# EVALUATING ALFALFA WEEVIL (HYPERA POSTICA) RESISTANCE TO MODE OF ACTION GROUP 3A PYRETHROID INSECTICIDES IN THE WESTERN UNITED STATES

by

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# DEDICATION

I would like to dedicate my dissertation to my family, Perryn Raymond, and friends who provided me with their love and support throughout this process. Especially, to my mother, Cecilia Maria Oballe, and my cousin Tom (Tommy) Peter Rodbell, who passed away while I was pursuing my graduate degrees. I would additionally like to dedicate my dissertation to my advisor Dr. Kevin Wanner without his guidance and vision, this research could never have been realized.

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# ABSTRACT

Alfalfa weevil (*Hypera postica* Gellenhal [Coleoptera: Curculionidae]) is an insect pest of forage alfalfa (*Medicago sativa* L. [Fabales: Fabaceae]) in the western United States. Over the last half-century, insecticides have been the primary control tactic used by alfalfa producers. However, in 2015 numerous reports of pyrethroid insecticide (mode of action (MoA) 3A) failure to control alfalfa weevil populations were made. In 2019, Montana producers were reporting the same failures in their production systems. Therefore, research efforts in the Wanner Lab commenced in 2020 with the exclusive research goal of identifying pyrethroid resistant alfalfa weevil populations in the western United States. The focus of the research is four-fold. The first was to identify alfalfa weevil lambda-cyhalothrin resistance and susceptibility in Montana. The second was to identify lambda-cyhalothrin resistance and susceptibility in Arizona, California, Montana, Oregon, Washington, and Wyoming. The third was to identify if resistance to lambda-cyhalothrin resulted in the loss of efficacy of other MoA 3A active ingredients. The fourth was to develop a case study addressing integrated resistance management recommendations for alfalfa weevil pyrethroid resistance mitigation. We conducted our research through contact bioassays, molecular genomics, and field trials, to corroborate our results and to identify if alfalfa weevil strain was a factor influencing our documented pattern of resistance. Cumulatively, our results suggest that alfalfa weevil lambda-cyhalothrin resistance is present in Arizona, California, Montana, Oregon, Washington, and Wyoming, and that susceptible populations remain in the western region. Our data further illustrate that regardless of alfalfa weevil strain, alfalfa weevils resistant to lambda-cyhalothrin will be resistant to other type II pyrethroid active ingredients and permethrin. A pattern seen in three distinct alfalfa production zones in the western United States (i.e., Arizona, Montana, and Washington), determined by both contact bioassays and field trials. In conclusion, our results illustrate a challenge that forage alfalfa production faces in the western United States and provides strategies that western forage alfalfa producers can employ to mitigate pyrethroid resistance from developing.

# CHAPTER ONE

# GENERAL INTRODUCTION

## Pyrethroid Origin and Development

Pyrethroids are the synthetic analogues of two naturally occurring insecticidal esters of chrysanthemic acid, defined as pyrethrin I, and pyrethric acid defined as pyrethrin II (Davies et al. 2007) (Table 1). Pyrethrins are derived from Chrysanthemum plants, and their dried flowers were likely used as an insecticide as early as the 1st century AD in China and in Persia and throughout the Middle Ages (Davies et al. 2007). In the 18th century Armenian traders brought 'Persian dust' derived from dried *Chrysanthemum roseum* to Europe. By the mid-19th century a marked increase in Chrysanthemum production occurred to meet the demand of pyrethrin I and II production (Davies et al. 2007). Around this time Tamari and Ueyama, two Japanese entrepreneurs, introduced pyrethrins in 1880 to control lice (lice powder) and mosquitoes through the use of a mosquito coil, a metal coil that is made with dried pyrethrin paste that controls mosquitoes for several hours (Matsuo 2019). However, the application of these compounds was limited due to their instability when exposed to light and wind (Davies et al. 2007).

Structural modifications to pyrethrins occurred between 1924 and 1970 resulting in the development of synthetic pyrethroids with better efficacy and persistence (Davies et al. 2007). Between 1968 and 1974 Michael Elliot developed synthetic pyrethroids that were photostable, had higher insect toxicity, less mammalian toxicity and reduced soil persistence (Elliot 1980, Davies et al. 2007). Overall, pyrethroids are highly hydrophobic and insoluble in water but are

soluble to alcohols and acetone (Coats et al. 1989, Chrustek et al. 2018). Of the synthetic pyrethroids, permethrin was first to be developed followed by cypermethrin and deltamethrin (Elliot 1980, Davies et al. 2007). During this period Sumitomo Chemical Company developed the first non-cyclopropane pyrethroids, such as fenvalerate (Ohno et al. 1976, Nakaayama et al. 1979). Today, pyrethroids are widely used across agricultural, animal, and human health sectors due to their efficacy, mammalian safety, and cost effectiveness. In all, pyrethroids account for 17% of the global market share of insecticides with annual sales greater than $1.5 billion (Davies et al. 2007, Yu. 2015).

Overall, pyrethroids effect insects through cuticular contact, as well as ingestion although to a lesser extent (Bond 2014, Yahouédo et al. 2017). Once ingested, pyrethroids affect the sodium ion channel resulting in prostration, paralysis, and eventual death through neurotoxicity (Davies et al. 2007). Generally, pyrethroids act very quickly, resulting in general loss of coordination and paralysis (i.e., knockdown effect). These symptoms are predominantly accompanied by repetitive activations of both sense organs and myelinated nerve fibers, resulting in the loss of extremities mainly legs and/or wings (Schleier and Peterson 2011). In all, pyrethroids are a diverse group of compounds that are widely used to control both agricultural insect pests as well as insects that have an impact on human health (Schleier and Peterson 2011).

## Introduction to the Sodium Ion Channel and Molecular Mode of Action

Generally, insect membranes contain three distinct passageways for cations, a potassium ($K^+$) ion channel, a sodium ion channel ($Na^+$), and a $K^+$/ $Na^+$ pump (Davies et al. 2007). These three features interact mainly because of the polarization difference between the extracellular fluid and the inside of the cell. The sodium ion channel has two gates the ‘m-gate’

(activation/voltage dependent) and 'h-gate' (inactivation) and the interruption of either of these gates will result in repetitive neural impulses (Davies et al. 2007). The extracellular fluid has a higher concentration of $Na^+$ and a lower concentration of $K^+$ than the cell. The cellular membrane is more permeable to $K^+$ than $Na^+$, resulting in the cell having a negative charge, a difference of approximately -60 millivolts (mV), relative to the extracellular fluid. The cellular membrane becomes permeable to $Na^+$, with nerve stimulation which opens the $Na^+$ channel, thus making the inside of the axon temporarily positively charged, resulting in increasing action potential. Sodium ion channel inactivation, or closure, opens the potassium channels resulting in the loss of $K^+$ triggering the reduction of action potential, resulting in a wave of depolarization along the axon (Davies et al. 2007) (Fig. 1). The $K^+$/ $Na^+$ pump maintains the ion gradient between the membrane and the extracellular fluid which restores the resting potential between the two. However, the reception of a chemical transmitter generates an impulse which depolarizes the membrane and triggers an increase in post-synaptic action potentials (Davies et al. 2007) (Fig. 1).

