F mutations (e.g. *super-kdr* genotype). The SBA-Boone-2019 isofemale line was wildtype at all loci (Table 1).

Sequence data from the DIIIS6-DIVS1 region had three of the eight total predicted mutations. Two involved transitions between purine (R) nucleotides (A and G) and one a transversion between T and G nucleotides (K), of which all were in a 3rd position (synonymous or non-amino acid changing). Furthermore, all lines were putatively heterozygous for these mutations, with the exception for the glycine 1545 codon in the SBA-Kanawha-2019 that was homozygous for the wildtype allele. These synonymous changes were located in or immediately downstream of the region encoding the DIII S6 helix. Comparisons within the Sanger sequenced DIVS4-S6 fragment predicted a single substitution mutation. This was either A nucleotide or co-

occurring A and G nucleotide signals in the 3rd position of codon encoding a leucine residue. Specifically, SBA-Nashua-2018, SBA-MN1-2017, and SBA-Kanawha-2019 were heterozygous with co-occurring purine (R) A and G nucleotides. This silent mutation was located upstream of DIVS6.

Pyrethroid toxicity bioassays

Bioassays to assess the susceptibility of each isofemale line to pyrethroids were performed between June and September 2020. We used two common commercially used active ingredients, bifenthrin (Type I) and lambda-cyhalothrin (Type II), that were also previously used in laboratory bioassays to estimate levels of resistance among field-collected *A. glycines*[30,53,57]. Type I pyrethroids do not have a cyano moiety at the α-position and are characterized by symptoms such as hyperactivity, uncoordination response to a single stimulus, and finally paralysis. Type II compounds have an α-cyano moiety, causing a pronounced convulsive phase, with membrane depolarization and suppression of the action potential[11,58]. We performed these bioassays with lambda-cyhalothrin (97.7% purity, Control Solutions Inc., Pasadena, USA) and bifenthrin (98% purity, Chem Service Inc., West Chester, USA) using a leaf-dip bioassay following recommendations by the Insecticide Resistance Action Committee (IRAC) for detecting resistance[59]. For this, stock solutions of lambda-cyhalothrin and bifenthrin were prepared separately using acetone, and then diluted with 0.05% (v/v) Triton X-100 (Alfa Aesar, Tewksbury, USA) in distilled water to prepare treatment concentrations (0.056-56 $\mu g\ ml^{-1}$). The control treatment consisted of 0.05% (v/v) Triton X-100 in distilled water, and acetone (< 0.05%) equal to the concentration in the treatment with the highest concentration of lambda-cyhalothrin or bifenthrin.

Leaves from the first and second trifoliate of *G. max* at V3-V4 growth stage[54] were cut in 3.8-cm diameter disks using a hole punch (Fiskars, Helsinki, Finland). Disks were individually submerged in one of the treatment solutions with gentle agitation for 10s and air dried abaxial side up on a paper towel. Plastic cups (29.6-ml, Choice Paper Company, New York, USA) were filled with approximately 20 ml of 1% w/v agar (Bacto™ Agar, Becton, Dickinson, and Company, Franklin Lakes, USA). Leaf disks were then placed abaxial side down onto the agar surface before congealing. A droplet of distilled water was added to the agar bed to increase leaf disk adherence when needed. Each leaf disk was infested with 20 apterous mixed-age adult aphids collected from *G. max* plants using paintbrushes, with each treatment concentration performed across three replicate leaf disks (triplicate; $n = 60$ aphids total). Plastic cups were sealed with a close-fitting ventilated lid, and incubated in a growth chamber (25 ± 2°C, 70% relative humidity [RH], and 16:8 [L:D]). Mortality was assessed 48h post-treatment, and aphids were considered moribund if unable to right themselves after 10s[57,59].

Mortality data were used to estimate slope and the LC_{50} of each isofemale line using a three-parameter log-logistic function of the 'drc' package[60], implemented in R version 3.6.2, and LC_{50} values were considered significantly different if 95% confidence intervals (CIs) did not overlap[61]. The resistance ratio (RR) was calculated relative to the susceptible line (SBA-Boone-2019) for each of the other populations following previously described methods[62]. The LC_{50} values of SBA-Nashua-2018, SBA-MN1-2017, SBA-Kanawha-2019, and SBA-Darwin-2019 were divided by the LC_{50} of the susceptible isofemale line (SBA-Boone-2019).

Life table analysis

Life history parameters from each isofemale line were measured in July and August 2020. From each *A. glycines* isofemale line, 45 apterous mixed-age adult aphids were

individually transferred onto an untreated V2-V3 stage *G. max* leaflet placed into a Petri dish containing a moistened filter paper at the bottom. Petri dishes were then sealed with parafilm to prevent aphid escape and stored in a growth room at 25 ± 2 °C, 70% RH and 16:8 (L:D). After 24 h, newly emerged nymphs (<24h-old) were transferred to new leaflets and maintained individually in a Petri dish as described above. A total of 45 nymphs were transferred for SBA-Boone-2019, SBA-Nashua-2018, SBA-MN1-2017 and SBA-Darwin-2019 isofemale lines, and 43 for SBA-Kanawha-2019. Leaflets were replaced every seven days and the filter paper was moistened as needed[63]. *Aphis glycines* morphological characteristics were used to determine developmental stages[64,65] along with the presence of exuviae, which was removed once detected. The developmental stages, fecundity and adult longevity were measured daily until the death of the aphid. The offspring were counted and removed daily.

Life table analysis was performed according to the age-stage, two-sex life table theory[66,67], within the TWO-SEX MSChart program[68]. Biological and demographic parameters were calculated according to Chi and Liu[66] and Chi[67]. The Bootstrap procedure[69] with 100,000 replicates[70] was used to estimate means and standard errors of each parameter. We used a paired bootstrap test at 95% significant level within the TWO-SEX MSChart program[68] to determine differences for each biological and demographic parameter among the five isofemale lines. We used Kaplan-Meier to estimate survival curves and Log-Rank tests to compare survival curves between isofemale lines. The R packages 'Survival'[71] and 'Surviminer'[72] were used for the survival analysis.

Results

Susceptibility to pyrethroids

Leaf-dip bioassays showed a range of susceptibilities to lambda-cyhalothrin and bifenthrin among the isofemale lines containing one or more *vgsc* mutations associated with pyrethroid resistance. Specifically, SBA-Darwin-2019 showed the highest LC_{50} estimated for both lambda-cyhalothrin [10.90 (8.71 – 13.09) μg ml^{-1}] and bifenthrin [12.40 (10.19 – 14.61) μg ml^{-1}]. SBA-Nashua-2017 had the lowest estimated LC_{50} for both lambda-cyhalothrin [0.90 (0.64 – 1.16) μg ml^{-1}], and bifenthrin [1.29 (0.93 – 1.66) μg ml^{-1}]. The LC_{50} estimated for SBA-Nashua-2017, SBA-MN1-2017, SBA-Kanawha-2019, and SBA-Darwin-2019 were significantly higher compared to SBA-Boone-2019 for both insecticides (Table 2). These results further showed that the estimated LC_{50} was significantly lower for SBA-Nashua-2017 compared with all other isofemale lines (SBA-MN1-2017, SBA-Kanawha-2019, and SBA-Darwin-2019) for both insecticides. The estimated LC_{50} was significantly higher for SBA-Darwin-2019 compared with all other isofemale lines for bifenthrin. The SBA-Boone-2019 isofemale line was considered susceptible, due to its estimated LC_{50} to lambda-cyhalothrin [0.29 (0.23 - 0.35) μg ml^{-1}] and bifenthrin [0.23 (0.18 - 0.28) μg ml^{-1}] (Table 2). The calculated RR varied ≥ 9.6-fold across the five *A. glycines* isofemale lines when compared to our standard susceptible line, SBA-Boone-2019, when exposed in leaf-dip bioassays to lambda-cyhalothrin (RR range 3.10 to 37.58) and bifenthrin (5.60 to 53.91; Table 2).

Based on these non-overlapping LC_{50} estimates and associated RR for each isofemale line, we defined those considered susceptible and to have significantly different levels of resistance from lambda-cyhalothrin and bifenthrin bioassays (Table 2). Specifically for lambda-cyhalothrin, we defined a susceptible group ($R0^L$) comprised of only SBA-Boone-2019, and used

this group to define resistance in the three other groups. The first significant incremental increase in resistance is observed in group consisting of only SBA-Nashua-2019 ($R1^L$). The second group ($R2^L$) consisted of SBA-MN1-2017, SBA-Kanawha-2019, and SBA-Darwin-2019 which have a significantly higher LC_{50} compared to $R0^L$ and $R1^L$, but no differences among each other. We used a similar nomenclature for these populations when exposed to bifenthrin in the leaf-dip bioassays. Correspondingly, four phenotypes with significantly different LC_{50} were defined (Table 2), with SBA-Darwin-2019 in group $R3^B$ having the highest level of resistance.

Life table parameters of susceptible and pyrethroid-resistant aphids

Time spent in the N2 and N4 developmental stages, adult pre-ovipositional period, total pre-ovipositional period, as well as adult longevity and number of offsprings varied significantly among the five isofemale lines. Significant differences in nymphal development time and reproduction were not consistent among pyrethroid-resistant isofemale lines when compared to the susceptible line (SBA-Boone-2019; Table 3). For example, the mean days in N2 were significantly lower for all lines compared to the susceptible aphids, but the corresponding mean number of days in N4 was significantly lower only between SBA-Darwin-2019 and all other lines. No differences were observed when comparing any of the N1 or N3 stages. The shortest adult pre-oviposition and total pre-oviposition periods were observed in SBA-MN1-2017, which also had the longest oviposition period and greatest adult longevity of all others except SBA-Darwin-2019 (Table 3). Fecundity was also significantly greater for SBA-MN1-2017 and SBA-Darwin-2019 compared to the other isofemale lines.