Voltage-gated sodium channels are structurally and functionally homologous with a subunit of mammalian voltage-gated sodium channels sodium ion channels (Catterall 2000, Davies et al. 2007). Structurally insect voltage-gated sodium channels include a single polypeptide chain consisting four domains, all of which containing six transmembrane helices. The domains jointly form a central aqueous pore lined by transmembrane (S5 and S6) linkers and helices which form the P-loops. The four voltage sensing domains are made of the remaining S1-S4 helices, with activation being triggered by the movement of the positively charged S4 segments (Davis et al. 2007). This movement allows for a confirmational change in the form of a

voltage-dependent, active gate, a second conformational change occurs when the inactivation gate opens. At resting potential, the gate is closed because the active gate is blocking the channel. The voltage-dependent active gate opens with the depolarization of the membrane, is only open for a few milliseconds allowing $Na^+$ to pass through the channel, this conformational change closes the inactive gate. With repolarization of the membrane, both active and inactive gates are closed. After a refractory period of two to five milliseconds, there is a conformational change within the active gate which reopens the inactive gate, closing the channel (Davies et al. 2007) (Fig. 1).

## Insecticides Disrupting the Sodium Ion Channel

The sodium ion channel is the target for a number of insecticides, many of which are commonly used today (Table 3). The first insecticide that targets the sodium ion channel was dichlorodiphenyltrichloroethane (DDT), a chlorinated hydrocarbon, whose insecticidal properties were first discovered in 1939 by Paul Müller, and is considered to be one of the more significant advancements in pest control using synthetic insecticides (Mellanby 1992, Davies et al. 2007). Initially, DDT was considered to be an ideal insecticide as it was highly toxic to most insect pests, retained its toxicity well and was relatively harmless to humans. Beginning in the 1970's DDT along with other hydrocarbon insecticides did not achieve renewed registrations, except for selective public health efforts, due to their persistence in the environment and their fat solubility resulting in bioaccumulation of the toxin in non-target organisms (Davies et al. 2007).

Mode of action (MoA) is the anatomical, physiological, and biochemical interactions and their responses that form the toxic action of a chemical, as well as the physical and molecular fate of the chemical within the exposed organism (Davies 2009). Pyrethroids and pyrethrins are

members of the MoA group 3A, and DDT is a member of MoA group 3B, and all affect the sodium ion channel (Davies 2017) (Table 3). These insecticides only need to affect/modify a small percentage of the population of sodium ion channels to cause repetitive neural discharges (Davies et al. 2007). However, DDT is relatively slow acting when compared to other insecticides, such as members of MoA group 3A (Davies et al. 2007). Regardless, these insecticide active ingredients impede the channel either by impeding the 'm-gate' or the 'h-gate' from closing, thus allowing for the continued conduction of sodium through the sodium ion channel and the disruption of regulation of the release of nerve action potentials. The affected nerve cells operate in a constant state of hyperexcitability initially resulting in knockdown, a sublethal effect. The sodium current continues until hyperexcitability overwhelms the cells such that their ability to maintain the sodium pump is compromised, resulting in paralysis and consequently death (Davies et al. 2007).

Exposure to pyrethrin and pyrethroid insecticides affects insect peripheral and central nervous systems, unlike DDT which only affects peripheral nervous system, meaning that pyrethroids and pyrethrins act faster than DDT (Davies et al. 2007). This affect triggers nerve cells to discharge causing a series of symptoms of exposure that is more pronounced than DDT. The absorption rates for these insecticides are improved with increased lipophilicity resulting in better knockdown rates. There are two types of pyrethroids known today and are distinguished by their chemical structure (Cayo et al. 2014) (Fig. 2). Despite the general diversity in chemical structure, there is one structural distinction between the pyrethroid types: Type I pyrethroids lack the μ-cyano-3-phenoxybenzyl group whereas Type II pyrethroids have it (Soderlund 2012). Type I pyrethroids, such as permethrin (Table 1), are considered to be a relatively effective

because of how quickly axons are triggered and result in mobility issues and end in prostration and paralysis (Davies et al. 2007). Type II pyrethroids (Table 1), such as deltamethrin, are distinct from Type I pyrethroids as Type II have a cyano group at the a-phenoxybenzylic position, which generally results in improved knockdown (Fig. 2). The reason for this distinct difference in efficacy is due to the difference in the modification of the sodium-ion channel, as Type I pyrethroid compounds cause disruptions for tens to hundreds of milliseconds, whereas Type II pyrethroid compound disruption lasts a minimum of a few seconds. Thus, resulting in a prolonged and severe convulsive phase and subsequent improved kill rates (Davies et al. 2007).

Type I and II pyrethroids have a number of features that impact their efficacy. Vais et al. (2001) identified that Type II pyrethroid binding sites are formed when the *para* channel is activated. This dependence on the *para* channel correlates with the negative temperature dependence of pyrethroids and the tendency of low temperatures to increase the time the *para* channel stays open (Motomura and Narahashi 2000). Once the pyrethroid (Type I and II) binds to the active site it maintains the open state of the *para* channel, however, binding is structure dependent which can influence the efficacy of the insecticide (Vais et al. 2001). For DDT, pyrethroids, and brevotoxin their binding sites on the *para* channel is allosterically coupled to a second site on the channel which bind numerous lipophilic neurotoxins (e.g., batrachotoxin). These allosteric interactions mean that the application of a pyrethroid along with a brevotoxin dramatically enhances the binding of lipophilic neurotoxins to the *para* channels compared with either compound alone, potentially improving pest mortality rates (Zlotkin 1999, Davies et al. 2007).

Variation in the effect of an insecticide on targeted insect populations is caused by several factors (Yu 2015). Insect gender is a source of susceptibility variance to an insecticide. Generally, female insects are more tolerant of insecticides than their male counterparts. This difference in tolerance based on gender is theorized to be due to differences in body weight, with female insects being generally larger than males (Yu 2015).

In addition, insect life stage has a profound effect on insecticide efficacy, with additional factors noted within each life stage. Generally, inactive life stages such as egg and pupae are less susceptible to insecticides than more active life stages such as the larval and adult life stages. However, within the larval or nymph stages molting and changes in metabolism explains variance in population susceptibility to an insecticide (Yu 1983). For adults, feeding habits, sexual maturity, and aging are all factors that additionally influence susceptibility to a given insecticide (Yu 2015).

Populations experiencing overcrowding, lower rearing temperatures, quantity and quality of food, and illumination are all factors that have been documented as having an effect on insecticide tolerance in a number of insect pests (Yust and Howard 1942, Kettle 1949, Munson 1953, Garms 1959, Sun 1960, Yu et al. 1979, Berry et al. 1980, Farnworth et al. 1981, Yu 1982, Yu 2015). Thus, if using laboratory colonies for bioassays, one should ensure proper in-lab protocols when rearing lab colonies, to limit distortions within the data.

## Pyrethroid Resistance

Understanding the mechanisms of pyrethroid resistance is important as it can inform the course of action taken to address emerging insecticide resistance. At its most basic, insecticide resistance is one of the best examples of evolutionary adaptation to environmental change

especially given how quickly it has emerged in the context of evolutionary time (Scott 1990). The benefits of studying resistance are enumerable as it can influence the sensitivity of future monitoring techniques, more efficiently identify a replacement pesticide, and identify management strategies to overcome it (Scott 1990).

## Mechanisms with which Resistance Occurs

For an insecticide to have an effect on an insect, it must encompass three levels of pharmacokinetic interactions (Welling and Peterson 1985). It first must penetrate the barrier tissues (e.g., insect cuticle), then the toxin must be distributed, stored, and metabolized in internal tissues, and finally it has a metabolic interaction with the target site (Welling and Peterson 1985, Soderlund and Bloomquist 1990). Therefore, the toxicity of an insecticide depends on its potency at the target site, the rate of uptake after contact, its distribution and partitioning between tissues as well as the metabolic activation and/or detoxification capabilities of these tissues (Soderlund and Bloomquist 1990).

There are diverse chemical structures and many insect pests, but there are comparatively few identified mechanisms of resistance (Georghiou 1986, Soderlund and Bloomquist 1990). These mechanisms include reduced cuticular penetration of the toxin; increased metabolism of the toxin by the enzyme cytochrome $P_{450}$-dependant monooxyenases, hydrolases, or glutathione-S- transferases; reduced sensitivity of acetylcholinesterases to organophosphates, carbamate insecticides and neuronal targets of pyrethroids, which includes chlorinated cyclodienes as well as DDT and its analogues (Oppenoorth 1985, Soderlund and Bloomquist 1990). It is important to emphasize that these mechanisms of resistance are nonspecific to an insect species or a chemical structure, and thus can confer cross-resistance to other toxins of a similar chemical structure, or

in extreme cases chemically unrelated toxins (Soderlund and Bloomquist 1990). As these mechanisms of resistance occur across all three levels of insect-insecticide interactions the likelihood of there being multiple mechanisms of resistance occurring at these different levels can produce synergistic interactions resulting in virtual immunity to a toxin within pest populations (Soderlund and Bloomquist 1990).

Reduced cuticular penetration is the result of modifications within the insect cuticle that reduce the rate of penetration, and this mode of resistance effects the first pharmacokinetic interaction of crossing the barrier tissues (Soderlund and Bloomquist 1990, Balabanidou et al. 2018). There are two mechanisms of this form of resistance that have been identified: cuticular thickening and changes within the composition of the cuticle (Balabanidou et al. 2018). Alterations within the cuticle are generally due to the over-expression of genes or proteins (e.g., cuticular proteins), changes in enzymes that catalyze enzymatic reactions (CYP4G16 and laccase 2), and transporters that aid in cuticular translocation (Balabanidou et al. 2018).

Increased metabolism of the toxin by the enzyme cytochrome $P_{450}$-dependant monooxyenases, hydrolases, or glutathione-S-transferases is conferred by a number of molecular mechanisms. These molecular mechanisms enhance oxidation through enzymatic activity in resistant insect strains (Soderlund and Bloomquist 1990). First, resistant populations may possess more of every component of the monooxygenase system. Second, oxidation may occur at a higher rate due to an overexpression of a single form of cytochrome $P_{450}$ which has high catalytic activity towards the insecticide. Third, alterations in gene regulation of cytochrome $P_{450}$ gene may affect its expression such that susceptible populations express the gene conversely to that of resistant populations. Finally, resistant populations may contain a mutation of the cytochrome

$P_{450}$ gene resulting increased catalytic activity towards the insecticide (Soderlund and Bloomquist 1990). A challenge with documenting enhanced oxidative metabolism in resistant populations through bioassays is that cytochrome $P_{450}$ genes are not identically susceptible to enzyme inhibitors, meaning that that the lack of synergism may not reflect the lack of oxidative metabolism. However, toxicity synergism within resistant populations is the first indicator that oxidative metabolism is contributing to resistance within a population (Soderlund and Bloomquist 1990).

Esterases in pyrethroid resistance has been linked to peach-potato aphids (*Myzus persicae*), Egyptian cotton leafworm (*Spodoptera littoralis*), houseflies (*Musca domestica* L.), and tobacco budworm (*Helicoverpa armigera*) (Devonshire and Moores 1982, Riskallah 1983, Sawicki et al. 1984, Dowd et al. 1987). Evidence from these documented species indicates that quantitative and qualitative changes are involved in increased hydrolytic capabilities of resistant populations (Soderlund and Bloomquist 1990). In short, even though esterase plays a pivotal role in the metabolism of pyrethroids in arthropods, relatively few species are clearly linked to increased esterase activities and resistance (Soderlund and Bloomquist 1990).

Target site resistance is commonly conferred by knockdown resistance (*kdr*). Knockdown resistance is caused by a recessive allele resulting in cross-resistance the whole class of pyrethrin, pyrethroids, and DDT as well as its analogues (Davis et al. 2007). This form of resistance was first documented by Busvine (1951) in houseflies. Since then, *kdr* has been recognized as conferring cross-resistance to DDT and to all known pyrethroids (Soderlund and Bloomquist 1990). Initially, *kdr* referred only to resistance conferring alleles in houseflies that map to the common locus on autosome 3, as the potential lack of genetic mapping in other insect species made it unclear

whether their resistance is the result from mutations at the *kdr* locus or from other mutations at other loci (Soderlund and Bloomquist 1990). Since then, additional economically important insect species have been reported as presenting with this form of resistance (Davies et al 2007). Another form of resistance has since been discovered, an additional recessive resistant trait referred to as *super-kdr*, a trait in which the resulting resistance to pyrethroids is much greater (Davies et al. 2007).