The demographic parameters reveal that the SBA-MN1-2017 had greater overall fitness (Table 4). Specifically, finite rate of increase and intrinsic rate of increase were significantly higher for SBA-MN1-2017 compared to all others, including the three lines with increased levels

of resistance. Furthermore, SBA-MN1-2017 had significantly higher net reproductive rate than SBA-Nashua-2018 and SBA-Kanawha-2019, and significantly higher gross reproductive rate than SBA-Darwin-2019.

The lowest net reproductive rate was estimated for SBA-Nashua-2018, while all remaining demographic parameters, with exception of mean generation time, were not significantly different from the susceptible line (Table 4). The age-specific survival rates (s_{xj}) overlapped among the developmental stages (N1–N4; Fig. 2). The earliest and greatest decline in adult female survival occurred after the 8th day for SBA-Nashua-2018, while analogous declines occurred for the other isofemale lines at day 12th. Likewise, SBA-Nashua-2018 had a lower survival (l_x) and net maternity ($l_x m_x$) rates. Net maternity peaks were highest for SBA-Boone-2019, SBA-MN1-2017 and SBA-Darwin-2018 isofemale lines (Fig. 3). Kaplan-Meier survival curves and log-rank tests showed differences in the survival probability among the isofemale lines. Significant pairwise differences were observed between SBA-Nashua-2018 and SBA-Darwin-2019 (*P-value*=0.036). No significant differences in survival probability were observed for any other comparisons.

Discussion

Differences in fitness among individuals in a population can impact their relative abundance and genetic contribution to future generations. When under selection, traits such as insecticide resistance are advantageous and genotypes conferring these phenotypes can rapidly increase in a population[73]. The persistence and overall success of these adaptations are dependent upon an interplay between selective advantage and any detrimental effects of the associated mutations on fitness (e.g., fitness costs)[74]. Resistance is generally considered to have associated fitness costs when individuals are competing in absence of the insecticide[33,46]. However, the

presence of modifier genes[75,76] and mechanisms to stop the production of detoxification enzymes in the absence of the selection agent[77] can ameliorate fitness in resistant individuals. One or more non-synonymous mutations in the *vgsc* are associated with *A. glycines* genotypes possessing varying levels of pyrethroid resistance[29,30]. This study demonstrates that these genetically distinct lines carry different levels of relative fitness in absence of pyrethroid exposure.

Among the five *A. glycines* isofemale lines initiated from collections made in Iowa and Minnesota soybean fields, we identified unique genotypes for each based on a combination of mutations in the DII S5-S6 region of the *vgsc* gene (Table 1). Among these genotypes, we defined phenotypic groups categorized by levels of resistance to lambda-cyhalothrin and bifenthrin (Table 2). The range of resistance we observed are analogous to estimates from previously published studies[30,57], revealing the presence of phenotypic variations within and between field locations. Although each of these mutations conferring the varied levels of resistance have arisen independently (e.g. at different loci of the *vgsc* gene), it remains unknown if resistance alleles arose *de novo* since or were extant within the population prior to widespread pyrethroid use[73,78]. Any direct implication of different amino acid changes encoded by the genotypes in the four isofemale lines in our study as completely causal of corresponding levels of lambda-cyhalothrin and bifenthrin resistance remains speculative. This is especially true given the potential for a portion of these resistance traits to be conferred by detoxification enzymes[79] or by interactions among *vgsc* mutations that alter pyrethroid binding in a non-additive fashion[80]. Although previous work showed that SBA-MN1-2017 (heterozygous *super-kdr* M918I + L1014F) did not present cross-resistance and the exposure to detoxification enzyme inhibitors did not affect its susceptibility to lambda-cyhalothrin[30], the genetic or biochemical basis for

estimated differences in the level of resistance and cross-resistance for the other isofemale lines remains unknown pending further investigations.

Our results suggest that different non-synonymous mutations in the *vgsc* might confer similar levels of resistance. For example, the heterozygous *super-kdr* (L1014F + M918I) of SBA-MN1-2017, homozygous *kdr* of SBA-Kanwha-2019, and heterozygous L925M + M918L of SBA-Darwin-2019, had similar LC_{50} when exposed to lambda-cyhalothrin (Group $R2^L$; Table 2). The SBA-Nashua-2018 (heterozygous, RS, for the L1014F *kdr* mutation) had the lowest LC_{50} for lambda-cyhalothrin and bifenthrin compared with homozygous L1014F (SBA-Kanawha-2019) and heterozygous *super-kdr* M918I + L1014F (SBA-MN1-2017; Table 2). These two comparisons agree with prior evidence that homozygous L1014F genotypes enhance aphid resistance to pyrethroids compared to heterozygous genotypes[13,81], and the increase resistance of super-*kdr* genotypes in aphids[82] and other insects[4]. Additionally, the presence of different mutations giving rise to similar and potentially field-relevant levels of resistance is challenging in the contest of IRM monitoring programs using genetic markers, where phenotypic effects of allele combinations likely need to be considered.

The L925M + M918L genotype (SBA-Darwin-2019) showed the highest level of bifenthrin resistance (group $R3^B$; Table 2), but no significant increase in resistance to lambda-cyhalothrin compared with SBA-Kanawha-2019, and SBA-MN1-2017 (group $R2^L$; Table 2). Individual or combinations of *vgsc* DIIS4-S6 target site mutations in resistant insect genotypes may also differentially affect the interaction with type I (e.g., bifenthrin) and type II pyrethroids (e.g., lambda-cyhalothrin)[43,82-84]. Our data suggests that an increase in bifenthrin resistance in SBA-Darwin-2019 could be a consequence of unique changes in the interactions between type I pyrethroids and a binding pocket with amino acids leucine and methionine at 918 and 925

positions, respectively. Pyrethroids may have a dual binding site, including the lipid interface in the DIIS4 to S5 linker region and a second putative receptor site in both S6DI and S6DII[84,85]. Specific differences in any change of lambda-cyhalothrin or bifenthrin to the *vgsc* L925M + M918L variant of SBA-Darwin-2019 remains speculative and requires further testing. Replicated trials of independent isofemale lines with the same *vgsc* genotype could improve our understanding how each mutation accounts for the levels of pyrethroid resistance observed in *A. glycines* in North America, or if other genetic factors are involved.

Given these caveats regarding the impact of the *vgsc* mutations on the observed phenotypes, we elected to focus on these five isolines as they allowed for an initial exploration of the impact of pyrethroid resistance on *A. glycines* fitness. We initially hypothesized that variation across the genotypes and phenotypes of *A. glycines* would produce a range of life-history parameters revealing resistance to pyrethroids is associated with a decline in fitness. We predicted that isolines with mutations in *vgsc* gene would negatively affect fitness. Our results did not reveal a trend across any of the various parameters measured to suggest that the susceptible isoline consistently outperformed the various resistant isolines (Tables 3 and 4). This may not be surprising given that fitness varies across different unrelated genetic backgrounds[86-88], or lines with other mechanisms or *vgsc* mutations conferring pyrethroid resistance[89]. Similarly, no clear association of reduced reproductive performance and insecticide resistance were shown among clones of *M. persicae*[90-92]. Regardless, our data indicate a significant reproductive advantage of one isofemale line carrying heterozygous *super-kdr* M918I + L1014F genotype (Table 3), observed in pre-oviposition period and increased overall fecundity. Likewise, a significant higher reproductive performance was observed in insecticide resistant *M. persicae*[90] and *Sitobion avenae*[93].

Although our results suggest that mutations in the *vgsc* genes of *A. glycines* did not confer a fitness cost, limitations within this study prevented us from reaching this conclusion. First, we lack a full complement of possible genotypes for the various *vgsc* mutations (i.e. SS, RS and RR). It is possible that the missing genotypes (e.g., RR for M918I, M918L, and L925M) and their combinations suffer a reduction in some parameters measured within the life table analysis. Such a reduction may help explain their absence from our samples. Second, variation in the genetic background of the five clonal isofemale lines used in this study may have prevented us from observing fitness costs associated with resistance. Specifically, selection for other biotic or abiotic factors unrelated to pyrethroid resistance might be responsible for the observed increased fitness of SBA-MN1-2017. Third, our observations were conducted under laboratory conditions and we did not perform density-dependent experiments to explore changes in the frequency of resistant alleles over time. Freeman et al[33] suggest that the association of fitness costs with resistance should be measured within congenic lines and include multiple measurements across different assays. Although highly useful, various challenges exist for generating aphid congenic lines through backcrossing due to their clonal nature and the low efficiency in generating outcrossed individuals[82,94]. Future studies involving replicated independent lines from different locations each carrying the same *vgsc* genotype in a diverse genetic background may likely address the potential influence of other genetic loci on fitness parameters measured in the *super-kdr* genotype of SBA-MN1-2017.