The mechanism of *kdr* and *super-kdr* is associated with the modification of an insect's voltage-gated sodium channel, with a close genetic linkage between *kdr* loci and the *para* channel. Evidence for this resistance mechanism was discovered over the course of numerous cross-resistance studies in house fly, tobacco budworm, and German cockroach (Soderlund and Bloomquist 1990, Taylor et al. 1993, Williamson et al. 1993, Dong and Scott 1994, Soderlund 2005, Davies et al. 2007). Since the genetic linkage was discovered, amino acid substitutions were noted in resistant insect populations that are responsible for the reduction in *para* channel sensitivity to pyrethroids and/or DDT in a number of insect pest species (Davies et al. 2007). The majority of amino acid substitutions occur on the S4/S5 linker and the S5 or S6 helices of domain II, or in the linker/helices of domains I or III (Davies et al. 2007, Dong 2007). The specific amino acid substitution has a profound impact on the level of resistance conferred (Davies et al. 2007). For example, with a *kdr* substitution in houseflies (L1014F) the voltage dependence of both the activation and inactivation is shifted towards more positive potentials (Davies et al. 2007, Yu 2015). Meaning, that a closed state of inactivation is promoted causing 70-80% of the *para* channel being closed, resulting in reduced action of pyrethroids as they bind to the open state of the channel (Davies et al. 2007). Additional sources of resistance located in

the intracellular and voltage-sensing regions of the *para* channel are responsible for altering the channel kinetics thus negatively impacting pyrethroid efficacy (Davies et al. 2007). Zlotkin (1999) highlighted the potential for two insecticides targeting different binding sites on the same *para* channel, when used synergistically may reduce the concentrations of the insecticides needed to control a pest population. This is credited as being a mechanism of reducing treatment costs and delaying resistance within the treated population (Davies 2007).

There are a diverse number of voltage-gated sodium channel regulatory protein sequences for *kdr* mutations (Punchihewa et al. 2019). Although the protein sequence for *kdr* is not known for alfalfa weevil *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) a close relative, maize weevil (*Sitophilus zeamais*) has been documented as having the *kdr* mutation T9291 located at IIS5. The T929I mutation can occur in conjunction with additional mutations such as L1014F (Location: IIS5+IIS6) or L932F (Location: IIS5+IIS5) (Punchihewa et al. 2019). Overall, the T929I mutation either alone or in combination mutations M827I and L932F eliminated permethrin sensitivity (Rinkevich et al. 2013). It stands to reason that the T929I mutation may be the *kdr* mutation responsible for pyrethroid resistance in other weevil species.

Behavioral avoidance is common form of insecticide-resistance. Such forms of behavioral resistance include increased walking activity, which allow insects to quickly evade contaminated areas, resulting in reduced exposure to the toxin (Haddi et al. 2018). Two members of the Curculionidae family (*Sitophilus zeamais* and *Sitophilus oryzae*) have shown increased walking activity as a form of behavioral resistance. It is important to note, that multiple mechanisms of pyrethroid resistance can occur simultaneously and have been noted for multiple weevil species (Haddi et al. 2018). This type of response can be divided in to two subsequent

groups: direct contact excitation (also referred to as irritancy) and non-contact spatial repellency (Roberts et al. 1997). Direct contact excitation is when an insect leaves the treated area after making physical (tarsal) contact with the chemical. Spatial repellency is when an insect moves away from the treated area without making contact with the chemical (Chareonviriyaphap et al. 1997, Chareonviriyaphap et al. 2013, Haddi et al. 2018).

## Insects with Resistance to Pyrethroids

As pyrethroids are used to control insect pests in agriculture and in human health, insecticide resistance to them has implications on both human health and agricultural yields. The occurrence of an insect population developing resistance is largely dependent on the number of generations per year and on the rate of insecticide use. For agricultural pests in the US, pyrethroid resistance has been documented for western corn rootworm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (Pereira et al. 2015, Pereira et al. 2017), corn earworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) (Jacobson et al. 2009), fall armyworm (Lepidoptera: Noctuidae) (Hardke et al. 2015), and soybean aphid (Hemiptera: Aphididae) (Hanson et al. 2017). However, there are many more that affect the top three most exported row crops in the US, corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merr.), and alfalfa (*Medicago sativa* L.) (Nelson 2019, Reitz 2020). In the western region, there as a number of common insect pests of alfalfa, each of which have their own management challenges (Table 2).

## Alfalfa Weevil Taxonomy, Biology, and Life History

The adult alfalfa weevil is a brown-grey beetle that has a characteristic snout located on the front of the head and can further be by its distinct black stripe along the dorsal side of its abdomen. It is 3.0-5.5-mm long (Fig. 3A, Pellissier et al. 2017). The adult stage overwinters

under leaf litter and becomes active in alfalfa fields when temperatures reach or exceed 15.56 °C. Once they emerge females chew holes in the stems of alfalfa and oviposit clusters of eggs in them (Pellissier et al. 2017). Eggs are bright yellow and often are found in clusters of five to 15 eggs. As the eggs mature, however, they darken and are dark yellow to brown. Eggs hatch within one to two weeks of being laid, the first instars feed on the plant terminals (Pellissier et al. 2017). The early instars feed almost exclusively on the developing plant terminals resulting in the characteristic shot-hole pattern which is noticeable as the leaves expand (Fig 4B and Fig. 3A). The small larvae have a black head capsule with a body that is light yellow to light green. Later instars acquire the characteristic green body color as well as a white stripe along the dorsal side of their abdomen (Fig. 3B). Across all stages, alfalfa weevil larvae retain their black head capsule.

Alfalfa weevils are native to Eurasia and North Africa and were introduced to North America a least three times, with each introduction being recognized as a distinct strain (Hsiao 1993, Bundy et al. 2005). These strains include eastern, western, and Egyptian. The western strain was first reported in near Salt Lake City Utah in 1904 (Titus 1910), Egyptian strain followed in 1939 in Yuma Arizona (Wehrle 1940), and the eastern strain in 1952 in Maryland (Poos and Bissell 1953, Wood et al. 1978). Regardless of the strain, alfalfa weevils cause economic injury to alfalfa and have developed resistance to insecticides in the past (Alder and Blickenstaff 1964, Bishop 1964).

Alfalfa weevil is the primary economically damaging pest of forage alfalfa, in the western region of the US (O'Neal et al. 2017). They are considered a pest due to their primary host being alfalfa and the feeding injury that is caused during the larval stage of their

development (Blodgett and Lenssen 2004). Alfalfa weevils cause economic injury to alfalfa by reducing yields, including forage and seed alfalfa, by 10 to 15% annually (alfalfa forage quality not taken into account) (University of Illinois Extension and Outreach 2020). Larvae feed on leaves and not the stems of alfalfa plants resulting in leaves with a frosted appearance when defoliation is severe, thus reducing the quantity and quality of alfalfa forage (Fig. 3B) (Herrnstadt et al. 1989, University of Illinois Extension and Outreach 2020). Forage quality is affected through larval feeding as alfalfa leaves contain more protein than stems, and a lower leaf to stem ratio reduces overall forage protein content. Adult alfalfa weevils feed mainly on alfalfa stems and are not considered to generate enough feeding damage to reduce yields.

Current management recommendations for alfalfa weevils are predominantly focused on insecticides. Chemical control is an integral part of integrated pest management of alfalfa weevil and in some situations, it is the only option available to producers to limit economic losses. Chemical control mechanisms are recommended when thresholds are met. These thresholds include a mixed management approach when 30% of plant terminals have feeding damage, it is recommended to cut and then treat the crop, or just treat the entire stand a decision largely dependent on location and production system. The decision between the two approaches is influenced by how close to cutting the alfalfa stand is (Reitz 2020). Additional thresholds include damage noticed within a week of cutting coupled with larvae exceeding 10 larvae per sweep, with a general threshold being larvae are 20 or more per sweep of 180° regardless of stand growth stage (Reitz 2020).