There are several points within the life-history of *A. glycines* when a fitness cost could be experienced beyond what we modelled in our life-table analysis. The frequency of resistant alleles might decline during the summer in the absence of the selection agent, when aphids migrate to their overwinter host, *Rhamnus cathartica* (buckthorn) to reproduce sexually, and

during the migration to soybean fields in the following spring. Measurements of several parameters under different environmental conditions that reflect the complex life-history of *A. glycines*, including frequency and survival of resistant alleles in the overwintering host, may be necessary for understanding the persistence of pyrethroid resistance in *A. glycines*. For now, our results suggest that in the simplest scenario modelled by the life table analysis, fitness costs were not observed.

Despite the limitations of our study, it does provide some insight into the potential of fitness cost associated with pyrethroid-resistant *A. glycines*, and their possible impacts on the evolution of resistance to pyrethroids in field populations. In general, a fitness cost is expected to delay the fixation of resistance within a population by reducing an increase in the sub-population with the corresponding genotype [36,88,95-97]. Understanding the occurrence and impacts of fitness costs are essential in developing and implementing IRM programs[88,98]. If pleotropic effects were associated with pyrethroid-resistant soybean aphids, using an insecticide with a different mode of action would be expected to decrease the frequency of resistant individuals. If this strategy is adopted, farmers could not only manage a resistant population, but also prevent the single mode selection and spread of pyrethroid resistant aphids. Such strategies would require farmers to increase the adoption of IPM and IRM programs throughout the northcentral United States. Regardless, switching to an alternative insecticide is challenging in the US. Other active ingredients are more costly[52], and one of the more commonly used active ingredients (chlorpyrifos) was recently banned by the Environmental Protection Agency[99]. Switching to another form of pest management (e.g. aphid-resistant varieties) is possible and cost effective, but is limited to varieties that are not currently glyphosate resistant which can require a substantial change to a farmer's weed management plan[52].

In absence of data revealing a pleotropic effect, models and predictions of resistance spreading should not assume a fitness cost. As demonstrated in our study, the opposite is possible. Not that resistance confers an increase in fitness, but that this trait could be within a genetic back-ground that has greater fitness than a susceptible/wild type sub-population. This in could explain a relatively sudden increase in a resistant population. This is likely stochastic and challenging to model.

Conclusions

Field-evolved resistance to pyrethroids highlights the need to adopt strategies to mitigate the effects of pyrethroid resistance and delay resistance evolution to other chemistries. To date, laboratory-selected pyrethroid-resistant *A. glycines* presented cross-resistance[100], but was not present in a field-collected resistance population[30], suggesting insecticides with different mode of action can still be used to manage outbreaks of *A. glycines*. Although our experiments were performed under laboratory conditions, the observed high levels of resistance associated with increased reproductive performance are concerning and require management strategies to prevent these clones from thriving throughout the growing season. Further studies on the distribution and consequences of field-evolved resistance to pyrethroids are needed to reduce the selection pressure and maintain the use of these insecticides in IPM programs for *A. glycines* management throughout the northcentral United States.

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Tables and Figures

Table 4.1. Location, year, and insecticide treatment status of isofemale lines. Non-synonymous (amino acid changing) mutations predicted for voltage-gated sodium channel (vgsc) genotype as determined by Sanger sequencing.# SBA- MN1-2017 sequence data from Valmorbida et al[30]. Field-collected susceptible line SBA-Boone-2019-ISO is highlighted grey.

Clonal line	Location	Year	Application	*vgsc* genotype			
				M918I	***M918L***	***L925M***	***L1014F***
SBA-Boone-2019-ISO	Boone, IA	2019	Before	SS	SS	SS	SS
SBA-Nashua-2018-ISO	Nashua, IA	2018	After	SS	SS	SS	RS
SBA-MN1-2017-ISO	Minnesota	2017	After	RS	SS	SS	RS
SBA-Kanawha-2019-ISO	Kanawha, IA	2019	After	SS	SS	SS	RR
SBA-Darwin-2019-ISO	Darwin, MN	2019	Before	SS	RS	RS	SS

Table 4.2. Toxicity of lambda-cyhalothrin and bifenthrin to *Aphis glycines* isofemale lines.

Clonal line	n	Slope ± SE[a]	LC_{50} (95% CI)[b]	RR[c]	χ^2 (d.f.)[d]	*P*-value	Group[e]
Lambda-cyhalothrin							
SBA-Boone-2019-ISO	480	1.36±0.16	0.29 (0.23 - 0.35) a	-	6.74 (5)	0.240	$R0^L$
SBA-Nashua-2018-ISO	480	1.48±0.27	0.90 (0.64 - 1.16) b	3.10	4.12 (5)	0.531	$R1^L$
SBA-MN1-2017-ISO	480	1.59±0.21	10.33 (7.70 - 12.96) c	35.62	1.43 (5)	0.920	$R2^L$
SBA-Kanawha-2019-ISO	480	2.26±0.32	10.75 (8.51 - 13.00) c	37.06	6.55 (5)	0.256	$R2^L$
SBA-Darwin-2019-ISO	480	2.35±0.34	10.90 (8.71 - 13.09) c	37.58	3.25 (5)	0.661	$R2^L$
Bifenthrin							
SBA-Boone-2019-ISO	420	2.77±0.54	0.23 (0.18 - 0.28) a	-	5.29 (4)	0.258	$R0^B$
SBA-Nashua-2018-ISO	420	1.90±0.46	1.29 (0.93 - 1.66) b	5.60	5.61 (4)	0.229	$R1^B$
SBA-MN1-2017-ISO	420	3.41±0.49	7.38 (6.31 - 8.45) c	32.08	1.22 (4)	0.873	$R2^B$
SBA-Kanawha-2019-ISO	420	2.20±0.29	7.26 (5.84 - 8.69) c	31.56	8.66 (4)	0.070	$R2^B$
SBA-Darwin-2019-ISO	480	2.98±0.51	12.40 (10.19 - 14.61) d	53.91	3.80 (5)	0.577	$R3^B$

Resistance

[a]SE, standard error.

[b]LC_{50} values followed by different letters within a column are significantly different from each other through non-overlap of 95% confidence intervals. CI, confidence interval.

[c]Resistance Ratio (RR), LC_{50} of a clonal lineage divided by the LC_{50} of the susceptible lineage (SBA-Boone-2019-ISO).

[d]Degrees of freedom.

[e]*Aphis glycines* isofemale lines with LC_{50} estimates that are not significantly different from one another.

Table 4.3. Biological parameters of pyrethroid susceptible and resistant *Aphis glycines* isofemale lines.

Biological parameter	Isofemale line				
	SBA-Boone-2019-ISO	**SBA-Nashua-2018-ISO**	**SBA-MN1-2017-ISO**	**SBA-Kanawha-2019-ISO**	**SBA-Darwin-2019-ISO**
N1 (days)	1.26±0.06a	1.26±0.06a	1.24±0.06a	1.20±0.06a	1.33±0.07a
N2 (days)	1.33±0.07a	1.20±0.06ab	1.13±0.05b	1.29±0.07ab	1.26±0.06ab
N3 (days)	1.22±0.06a	1.18±0.05a	1.25±0.06a	1.17±0.06a	1.32±0.07a
N4 (days)	1.29±0.02a	1.40±0.07a	1.44±0.07a	1.46±0.08a	1.12±0.05b
APOP	0.23±0.06a	0.17±0.05a	0.07±0.000b	0.24±0.08a	0.22±0.07a
TPOP	5.32±0.071a	5.14±0.081a	5.09±0.000b	5.29±0.100a	5.25±0.077a
Oviposition period (days)	10.62±0.55b	8.58±0.77c	12.64±0.52a	11.32±0.71ab	12.40±054a
Adult longevity (days)	17.71±0.84ab	15.44±1.03b	19.71±0.82a	17.60±1.16ab	19.40±0.98a
Fecundity	43.11±2.49bc	35.16±3.59c	52.00±2.46a	43.53±3.42bc	48.09±2.62ab

Mean ± standard error (SE) was estimated using 100 000 bootstrap replications. Different letters within the same row indicate significant differences among the clonal lines at $P < 0.05$ level, with a paired bootstrap test. APOP, adult pre-oviposition period; TPOP, total pre-oviposition period.

Table 4.4. Demographic parameters of pyrethroid susceptible and resistant *Aphis glycines* isofemale lines.

Demographic parameter	*Aphis glycines* **isofemale line**				
	SBA-Boone-2019-ISO	**SBA-Nashua-2018-ISO**	**SBA-MN1-2017-ISO**	**SBA-Kanawha-2019-ISO**	**SBA-Darwin-2019-ISO**
Net reproductive rate (Ro)	41.15±2.61ab	32.80±3.59c	49.68±2.84a	39.48±3.65bc	43.82±3.13ab
Finite rate of increase (λ, d^{-1})	1.46±0.00b	1.45±0.01b	1.49±0.01a	1.44±0.01b	1.45±0.01b
Intrinsic rate of increase (r, d^{-1})	0.38±0.00b	0.37±0.01b	0.40±0.00a	0.36±0.00b	0.37±0.00b
Mean generation time (T, days)	9.77±0.11ab	9.30±0.17c	9.65±0.10bc	9.97±0.14ab	9.99±0.10a
GRR	52.97±2.06ab	52.40±3.94ab	57.52±1.44a	54.23±1.60ab	53.19±1.42b

Mean ± standard error (SE) was estimated using 100,000 bootstrap replications. Different letters within the same row indicate significant differences among the isofemale lines at $P < 0.05$ level, with a paired bootstrap test. GRR, gross reproductive rate.

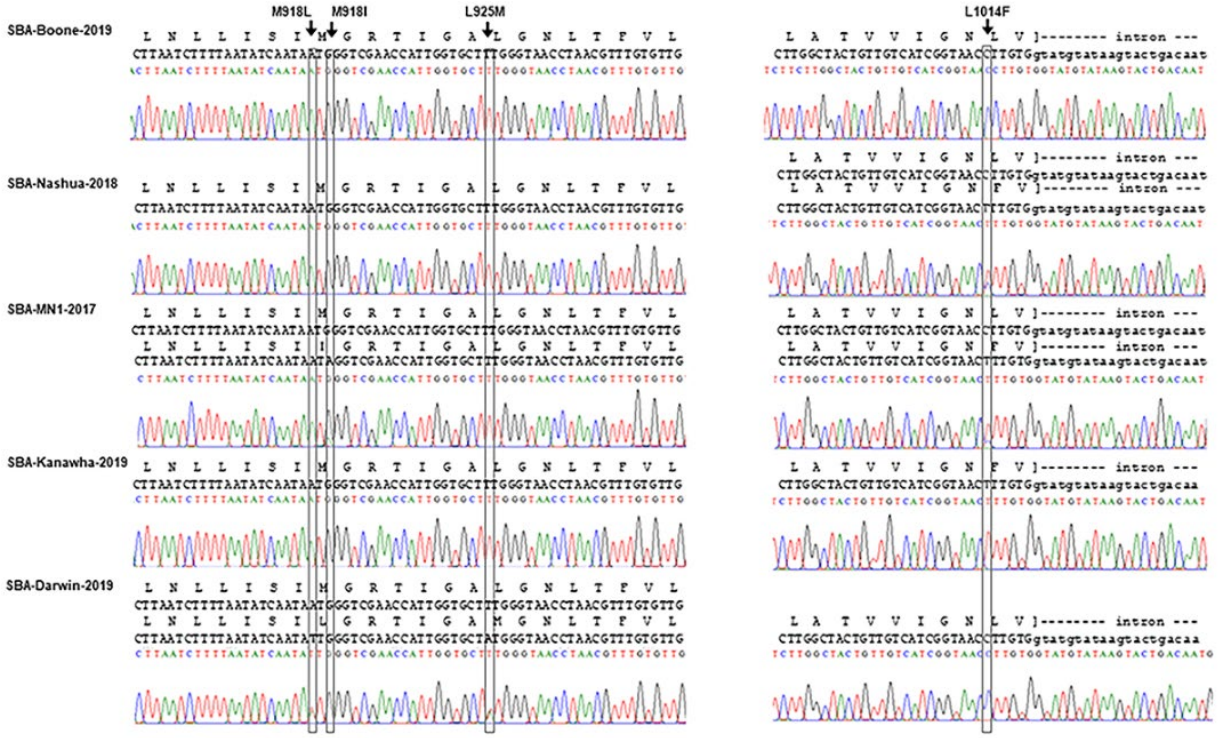

Figure 4.1. Electropherogram from Sanger sequence reads from the *Aphis glycines* voltage-gated sodium channel (*vgsc*) gene. Substitution mutations predicted to cause amino acid changes M918I, M918L, L925M, and L1014F in translated amino acid sequence in one or more pyrethroid-resistant *A. glycines* isofemale lines are indicated by arrows. Heterozygote genotype present at co-occurring nucleotide signals in SBA-Nashua-2018 for the L1014F mutation, SBA-MN1-2017 for M918I and L1014F mutations, and in SBA-Darwin-2019 for M918L and L925M mutations. Data from SBA-Kanawha-2019 indicate homozygosity for the mutant 1014F allele. In contrast, these mutations are not predicted in the susceptible SBA-Boone-2019 isofemale line.

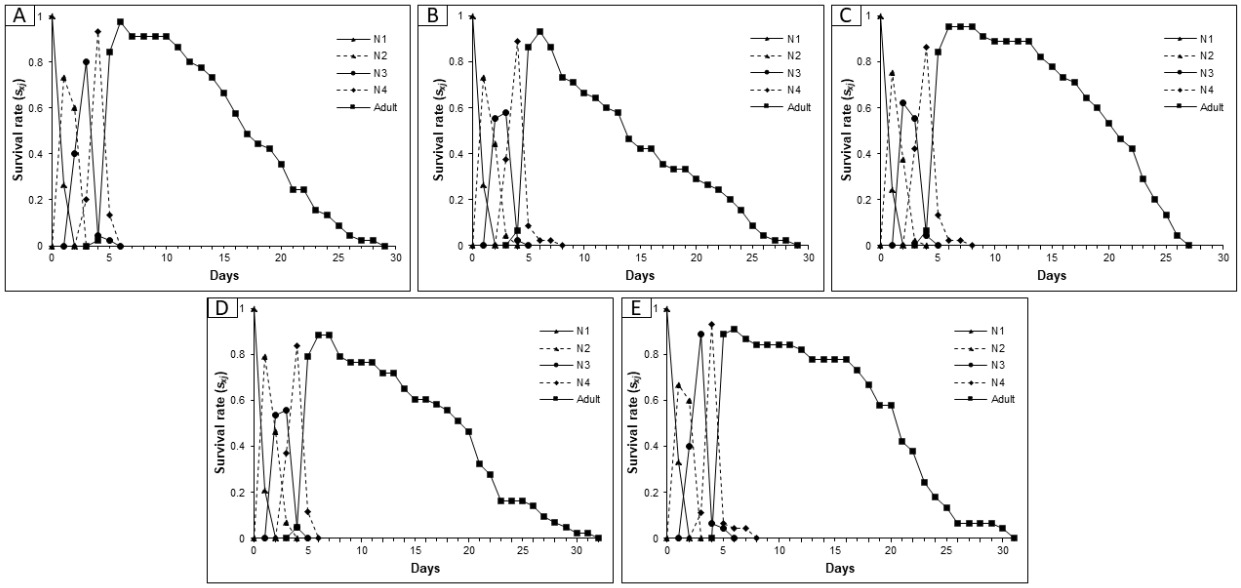

Figure 4.2. Age-stage specific survival rate (s_{xj}). A) SBA-Boone-2019; B) SBA-Nashua-2018; C) SBA-MN1-2017; D) SBA-Kanawha-2019; E) SBA-Darwin-2019.

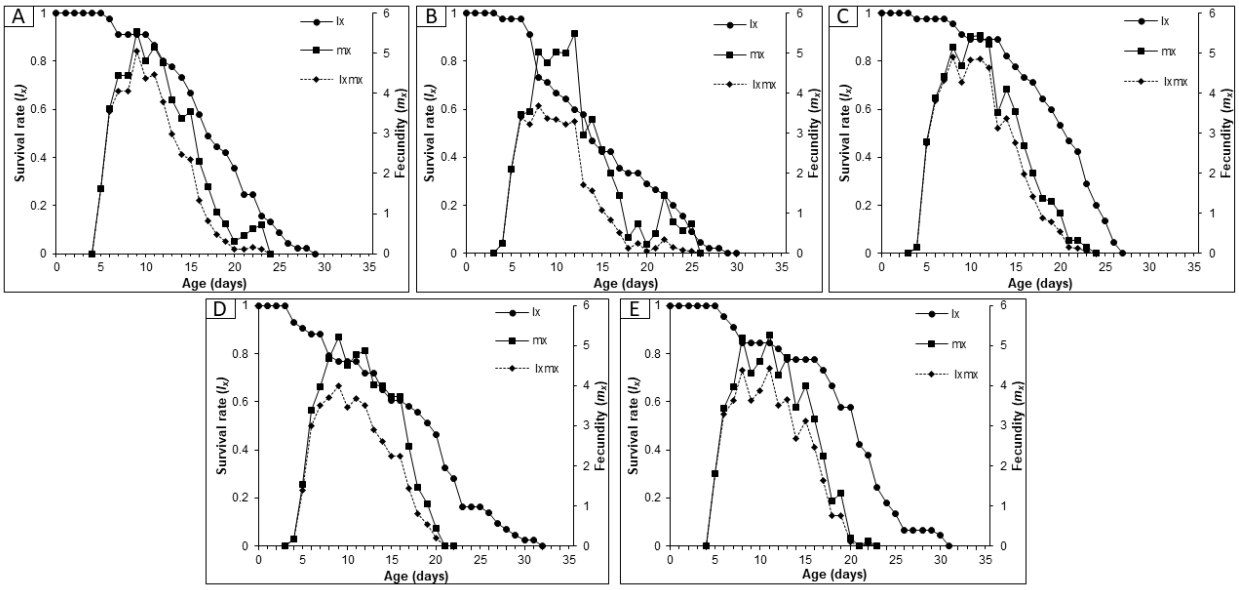

Figure 4.3. Age-specific survival rate (l_x), age-specific fecundity (m_x) and age-specific maternity ($l_x m_x$) of susceptible and pyrethroid-resistant *Aphis glycines*. A) SBA-Boone-2019; B) SBA-Nashua-2018; C) SBA-MN1-2017; D) SBA-Kanawha-2019; E) SBA-Darwin-2019.