Since alfalfa weevils were accidentally introduced in the US in the early 20$^{th}$ century, the USDA Agricultural Research Service (USDA-ARS) along with the Animal and Plant Health

Inspection Service (APHIS) introduced parasitoid wasps as biocontrol agents of the three strains of alfalfa weevils (Pellissier et al. 2017). In all, these agencies introduced eight parasitoid wasp species. In the western region of the US, the most common parasitoid wasps include ichneumonid wasps: *Bathyplectes curculionis* (Thomson), *Bathyplectes anurus* (Thomson), and *Bathyplectes stenostigma* (Thomson) (Pellissier et al. 2017). Additional biocontrol agents include lady beetles (Coccinellidae), damsel bugs (Nabidae), and lacewings (Chrysopidae and Hemerobiidae), but these biocontrol agents often prefer aphids over alfalfa weevil larvae (Kalaskar and Evans 2001).

Host plant resistance, a key aspect of integrated pest management is incredibly limited, however, research has shown the efficacy of resistant cultivars and their control of alfalfa weevil populations (Busbice et al. 1977, Summers 1998, Tohidfar et al. 2013). Cultural control, however, is commonly used. Cultural control mechanisms include harvesting date, hay removal as well as early application of an insecticide followed by continuous spring grazing (Summers 1998). Harvest date is important as harvest in the winter (southern climates) or spring (northern climates) to greatly reduces larval populations due to mortality caused by desiccation or starvation, with eggs located in the stems removed from the field once baled (Casagrande and Stehr 1973). The timing of harvest is critical as harvest prior to oviposition results in injury to the second crop regrowth derived from larvae hatched from eggs located in the stubble below the height of the cutter bar (Summers 1998). Furthermore, the combination of an early application of an insecticide followed by continuous spring grazing provided control of larval populations (Buntin and Bouton 1996).

## History of Alfalfa Weevil Insecticide Resistance

During the 1950s and 1960s the insecticides used to control alfalfa weevil were primarily organochlorines, heptachlor, and dieldrin (Bishop 1964). In 1962, however, reports of resistance to either heptachlor or dieldrin occurred in Virginia and in Utah and indicated poor control of adults and larvae (Bishop 1964). Since the initial reports of resistance, data collected from uncontrolled populations showed that repeated doses of heptachlor failed to kill resistant adults in laboratory bioassays (Bishop 1964). This first documentation of insecticide resistance in alfalfa weevils in the US indicated that the lethal dose (LD50) required to kill resistant adults was 4,420 µg/g, or 170 times higher than the LD50 value of 26 µg/g for susceptible adults of heptachlor (Bishop 1964). Cross-resistance to dieldrin was noted for populations never exposed to the insecticide, indicating cross-resistance to multiple active ingredients of the same mode of action group (Bishop 1964). In 1969, Furadan, a carbofuran, was considered an effective alternative to heptachlor and dieldrin (DePew 1977, Rethwisch 2019). However, in 2009 the US Environmental Protection Agency (EPA) announced a tolerance revocation of all products containing carbofuran (EPA 2016A). Today, the main source of alfalfa weevil control is MoA 3A pyrethroid insecticides but reports of insecticide failures from concerned producers indicate an emerging issue in the US and Canada (Glen 2015).

## Alfalfa Weevil Pyrethroid Resistance in the United States and Alternative Insecticides

There are two types of pyrethroids, type I and type II, and they generally differ on the absence or presence of a cyano group respectively (EPA 2016B) (Table 1) (Fig. 2). With the most effective and affordable insecticides consisting of MoA group 3A, which includes alpha-

cypermethrin, zeta-cypermethrin, beta-cyfluthrin, lambda-cyhalothrin, permethrin, and bifenthrin (IRAC 2020) (Table 1).

In 2015, growers in Alberta Canada reported repeated failures of insecticides containing pyrethroid as the active ingredient (AI) such as Decis® (AI deltamethrin) and Matador (AI lambda-cyhalothrin) (Glen 2015, Bayer 2020, Syngenta 2020). Repeated applications of Baythroid® (AI beta-cyfluthrin) and Warrior II® (AI lambda-cyhalothrin) failed to provide effective control of alfalfa weevil populations in Scott Valley, CA. Weevils collected from areas suspected of insecticide resistance and individuals from organic fields were collected and shipped to UC Davis where in-lab bioassay was conducted and the results showed that alfalfa weevils had developed insecticide resistance compared to their susceptible counterparts (Orloff et al. 2016).

Although we are only beginning to understand the scope of pyrethroid resistance in the western region, it is clear that very little is known about the level of resistance. Despite the lack of official documentation of resistance, reports of repeated insecticide failures to control alfalfa weevils were documented from Rosemary, Alberta, and northern, central, and southern California, with collaborators also noting potential failures in Washington and Colorado (Glen 2015). What these reports suggest is that pyrethroid resistance in alfalfa weevil populations have been increasing for several years and are now reaching levels that are now apparent. As pyrethroids (MoA 3A) are more effective and affordable insecticides to control alfalfa weevil populations, region-wide reduction in their efficacy would be catastrophic to alfalfa producers, as there is limited alternative insecticides with different MoA groups available for use in the US.

Alternative insecticides available for producers in the US are chlorpyrifos (Lorsban®, MoA 1B) and indoxacarb (Steward®, MoA 22A). Chlorpyrifos is currently facing a national revocation of its tolerances in the US. In 2017, the EPA denied a petition to remove all pesticide tolerances and to cancel registrations of chlorpyrifos, as the results of investigation of the impact that chlorpyrifos may have on neurodevelopment were unresolved and that more data were warranted (EPA 2019). In 2018, the US Ninth Circuit Court of Appeals demanded the ban of chlorpyrifos within 60 days, however, the U.S. Department of Justice was granted a rehearing with an 11-judge panel, thus overruling the Ninth Circuit Court of Appeals. The panel heard arguments in spring of 2019, and on April 19, 2019, the panel ordered the EPA to make a final decision on the petition objections within 90 days (EPA 2019). Currently, the EPA allows the use of chlorpyrifos under strict label instructions until at least 2020 (Flynn 2019). In 2022 the EPA will complete a review of neurodevelopmental effects of chlorpyrifos (Flynn 2019). However, states like New York, Hawaii, and California have banned the use chlorpyrifos in many aspects of their agricultural production, and it stands to reason that other states will do the same (Flynn 2019, NPR 2019). Given the uncertainty of chlorpyrifos status on the state and federal level producers may wish to hold off on chlorpyrifos until the conclusion of the 2022 EPA review.

Indoxacarb is an additional insecticide that blocks the sodium channels in the nervous system of insects, and currently is established under time-limited tolerances, due to the EPA granting an emergency exemption for the use of the pesticide in alfalfa and grasses. This exemption establishes maximum permissible levels of indoxacarb in or on grass forage and hay. However, the emergency exemption will expire on December 31, 2022 (Federal Register 2019).

As alfalfa is the nation's third most valuable field crop, it is important to match national tolerances to international tolerances dictated in the Codex Alimentarius Commission (Codex), but there are currently no established international maximum residue limits (Nelson 2019, Federal Register 2019). Therefore, preserving the efficacy of indoxacarb for alfalfa weevil control is critical until new MoAs can be developed and registered.

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## Tables and Figures

Table 1. Types of pyrethrins and pyrethroids.

| Active ingredient | Pyrethrins: constituents of natural pyrethrum extracts | | Synthetic derivatives of pyrethrin: pyrethroids | |
|---|---|---|---|---|
| | Esters of Chrysanthemic acid | Esters of Pyrethric acid | Type I Esters without alpha-cyano group | Type II Esters with alpha-cyano group |
| Types of pyrethrins/pyrethroids gradually developed | Pyrethrin-I | Pyrethrin-II | Allethrin 1st generation | Fenvalerate 3rd generation |
| | Cinerin-I | Cinerin-II | Resmethrin 2nd generation | Cyhalothrin 4th generation |
| | Jasmolin-I | Jasmolin-II | Permethrin 3rd generation | Cypermethrin 4th generation |
| | | | Bifenthrin 4th generation | Deltamethrin 4th generation |

*Table sourced from Roy 2002, Soderlund et al. 2002, and Kaviraj and Gupta 2014

Table 2. Common pests of alfalfa hay in the western region of the United States.

| Common Name | Scientific Name | Recommended Control Strategies |
|---|---|---|
| Alfalfa Looper | *Autographa californica* | Chemical Control |
| Alfalfa Caterpillar | *Colias eurytheme* | Chemical Control |
| Alfalfa Aphid | *Macrosiphum creelii* | Biological, Chemical and Cultural Control |
| Blue Alfalfa Aphid | *Acyrthosiphon kondoi* | Biological, Chemical and Cultural Control |
| Pea Aphid | *Acyrthosiphon pisum* | Biological, Chemical and Cultural Control |
| Spotted Alfalfa Aphid | *Therioaphis maculata* | Biological, Chemical and Cultural Control |
| Beet Armyworm | *Spodoptera exigua* | Chemical Control |
| Bertha Armyworm | *Mamestra configurata* | Chemical Control |
| Western Yellowstriped Armyworm | *Spodoptera praefica* | Chemical Control |
| Blister Beetle | *Epicauta spp.* | Chemical Control |
| Clover Leaf Weevil | *Hypera punctata* | Biological and Chemical Control |
| Alfalfa Weevil | *Hypera postica* | Chemical Control |
| Clover Root Curculio | *Sitona hispidulus* | Chemical control for adult weevils only |
| Army Cutworm | *Euxoa auxiliaris* | Chemical Control |
| Clover Cutworm | *Scotogramma trifolii* | Chemical Control |
| Granulate Cutworm | *Feltia subterranea* | Chemical Control |
| Redbacked Cutworm | *Euxoa ochrogaster* | Irrigate fields prior to application of chemical control |
| Variegated Cutworm | *Peridroma saucia* | Chemical Control |

| | | |
|---|---|---|
| Grasshoppers | *Melanoplus spp.* | Chemical Control |
| Meadow Spittlebug | *Philaenus spumarius* | Chemical control is not warranted. |
| Pea Leaf Weevil | *Sitona lineata* | Chemical Control |
| Western Spotted Cucumber Beetle | *Diabrotica undecimpunctata* | Chemical Control |

*Sourced from Reitz 2020

Table 3. Mode of action (MoA) classification into group and sub-group as well as their associated active ingredients (IRAC 2020).

| Main Group & Primary Site of Action | Sub-group or Active Ingredient | Active Ingredients |
|---|---|---|
| 1<br>Acetylcholinesterase inhibitors<br><br>Nerve Action | 1A<br>Carbamates | Alanycarb, Aldicarb, Bendiocarb, Benfuracarb, Butocarboxim, Butoxycarboxim, Carbaryl, Carbofuran, Carbosulfan, Ethiofencarb, Fenobucarb, Formetanate, Furathiocarb, Isoprocarb, Methiocarb, Methomyl, Metolcarb, Oxamyl, Pirimicarb, Propoxur, Thiodicarb, Thiofanox, Triazamate,Trimethacarb, XMC, Xylylcarb |
| | 1B<br>Organophosphates | Acephate, Azamethiphos, Azinphos-ethyl, Azinphos- methyl, Cadusafos, Chlorethoxyfos, Chlorfenvinphos, Chlormephos, Chlorpyrifos, Chlorpyrifos-methyl, Coumaphos, Cyanophos, Demeton-S-methyl, Diazinon, Dichlorvos/ DDVP, Dicrotophos, Dimethoate, Dimethylvinphos, Disulfoton, EPN, Ethion, Ethoprophos, Famphur, Fenamiphos, Fenitrothion, Fenthion, Fosthiazate, Heptenophos, Imicyafos, Isofenphos, Isopropyl O-(methoxyaminothio-phosphoryl) salicylate, Isoxathion, Malathion, Mecarbam, Methamidophos, Methidathion, Mevinphos, Monocrotophos, Naled, Omethoate, Oxydemeton-methyl, Parathion, Parathion-methyl, Phenthoate, Phorate, Phosalone, Phosmet, Phosphamidon, Phoxim, Pirimiphos- methyl, Profenofos, Propetamphos, Prothiofos, Pyraclofos, Pyridaphenthion, Quinalphos, Sulfotep, Tebupirimfos, Temephos, Terbufos, Tetrachlorvinphos, Thiometon, Triazophos, Trichlorfon, Vamidothion |
| 2<br>GABA- gated chloride channel<br><br>blockers | 2A<br>Cyclodiene<br><br>Organichlorines | Chlordane, Endosulfan |

| | | |
|---|---|---|
| | | |
| Nerve Action | 2B<br>Phenylpyrazoles | Ethiprole, Fipronil |
| 3<br>Sodium Channel<br>Modulators<br>Nerve Action | 3A<br>Pyrethroids<br>Pyrethrins | Acrinathrin, Allethrin, d-cis-trans Allethrin, d-trans Allethrin, Bifenthrin, Bioallethrin, Bioallethrin S-cyclopentenyl isomer , Bioresmethrin, Cycloprothrin, Cyfluthrin, beta-Cyfluthrin, Cyhalothrin, lambda-Cyhalothrin, gamma-Cyhalothrin, Cypermethrin, alpha-Cypermethrin, beta-Cypermethrin, theta- cypermethrin, zeta-Cypermethrin, Cyphenothrin , (1R)-trans- isomers], Deltamethrin, Empenthrin (EZ)- (1R)- isomers], Esfenvalerate, Etofenprox, Fenpropathrin, Fenvalerate, Flucythrinate, Flumethrin, tau-Fluvalinate, Halfenprox, Imiprothrin, Kadethrin, Permethrin, Phenothrin [(1R)-trans- isomer], Prallethrin, Pyrethrins (pyrethrum), Resmethrin, Silafluofen, Tefluthrin, Tetramethrin, Tetramethrin [(1R)-isomers], Tralomethrin, Transfluthrin, |
| Sodium Channel<br>Modulators<br>Nerve Action | 3B<br>DDT<br>Methoxychlor | DDT, Methoxychlor |
| 4<br>Nicotinic Acetylcholine<br>Receptor Allosteric Competitive<br>Modulators | 4A<br>Neonicotinoids | Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid, Thiamethoxam, |
| | 4B<br>Nicotine | Nicotine |
| | 4C<br>Sulfoximines | Sulfoxaflor |
| Nerve Action | 4D<br>Butenolides | Flupyradifurone |