CHAPTER 5. HOW EFFECTIVE IS IRM FOR ANNUAL CROPS: TWO INSECT PESTS THAT THREATEN CROP PRODUCTION IN IOWA

Ivair Valmorbida[1], Erin W. Hodgson[1] and Matthew E. O'Neal[1]

[1] Department of Entomology, Iowa State University, Ames, IA, USA

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Abstract

Corn and soybean account for most of the agricultural land use in Iowa. Farmers have to manage several insect pests that can attack these crops throughout the growing season. Notably in Iowa, western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) and soybean aphid (SBA), *Aphis glycines* Matsumura (Hemiptera: Aphididae), are considered the most important insect pests of corn and soybean, respectively. We explore the rates at which resistance is developing in these pests, tactics to delay resistance evolution and the relative success of these methods. Interestingly, the documented occurrence of Bt-resistant WCR in Iowa is greater than for pyrethroid-resistant aphids. The perception of farmers to the pace at which resistance occurred, and adoption of insect resistance management (IRM) strategies by farmers for each pest may be more important than which pest has evolved resistance faster. While farmers are required to practice IRM when using Bt corn for WCR, similar requirements do not exist for insecticides used to suppress SBA. We suggest that IRM strategies along with integrated pest management (IPM) practices need to be adopted for both species. Specifically,

for SBA, scouting and monitoring soybean fields throughout the summer, and following the economic threshold to decide on the need for foliar insecticide applications is strongly recommended. For WCR, IPM strategies are recommended along with the adoption of refuge when using Bt corn as part of an IRM program. Farmers compliance to IRM and IPM practices impacts resistance evolution rates, and without an IRM requirement, SBA resistance is likely to increase in frequency.

Keywords: resistance, rootworm, soybean aphid, corn

Crop production and pest management in Iowa corn and soybean fields

Localized within the Corn Belt in the Midwest, Iowa is the largest corn (*Zea mays* L.) and the second largest soybean (*Glycine max* [L.] Merr.) producer in the United States (USDA-NASS 2021a). The total harvested area of corn and soybean in Iowa in 2020 was approximately 5.20 and 3.80 million ha, respectively (USDA-NASS 2021a). These two crops account for the majority of land use in the state. The predominant cropping systems are corn alternated with soybeans annually (corn-soybean), two years of corn followed by one year of soybean (corn-corn-soybean) or continuous corn (Padgitt et al. 2000, Wright and Lenssen 2013).

The management of insect pests is one of several issues farmers face when growing corn and soybean. Insect pests can cause direct and indirect injury to these crops (Pedigo and Rice 2014). The lepidopterans corn earworm, *Helicoverpa zea* (Boddie), armyworm, *Mythimna unipuncta* (Haworth), and black cutworm, *Agrotis ipsilon* (Hufnagel) overwinter in the southern states and their potential to damage corn depends upon the time they arrive in Iowa (Hodgson et al. 2012a, Dean et al. 2021). European corn borer, *Ostrinia nubilalis* (Hübner), Japanese beetle, *Popillia japonica* Newman, corn leaf aphid *Rhopolosiphum maidis* (Fitch), and bird cherry-oat aphid *Rhopalosiphum padi* (Linnaeus) can be common in Iowa, but their capacity to injury corn

can vary in time and space (Park and Obrycki 2004, Shanovich et al. 2019, Dean et al. 2021). On the other hand, northern corn rootworm, *Diabrotica barberi* Smith & Lawrence and western corn rootworm, *Diabrotica virgifera virgifera* LeConte (WCR), are major concerns for corn production throughout the state (Hurley and Mitchel 2020, Dean et al. 2021, Gassmann et al. 2021).

In Iowa soybeans, several arthropods can threaten soybean production, including bean leaf beetle, *Cerotoma trifurcata* Förster, Japanese beetle, spider mites, caterpillars, and soybean aphid (SBA), *Aphis glycines* Matsumura (Hodgson et al. 2012a, Hartman et al. 2015, Hesler et al. 2018, Hurley and Mitchell 2020, Huseth et al. 2021). Recently, a new pest, soybean gall midge, *Resseliella maxima* Gagné, was reported causing injury to soybeans (Gagné et al. 2019, McMechan et al. 2021). Despite the possible occurrence of several pests throughout the growing season, surveys revealed that SBA control is a priority for farmers when making management decisions (Hodgson et al. 2012a, Hurley and Mitchell 2017; Hurley and Mitchell 2020) and this pest is actively managed by farmers in the north central United States (Hurley and Mitchell 2020).

Biology, ecology, and management

Western corn rootworm

Western corn rootworm is native to Central America (Melhus et al. 1954, Lombaert et al. 2018). In the United States, WCR adaptation and expansion range was greatly favored by the modern corn cultivation system (Gray et al. 2009). This pest has one generation per year (Hein and Tollefson 1985, Levine and Oloumi-Sadeghi 1991) and females lay eggs in the soil late in the summer, and egg hatching occurs in the following year (Hein and Tollefson 1985). Larvae feed on corn root tissue after emerging in the spring (Chiang 1973, Spencer et al. 2009). Injury

caused by larvae can severely reduce corn yields, with an estimate of approximately 15% yield loss for each node of roots that is pruned due to WCR feeding (Dun et al. 2010, Tinsley et al. 2013). Corn root injury caused by larvae can also result in indirect damage such as lodging of the corn plants. Additional yield losses can occur when lodging complicates harvest (Riedell 1990, Spike and Tollefson 1991). Western corn rootworm adults feed on corn silk, pollen and leaves, and high densities might reduce pollination. Regardless, injury caused by WCR adults does not always result in yield reduction (Culy et al. 1992, Gyeraj et al. 2021).

In the United States, costs to manage WCR are estimated to be over $1 billion US dollars annually (Gray et al. 2009, Wechsler et al. 2018). In Iowa, WCR is considered one of the most concerning pests of corn (Dean et al. 2021). Due to its potential to result in yield reduction, WCR has been managed by farmers using several strategies. Crop rotation such as corn-soybean alternation can help reduce WCR populations (Levine and Oloumi-Sadeghi 1991, Levine et al. 2002). Insecticides applied in the furrow at planting can help manage larvae, while foliar insecticide applications target adults (Levine and Oloumi-Sadeghi 1991). In 2003, transgenic corn hybrids expressing a protein (Cry3Bb1) derived from the bacteria *Bacillus thruringiensis* (Bt) were commercialized in the United States (EPA 2003). The adoption of this technology changed the way farmers managed WCR in the Midwest (Rice 2004, Hellmich and Hellmich 2012). Farmers rapidly adopted this management practice which led to a widespread adoption of Bt hybrids to control WCR (Rice 2004, James 2011). Stacked biotechnology traits, including insect-resistant (Bt) traits accounted for 80% of the area planted with corn in Iowa in 2021 (USDA - NASS 2021b).

Soybean aphid

Soybean aphid, an invasive species from Asia, was first reported in the new world in 2000 (Alleman et al. 2002, Ragsdale et al. 2011). The presence of its overwintering host, buckthorn (*Rhamnus* spp.) (Ragsdale et al. 2004), facilitated the rapid spread of SBA, which became the dominant pest in soybean fields throughout the north central United States (Ragsdale 2011, Timon et al. 2011). Soybean aphid has a heteroecious holocyclic life cycle that utilizes a primary host plant for overwintering and a secondary host plant during the summer, with sexual and asexual reproduction occurring at different times (Ragsdale et al. 2011). Asexual reproduction occurs during the spring on common buckthorn, and then aphids migrate to soybeans, where they reproduce asexually throughout the summer. In the fall, SBA migrate to buckthorn where they reproduce sexually (Ragsdale et al. 2011). Soybean aphid develops quickly on soybeans with populations doubling in 6-7 days (Ragsdale et al. 2007). Throughout the growing season, this species can have as many as 15 overlapping generations (Tilmon et al. 2011).

Soybean aphid feeding can reduce pods per plant, seeds per pod, and individual seed weight, and high population can result in yield losses of up to 40% (Beckendorf et al. 2008, Ragsdale et al. 2007, Pierson et al. 2010). Despite the high potential to reduce soybean yield, SBA outbreaks vary spatially and temporally (Bahlai et al. 2015, Dean et al. 2020a). Given this non-uniform occurrence of aphids throughout the growing season and within a region, farmers are encouraged to monitor SBA populations and follow the economic threshold of 250 aphids per plant with 80% of plants infested to apply control measures (Ragsdale et al. 2007, Koch et al. 2016).

Farmers in the north central region take SBA management seriously and consider it a priority during the growing season (Hurley and Mitchell 2017, Hurley and Mitchell 2020). Several strategies can be used to suppress SBA populations, including natural biological control (Fox et al. 2004, Costamagna and Landis 2006, Ragsdale et al. 2011), host plant resistance (Hesler et al. 2013, Dean et al. 2020b, Tilmon et al. 2021), and chemical control (Myers et al. 2005, Magalhães et al. 2009, Hodgson et al. 2012b). Limitations of biocontrol associated with the diversity and composition of landscapes can affect SBA suppression (Gardiner et al. 2015), and the presence of virulent aphids and limited availability of soybean varieties containing resistance to *Aphis glycines* (*Rag*) genes (Hesler et al. 2013, Tilmon et al. 2021), interfere with the adoption of these control tactics.

The use of soybean seeds treated with insecticides (e.g. neonicotinoids) to suppress SBA populations early in the season are also available. Nevertheless, the timing of the occurrence of this pest in soybean may not align well with the control period provided by seed treatments and threshold-based foliar insecticide applications are a more economical alternative to manage SBA (Krupke et al. 2017). In the north central United States, management of SBA outbreaks still relies on foliar applications of pyrethroids and organophosphates (Johnson et al. 2009, Hodgson et al. 2012b). These broad-spectrum insecticides effectively suppressed SBA in the first decade after detection in the United States (Chandrasena et al. 2011), and a notable increase in insecticide use in soybean fields was observed (Hodgson et al. 2012b, Coupe and Capel 2016).