| | | |
|---|---|---|
| | 4E<br>Mesoionics | Triflumezopyrim |
| 5<br>Nicotinic Acetylcholine Receptor<br>Allosteric Modulators- Site I<br>Nerve Action | Spinosyns | Spinetoram, Spinosad |
| 6<br>Glutamate-Gated Chloride Channel<br>Allosteric Modulators<br>Nerve and Muscle Action | Avermectins, Milbemycins | Abamectin, Emamectin benzoate, Lepimectin, Milbemectin |
| 7<br>Juvenile Hormone Mimics<br>Growth Regulation | 7A<br>Juvenile hormone analogues | Hydroprene, Kinoprene, Methoprene |
| | 7B<br>Fenoxycarb | Fenoxycarb |
| | 7C<br>Pyriproxyfen | Pyriproxyfen |
| 8<br>Miscellaneous Non-Specific<br>Inhibitors | 8A<br>Alkyl halides | Methyl bromide and other alkyl halides |
| | 8B<br>Chloropicrin | Chloropicrin |
| | 8C<br>Fluorides | Cryolite (Sodium aluminum fluoride), Sulfuryl fluoride |
| | 8D<br>Borates | Borax, Boric acid, Disodium octaborate, Sodium borate, Sodium metaborate |
| | 8E | Tartar emetic |

| | | |
|---|---|---|
| | Tartar emetic | |
| | 8F<br>Methyl Isothiocyanate<br>Generators | Dazomet, Metam |
| 9<br>Chordotonal Organ TRPV Channel<br>Modulators | 9B<br>Pyridine azomethine<br>derivatives | Pymetrozine, Pyrifluquinazon |
| | 9D<br>Pyropenes | Afidopyropen |
| 10<br>Mite Growth Inhibitors Affecting<br>CHS1<br><br>Growth Regulation | 10A<br>Clofentezine Diflovidazin<br>Hexythiazox | Clofentezine Diflovidazin Hexythiazox |
| | 10B<br>Etoxazole | Etoxazole |
| 11<br>Microbial disruptors of insect<br>midgut membranes | 11A<br>Bacillus thuringiensis and their<br>insecticidal proteins | Bacillus thuringiensis subsp. israelensis<br>Bacillus thuringiensis subsp. aizawai<br>Bacillus thuringiensis subsp. kurstaki<br>Bacillus thuringiensis subsp. tenebrionis |
| | 11B<br>Bacillus sphaericus | Bacillus sphaericus |
| 12<br>Inhibitors of Mitochondrial ATP<br>Synthase | 12A<br>Diafenthiuron | Diafenthiuron |

| | | |
|---|---|---|
| Energy Metabolism | | |
| | 12B<br><br>Organotin miticides | Azocyclotin, Cyhexatin, Fenbutatin oxide |
| | 12C<br><br>Propargite | Propargite |
| | 12D<br><br>Tetradifon | Tetradifon |
| 13<br><br>Uncouplers Of Oxidative Phosphorylation via Disruptive of the Proton Gradient<br><br>Energy Metabolism | Pyrroles Dinitrophenols<br><br>Sulfluramid | Chlorfenapyr DNOC Sulfluramid |
| 14<br><br>Nicotinic Acetylcholine Receptor Channel Blockers<br><br>Nerve Action | Nereistoxin analogues | Bensultap, Cartap hydrochloride, Thiocyclam, Thiosultap-sodium |
| 15<br><br>Inhibitors of Chitin Biosynthesis Affecting CHS1<br><br>Growth Regulation | Benzoylureas | Bistrifluron, Chlorfluazuron, Diflubenzuron, Flucycloxuron, Flufenoxuron, Hexaflumuron, Lufenuron, Novaluron, Noviflumuron, Teflubenzuron, Triflumuron |
| 16<br><br>Inhibitors of Chitin Biosynthesis, Type 1<br><br>Growth Regulation | Buprofezin | Buprofezin |

| | | |
|---|---|---|
| 16<br>Inhibitors of Chitin Biosynthesis,<br>Type 1<br>Growth Regulation | Buprofezin | Buprofezin |
| 17<br>Molting Disruptor, Dipteran<br>Growth Regulation | Cyromazine | Cyromazine |
| 18<br>Ecdysone Receptor Agonists<br>Growth Regulation | Diacylhydrazines | Chromafenozide, Halofenozide, Methoxyfenozide, Tebufenozide |
| 19<br>Octopamine Receptor Agonists<br>Nerve Action | Amitraz | Amitraz |
| 20<br>Mitochondrial Complex III<br>Electron Transport Inhibitors<br>Energy Metabolism | 20A<br>Hydramethylnon | Hydramethylnon |
| | 20B<br>Acequinocyl | Acequinocyl |
| | 20C<br>Fluacrypyrim | Fluacrypyrim |
| | 20D<br>Bifenazate | Bifenazate |
| 21<br>Mitochondrial Complex I Electron<br>Transport Inhibitors | 21A<br>METI Acaricides and<br>Insecticides | Fenazaquin, Fenpyroximate, Pyridaben, Pyrimidifen, Tebufenpyrad, Tolfenpyrad |

| | | |
|---|---|---|
| Energy Metabolism | | |
| | 21B<br>Rotenone | Rotenone (Derris) |
| 22<br>Voltage-Dependent Sodium<br>Channel Blockers | 22A<br>Oxadiazines | Indoxacarb |
| | 22B<br>Semicarbazones | Metaflumizone |
| 23<br>Inhibitors of Acetyl CoA<br>Carboxylase<br>Lipid Synthesis, Growth Regulation | Tetronic and Tetramic acid<br>derivatives | Spirodiclofen, Spiromesifen, Spiropidion, Spirotetramat |
| 24<br>Mitochondrial Complex IV<br>Electron Transport Inhibitors | 24A<br>Phosphides | Aluminium phosphide, Calcium phosphide, Phosphine, Zinc phosphide |
| Energy Metabolism | 24B<br>Cyanides | Calcium cyanide, Potassium cyanide, Sodium cyanide |
| 25<br>Mitochondrial Complex II Electron<br>Transport Inhibitors | 25A<br>Beta-ketonitrile derivatives | Cyenopyrafen, Cyflumetofen |
| Energy metabolism | 25B<br>Carboxanilides | Pyflubumide |
| 28<br>Ryanodine Receptor Modulators<br>Nerve and Muscle Action | Diamides | Chlorantraniliprole, Cyantraniliprole, Cyclaniliprole Flubendiamide, Tetraniliprole |
| 29 | Flonicamid | Flonicamid |