Mechanisms of resistance to control tactics

Overview of resistance mechanisms

Insecticides continue to be a valuable tool in IPM programs to control agricultural pests (Perry et al. 2011, Bass and Jones 2018). However, evolution of resistance to management

practices, especially insecticides and insecticidal plant-incorporated protectants (e.g. Bt corn), by insects can threaten the control of pests in agroecosystems (Knight and Norton 1989, Soderlund and Knipple 2003, Tabashnik et al. 2008, Bass and Jones 2018, Gould et al. 2018, Jurat-Fuentes et al. 2021). Insecticide resistance evolution can be driven by one or more mechanisms. This includes biochemical mechanisms such as target-site mutations, increased detoxification, enhanced efflux, and reduced penetration through the cuticle (Casida and Quistad 1998, ffrench-Constant 2013, Yu 2014, Feyereisen et al. 2015, Bass and Jones 2018, Nauen et al. 2022).

Target site resistance refers to point mutations on the sequences of insecticide target genes, reducing the binding or affinity of the insecticide to the target proteins. These mutations have been detected in genes encoding for the voltage-gated sodium channel (*vgsc*), the enzyme acetylcholinesterase (AChE), the nicotinic acetylcholine receptor (nAChR), the γ-aminobutyric acid receptor (GABA), and ryanodine receptors (RyRs) (Hollingworth and Dong 2008, Yu 2014, Liu 2015). Enhanced metabolism or detoxification that metabolizes, sequesters or eliminates the chemical compounds before they reach the target site involve cytochrome P450-dependent monooxygenases, carboxyl/cholinesterases (CCEs), glutathione S-transferases (GSTs), UDP-glucosyltransferases (UGTs) enzyme families, and ATP-binding cassette proteins (ABC transporters) (McKenzie 1996, Scott 1999, Hemingway 2000, Dermauw and Van Leeuwen 2014, Merzendorfer 2014, Yu 2014, Liu et al. 2015, Nauen et al. 2022). Resistance mechanisms to insecticidal proteins from Bt including those expressed in corn are associated with reduction in midgut target site binding, alterations in the capacity to activate protoxins, sequestration and enhanced immunity (Yu 2014, Jurat-Fuentes et al. 2021).

Western corn rootworm resistance to management strategies

The selection pressure imposed by management tactics has resulted in the evolution of resistance in WCR to multiple strategies (Fig. 1), including insecticides, crop rotation and Bt corn (Levine et al. 2002, Spencer et al. 2014, Gassmann 2016, Gassmann 2021, Meinke et al. 2021). Specifically for insecticides, the first report of field-evolved resistance to soil-applied cyclodienes occurred in the 1960s (Ball and Weekman 1962). In the 1990s, adult control failures were reported and resistance to carbamate and organophosphate was confirmed (Meinke et al. 1998). Recently, WCR adult populations were found to have evolved resistance to pyrethroids (Souza et al. 2019). Intense use of corn-soybean cropping system in some regions of the Midwest has selected WCR capable of laying eggs in soybean fields, where farmers would grow corn in the following year. This behavioral resistance threatens crop rotation as a strategy to control WCR populations (Levine et al. 2002, O'Neal et al. 2002, Spencer et al. 2014). However, rotation-resistant genotypes were rare in northeastern Iowa (Dunbar et al. 2013).

Field-evolved resistance of WCR to transgenic Bt proteins expressed in corn was detected within six years after its commercial release (Gassmann et al. 2011). Specifically, laboratory bioassays confirmed resistance to Bt corn in populations collected in 2009 from northeastern Iowa fields with severe injury to Cry3Bb1 by WCR (Gassmann et al. 2011). In 2011, resistance to Cry3Bb1 was detected in other locations in Iowa, with WCR presenting cross-resistance between Cry3Bb1 and mCry3A (Gassmann et al. 2014). In 2013, severe injury was observed in Iowa cornfields planted with Gpp34/Tpp35Ab1 (Cry34/35Ab1). Using WCR collected from these fields, bioassays confirmed resistance to Gpp34/Tpp35Ab1 (Gassmann et al. 2016). Bioassays with populations collected in 2017 demonstrated that WCR has evolved resistance to all four Bt traits, including eCry3.1Ab (Gassmann et al. 2020). WCR resistance to

Bt traits is not restricted to Iowa cornfields and has also been observed in other states throughout the Midwest (Wangila et al. 2015, Schrader et al. 2016, Zukoff et al. 2016).

Soybean aphid resistance to insecticides

The first assessment of susceptibility of SBA populations to insecticides in the United States was performed using field-collected aphids in 2007 and 2008 from two counties in Michigan. Field-collected aphids were exposed to pyrethroids, organophosphates and neonicotinoids, and no evidence of reduced susceptibility to these insecticides was observed when compared with a laboratory population collected in 2000 (Chandrasena et al. 2011). A few years later, 25 SBA populations collected from 2012 to 2014 in the northcentral United States were used to characterize their susceptibility to thiamethoxam. Overall, field-collected SBA were highly susceptible to thiamethoxam with resistance ratios characterized as very low (2-10-fold; Ribeiro et al. 2018). Field-collected populations of SBA from 2013 to 2016 demonstrated a significant reduction in susceptibility to pyrethroids compared with susceptible laboratory aphids. The most resistant population presented almost 40-fold reduction in susceptibility, and some populations were collected from fields with lambda-cyhalothrin and bifenthrin control failures (Fig. 1; Hanson et al. 2017). Continuous monitoring in the north central United States has revealed expansion of pyrethroid-resistant SBA (Koch et al. 2018a,b Menger et al. 2020, Valmorbida et al. 2022a).

Western corn rootworm *vs* soybean aphid

As noted in Figure 1, WCR evolved resistance to Bt corn hybrids that express at least one pesticidal protein 4-7 years after their commercial release. In contrast, pyrethroid resistance was first documented in soybean aphids 13 years after arriving in the United States. From this, one may think that the WCR evolved resistance to Bt corn faster than the SBA did to pyrethroids.

This perception is influenced by WCR status as a more common pest of a more valuable crop, and Bt-hybrids have a federal-regulated IRM plan that includes annual monitoring. We explore how differences in management practices may affect the selection pressure they experience in light of operational practices. When the biology of the SBA is fully considered, there is reason to be concerned that they are experiencing a rate of resistance to pyrethroids that is similar to what WCR have experienced for Bt-corn. Below, we discuss how factors associated with the biology, ecology and management practices affect rates of resistance evolution in both insects. In addition, we discuss management tactics that can reveal the degree of selection pressure each insect would experience on the same farm in Iowa.

Several factors affect the rates at which an insect evolves resistance to a given management practices such as genetic, ecological, biological, and operational factors (Georghiou and Taylor 1977, Roush and McKenzie 1987, Crowder and Carrière 2009). These include initial frequency and number of resistant alleles, degree of dominance of resistance, generations per year, offspring per generation, and reproductive mode (e.g. sexual and asexual) (Georghiou and Taylor 1977a, Roush and McKenzie 1987). In general, a high initial frequency of resistant alleles, dominant inheritance mode, more than one generation(s) per year, limited gene flow and lack of fitness costs associated with resistant alleles accelerate resistance evolution (Georghiou and Taylor 1977a, Roush and McKenzie 1987, Crowder and Carrière 2009). The initial frequency and number of resistant alleles, number of generations per year and offspring per generation are examples of factors associated with resistance evolution that cannot be controlled. On the other hand, factors such as the chemical compound, formulation, persistence, application mode, concentration and timing can be managed to delay resistance evolution (Georghiou and Taylor 1986). Increased selection pressure with repeated use of the same mode of action, active

ingredients with long residual protection, and applications made when pests have not reached economic threshold level can accelerate resistance evolution (Georghiou and Taylor 1986).

The factors associated with WCR evolution to Bt corn hybrids have been recently reviewed (Gassmann 2021). Both biological and operational factors are considered significant drivers of the rapid evolution of WCR to Bt-corn. Specifically, the movement of adults is limited and females are likely to mate near their emergence site (Hughson et al. 2015). This limited gene flow hastens resistance evolution within-field (Denholm et al. 1992, Gassmann 2021). Yet, the limited mobility of adults, WCR mating behavior, and likelihood of laying eggs near their emergence site increase selection pressure when farmers adopt continuous corn as a cropping system (Gassmann 2021).

Initial frequency and geographic distribution of resistance alleles, inheritance pattern, and fitness costs associated with the resistant genotypes also affect resistance evolution (Roush and McKenzie1987, Gassmann et al. 2009, Gassmann 2021). For example, the initial frequency of WCR alleles conferring resistance to Bt corn was suggested to be ~0.01 (Onstad and Meinke 2010), higher than the expected 0.001 frequency (Georghiou and Taylor 1977a, Roush 1993). Modeling approaches have suggested that this high frequency of resistance alleles in WCR facilitated the rapid evolution of resistance to Bt hybrids (Onstad and Meinke 2010). Studies suggested that inheritance of resistance to Bt corn is non-recessive and that hybrids with rootworm-active Bt proteins are not providing a high-dose to WCR (Meihls et al. 2008, Petzold-Maxwell et al. 2012, Geisert et al. 2016). Delivery of a high-dose Bt is an essential component of an IRM plan as it is expected to kill both homozygous susceptible and heterozygous individuals, thus delaying resistance evolution (Gould 1998). Finally, fitness costs in resistant individuals are expected to delay evolution of resistance when selection pressures are relaxed (Gassmann et al.

2009, Kliot and Ghanin 2012, Freeman et al. 2021). Fitness costs in WCR that are resistant to Bt corn are inconsistent and may not be sufficient to delay resistance evolution (Gassmann 2021).

Beyond these biological factors is a key operational factor that affects the potential to limit the evolution of Bt-resistant, the planting of a refuge comprised of corn hybrids susceptible to WCR. The planting of a refuge area allows susceptible individuals to survive and mate with resistant individuals, which is expected to reduce the frequency of resistant alleles (Gould 1998). Refuge areas can consist of blocks of plants separate from the crop, field stripes, natural areas, and refuge plants (e.g., seed blends) (Onstad 2014). Current refuge strategies for Bt corn targeting WCR consist of structured refuges and seed blends. The size of structured refuges and proximity to Bt corn fields vary according to the Bt corn type (e.g., single pesticidal protein vs pyramid) (EPA 2022a). Similarly, the percentage of not-Bt seeds mixed with Bt seeds in the same bag varies from 5% (pyramid) to 10% (single pesticidal protein) (EPA, 2022).

Farmer compliance to the adoption of refuge has been shown to affect evolution of resistance (Gray 2011, Hurley and Mitchell 2014). One advantage of seed blends refuge compared with structured refuges is that farmers do not need to plant a separate area with non-Bt corn, thus assuring compliance to the refuge strategy (EPA 2022a). In addition, areas with high levels of continuous corn cultivation can favor resistance evolution (St. Clair et al. 2020, St. Clair and Gassmann 2021). In summary, continuous corn cropping systems coupled with Bt-varieties that, because of the biology of the target pest, did not provide a high dose contributed to the evolution of WCR that are resistant to Bt-toxins.

While the factors affecting the evolution of WCR to Bt hybrids have been explored in detail, a fewer studies regarding SBA resistance to pyrethroids have been completed since resistance was first detected. Unlike the WCR, SBA does not reside in crop fields and have a

much larger dispersal range (Ragsdale et al. 2011). They also produce multiple, asexual generations within soybean fields. These factors would facilitate the evolution of resistance. Although pyrethroids were one of the first active ingredients used to manage this pest, pyrethroid control failures were observed only within 15 years after SBA was identified in the United States (Koch et al. 2018b). Laboratory bioassays confirmed that aphids collected from fields in Minnesota that experienced control failures had lower susceptibility to pyrethroids compared with a laboratory population. (Fig. 1, Hanson et al. 2017). A few years later, pyrethroid resistance was detected in key states of north central United States (Menger et al. 2020, Valmorbida et al. 2022a). Recent studies showed that pyrethroid resistance is primarily associated with detoxification enzymes and the presence of nonsynonymous mutations in the *vgsc* (Paula et al. 2020, Paula et al. 2021, Valmorbida et al. 2022a,b).

Unlike the WCR, we do not have good estimates of the frequency of mutations to pyrethroids present before the SBA arrived in the United States. In a study performed with aphids collected in Iowa and Minnesota, Valmorbida et al. (2022a) observed a high frequency of individuals carrying at least one of the mutations associated with pyrethroid resistance. The frequency of resistant alleles in some locations exceeded 50%, suggesting that control failures were likely to occur. In addition, the frequency of resistant alleles significantly increased after an application of field rate of lambda-cyhalothrin (Valmorbida et al. 2022a). No evidence of fitness costs was observed among pyrethroid- resistant aphid clones collected from Iowa and Minnesota populations. Specifically, clonal lines with various levels of resistance and mutations were used to evaluate reproductive performance, rates of population increase, aphid size and asymmetry (Menger et al. 2022, Valmorbida et al. 2022b). These studies demonstrated no clear association of reproductive fitness costs and pyrethroid-resistant phenotypes. On the contrary, some of the

resistant clonal lines had significantly higher reproductive performance compared with susceptible ones (Menger et al. 2022, Valmorbida et al. 2022b).

At least three operational factors may have contributed to the evolution of SBA to pyrethroids in the north central United States: 1) Pest pressure in some locations requiring foliar insecticide applications, 2) use of a limited number of insecticides with different modes of action, primarily reliance on pyrethroids, and 3) prophylactic application and overuse of insecticides from lack of scouting (Koch et al. 2018 for details). Specifically for Iowa, pyrethroids were used on approximately 15% of planted soybeans in 2020 (USDA-NASS 2021c). Although this number might suggest few acres of soybean were treated with pyrethroids in Iowa, soybean aphid outbreaks in the state are observed primarily in the northern counties (Dean et al. 2020a). In addition, the use of prophylactic, calendar-based insecticide applications, especially applications performed late in the season, might favor the selection of resistant aphids that will migrate back to their overwintering host and reproduce sexually, favoring the spread of resistant alleles. Rapid, asexual reproduction during the summer combined with selection pressure due to insecticide applications favor the multiplication of resistant clones.

Despite farmers experiencing a longer period of time between the arrival of the SBA and the occurrence of control failures to pyrethroids, there are as many selection pressures acting on this invasive pest as there are the WCR. To what extent the sexual reproduction of SBA occurring on the overwintering host affect resistance evolution and spread of resistance alleles remains unknown. Regardless of what biological factors are contributing to delaying the spread and frequency of pyrethroid resistance, there is a need for IRM for SBA and foliar insecticides. These include the use of economic threshold levels, rotation of insecticide mode of action if more than one spray is needed, and appropriate sprayer equipment.

In Iowa, this may be especially challenging as farmers grow both corn and soybeans, with both being attacked by pests such as WCR and SBA. Although resistance to management tactics were detected for WCR and SBA, what farmers do to manage these pests varies. Insect resistance management strategies to prevent or delay insect resistance to control tactics need to address ecological and operational factors associated with genetic approaches to monitor and track resistance evolution to have effective and lasting management strategies (Walsh et al. 2022). Farmers will have to consider how each pest may respond to management practice to implement IRM plans for WCR and SBA.

IRM and IPM for each pest

Insect resistance management tactics to delay or prevent WCR resistance to Bt corn involves using a refuge. This refuge strategy is mandatory by the Environmental Protection Agency (EPA) and consists of using a non-Bt host to provide a source of susceptible individuals to mate with Bt-resistant counterparts (EPA 2022a). In addition, the EPA outlines specific integrated pest management practices to delay WCR resistance evolution. These include rotation with a non-rootworm host crop, use of pyramided Bt corn, and planting non-Bt corn with soil-applied insecticide (EPA 2022b). These strategies were also recently reviewed by Gassmann (2021), who also suggested that multiple approaches are needed to improve IRM programs for WCR and manage resistant populations.

Insect resistance management plans to delay SBA resistant to pyrethroids and other insecticides were not discussed until control failures occurred throughout the north central United States. It has been suggested that growers should scout and spray only when needed, follow recommendations from the insecticide label, and rotate modes of actions if a second application is required within the same growing season and among years (Koch et al. 2018a,b).

Recent studies suggested no evidence of cross-resistance to other mode of action (Valmorbida et al. 2022a), and these new chemistries demonstrated efficacy similar to lambda-cyhalothrin against SBA (Koch et al. 2020, Queiroz et al. 2020). However, higher costs might discourage the use of insecticides with different modes of action (Dean et al. 2020a). Bifenthrin and lambda-cyhalothrin were the insecticides most used in Iowa soybean fields in 2020 (USDA - NASS 2021c), indicating that pyrethroids continue being the primary choice to manage insect pests in soybeans. Host-plant resistance containing *Rag* genes (resistance to *Aphis glycines*) can also be incorporated into programs to manage pyrethroid-resistant SBA (Dean et al. 2020a, Tilmon et al. 2021). However, the use of soybean varieties containing *Rag* genes is limited due to restricted availability (Tilmon et al. 2021). To what extent these practices can delay the spread of pyrethroid-resistant aphids, cross resistance to other chemistries and evolution of aphids capable of overcoming insecticides and aphid-resistant soybean varieties is unknown.

Adopting IRM and IPM strategies for both pests can help delay resistance evolution. However, farmer compliance with IRM and IPM recommendations also relies on the profitability of the strategy and how much effort is needed to implement them (Hurley and Mitchell 2014). If a management strategy results in higher profit, it is more likely to be adopted by the majority of farmers. Conversely, suppose the management strategy is not profitable in the short term and/or requires many efforts to be implemented. In that case, adoption rates are slower and delay in resistance evolution may not occur (Hurley and Mitchell 2014). Strategies to delay resistance evolution and to evaluate insect resistance management tactics should include models to predict resistance evolution and resistance monitoring programs (McGaughey and Whalon 1992). Modeling allows to better understand resistance evolution and to develop and implement resistance management tactics in complex ecological systems (Tabashnik 1990, Onstad 2014).

Monitoring resistance evolution allows the assessment of the susceptibility of an insect population to a toxin and provides information on whether the susceptibility of a population is shifting over time (Roush and Miller 1986, Stanley 2014).

Conclusions

Although WCR and SBA present several differences in their biology (e.g. reproduction) and ecology (e.g. migratory behavior), evidence of resistance evolution to management tactics were documented for both pests. Interestingly, there are similarities when considering factors associated with resistance evolution, mostly driven by operational factors such as pest management issues, IPM and IRM practices. Because SBA outbreaks in Iowa are sporadic, vary in time and space, and the migratory behavior of the aphid, predicting the occurrence of resistant aphids during the growing season can be difficult. On the other hand, the presence of high densities of WCR in one year could indicate an issue in the next growing season, especially in areas with continuous corn production systems. Recent studies demonstrated landscape use (e.g. continuous corn cultivation) facilitated resistance evolution (St. Clair and Gassmann 2021). The success of IRM and IPM plans for WCR and SBA in Iowa have to address genetic, biological, and ecological aspects of pests and social processes such as adoption and compliance of these strategies throughout the state. Implementing strategies to delay resistance evolution is complex and involves several sociobiological factors. Effective pesticide resistance management relies on understanding farmers' individual, community, and landscape decisions (Gould 2018).

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Figures

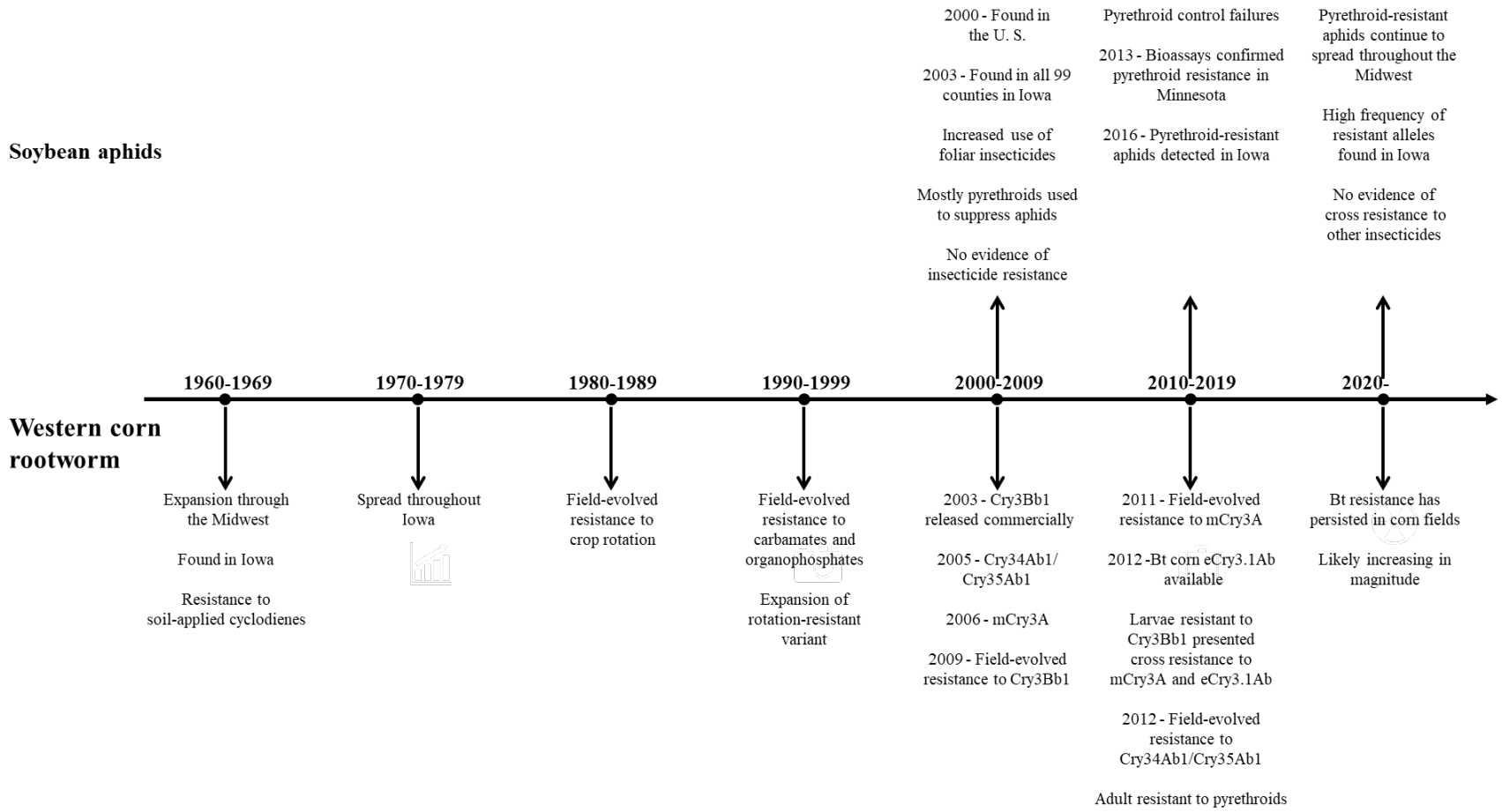

Figure 5.1. A brief history of soybean aphid and western corn rootworm evolution to management tactics in the Midwest.

CHAPTER 6. GENERAL CONCLUSIONS

Soybean aphid is an invasive species first identified in Wisconsin in 2000, which rapidly spread throughout the major soybean production areas in the U. S. (Ragsdale et al. 2011). This pest is considered one of most important insect pests of soybeans in North America. Prior to the soybean aphid introduction, famers rarely used foliar insecticide applications in soybeans in the North Central region (Costamagna and Landis 2006, Ragsdale et al. 2011). Currently, soybean aphid management is a priority for farmers in North America (Hurley and Mitchell 2017), and pyrethroid use in soybean fields increased (Hodgson et al. 2012, Coupe and Capel 2016). This overreliance of foliar insecticides with the same mode of action resulted in subpopulations of soybean aphids evolving resistance to pyrethroids (Hanson et al. 2017, Menger et al. 2020), which can affect the effectiveness of foliar applications to suppress soybean aphid outbreaks (Koch et al. 2018).

Exposure to lower insecticide concentrations can occur in several ways in the agroecosystems (Duke 2014). These exposures can lead to hormesis, which can favor pest resurgence and insecticide resistance evolution (Guedes et al. 2014, Guedes et al. 2017). In addition, studies have suggested that insect adaptation to plant defenses (e.g. host plant resistance) can affect their susceptibility to insecticides (Alyokhin and Chen 2017). The objectives of chapter 2 were to evaluate the susceptibility of two distinct biotypes of soybean aphid to lambda-cyhalothrin, a common foliar insecticide used to suppress soybean aphid populations. We also explored how both virulent and avirulent aphids responded to the exposure to a lower concentration and if this induced hormesis. We found that the LC_{50} for lambda-cyhalothrin was significantly higher for the virulent biotype than the LC_{50} for the avirulent aphids. Our results also demonstrated that exposure to the LC_{25} of lambda-cyhalothrin can

trigger hormesis in the soybean aphid. In addition, our results suggested that the virulent aphid may have advantages compared with the avirulent aphids. This is concerning because it can inadvertently favor the selection of virulent aphids and impact other management strategies such as the use of host plant resistance.

In chapter 3, we explored the mechanism associated with pyrethroid-resistant soybean aphids. Resistance to pyrethroids is associated with non-synonymous mutations in the voltage-gated sodium channel (*vgsc*) genes (Dong et al. 2007, Rinkevich et al. 2013, Scott 219). We confirmed the presence of two mutations in the *vgsc* of pyrethroid-resistant soybean aphids. A leucine to phenylalanine at *vgsc* position 1014 (L1014F), and a methionine to isoleucine change (M918I), conferring knockdown (*kdr*) resistance to pyrethroids. In addition, we developed molecular markers to assess resistant allele frequency (RAF) changes before and after a foliar application of lambda-cyhalothrin. We observed a significant increase of *kdr* alleles in field populations following the application of lambda-cyhalothrin. The use of molecular markers for the L1014F and M918I revealed an association between aphids surviving field rate of lambda-cyhalothrin and the presence of the *kdr* mutations, particularly a *super-kdr* (L1014F + M918I) genotype. These molecular markers can be used to monitor RAF changes in soybean aphid populations and can be incorporated to develop and implement insecticide resistance management (IRM) plans.

In chapter 4, we explored fitness costs associated with pyrethroid-resistant soybean aphids. Although resistance evolution can confer a selective advantage to soybean aphids when exposed to pyrethroids, fitness costs can impact the frequency of resistant individuals and their persistence in the agroecosystems (Freeman et al. 2021). Leaf dip bioassays demonstrated resistance ratios varied significantly among soybean aphid isofemale lines exposed to lambda-

cyhalothrin and bifenthrin. Our results demonstrated no clear association of levels of pyrethroid resistance and decreased reproductive performance. Conversely, we found that a clone with the *super-kdr* (L1014F + M918I) genotype presented significantly higher reproductive rates than pyrethroid-resistant and -susceptible isofemale lines. In addition, we observed that different non-synonymous mutations in the *vgsc* might confer similar levels of resistance to bifenthrin and lambda-cyhalothrin. The evidence of pyrethroid-resistance aphids with high reproductive capacity is concerning and will require IPM and IRM plans to prevent these clones from spreading throughout the major soybean production areas in the U. S.

Understanding factors associated with resistance evolution are important to develop and implement IPM and IRM strategies. In Iowa, soybean aphid and western corn rootworm are insect pests that threaten soybean and corn production, respectively. Both pests have evolved resistance to management tactics. We explore the rates at which resistance is developing in these pests, tactics to delay resistance evolution and the relative success of these methods. In addition, we suggest that IRM and IPM for the soybean aphid should include scouting and monitoring, following economic threshold level, and rotation of insecticides with a different mode of action when more than one foliar application is needed during the growing season. For WCR, IPM strategies are recommended along with the adoption of refuge when using Bt corn as part of an IRM program. Adopting IRM and IPM and understanding factors driving resistance evolution are important to inform future approaches for extending the efficacy of management strategies for both pests.

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