| | | |
|---|---|---|
| Chordotonal organ Modulators - undefined target site<br>Nerve Action | | |
| 30<br>GABA-gated chloride channel allosteric modulators<br>Nerve Action | Meta-diamides Isoxazolines | Broflanilide Fluxametamide |
| 31<br>Baculoviruses<br>Host-Specific Occluded Pathogenic Viruses | Granuloviruses (GVs)<br>Nucleopolyhedroviruses (NPVs) | Cydia pomonella GV Thaumatotibia leucotreta GV<br>Anticarsia gemmatalis MNPV<br>Helicoverpa armigera NPV |
| 32<br>Nicotinic Acetylcholine Receptor (nAChR) Allosteric Modulators–Site II<br>Nerve Action | GS-omega/kappa HXTX-Hv1a peptide | GS-omega/kappa HXTX-Hv1a peptide |

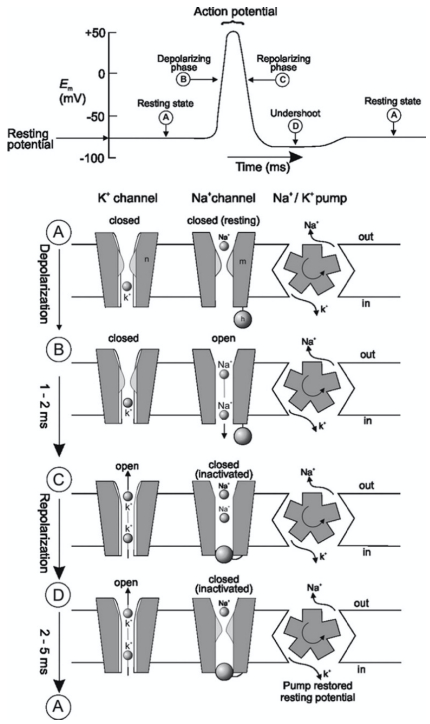


Figure 1. Sourced from Davies et al. (2017) illustrating the difference phases in action potential. Starting with resting potential (A), the axonal membrane is permeable to $K^+$ but is not permeable to $Na^+$, rending the interior of the membrane being negatively charged with respect to the outside with a difference in potential around -60mV. Step B is nerve stimulation, this triggers the membrane to become permeable to $Na^+$, due to the opening of the sodium ion channel. Resulting in the interior of the axon becoming momentarily positively charged causing the rising phase of action potential. Step C is sodium channel closure/inactivation, this triggers the opening of the potassium channels resulting in a falling action potential. This shift in action potential causes the subsequent depolarization of other regions of the axon. The ion gradient between the extracellular fluid and axonal membrane is maintained by an $Na^+$- $K^+$ pump (Step D) which restores the resting potential. This series of steps is repeated to allow for the influx of $Na^+$ into the axon.

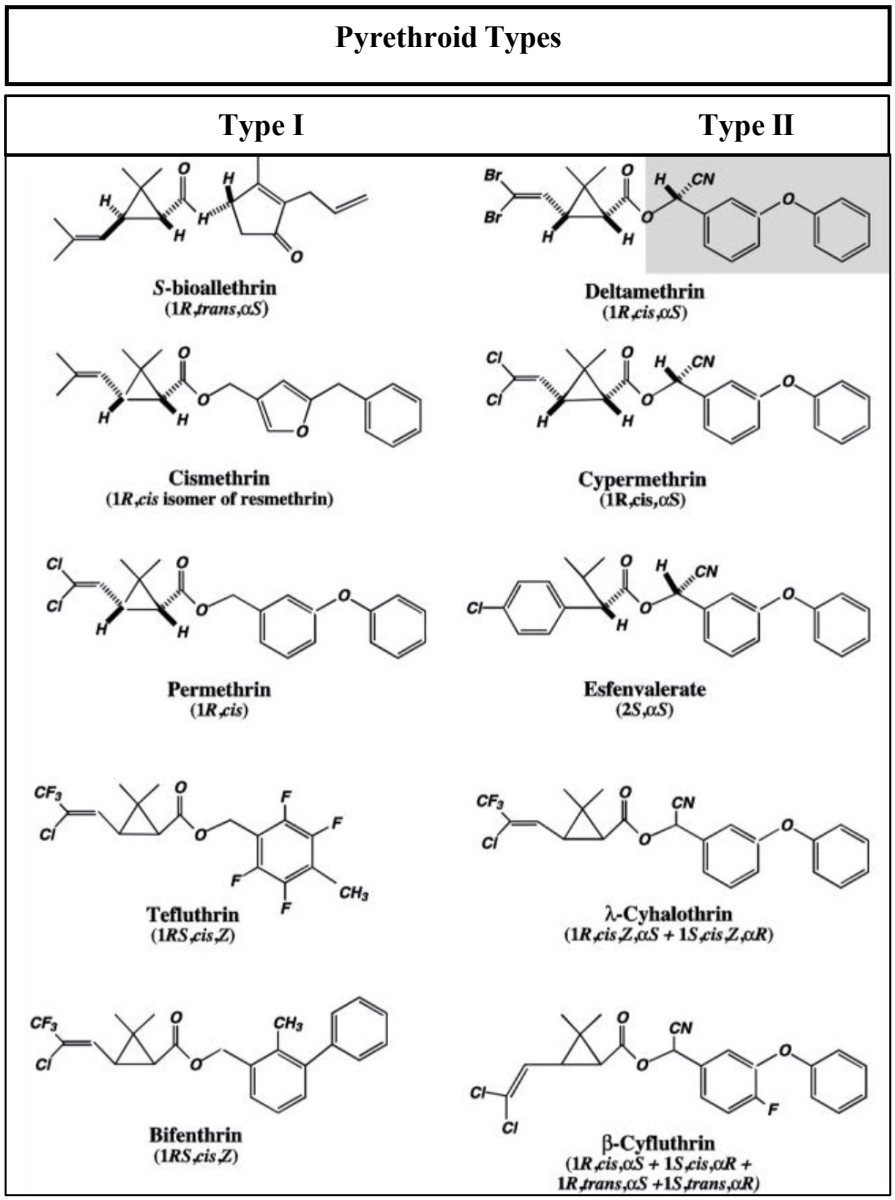


Figure 2. Sourced from Soderlund (2012). The figure illustrates the structures of Type I and Type II pyrethroids. Despite the general diversity in chemical structure, there is one structural distinction between the pyrethroid types: Type I pyrethroids lack the μ-cyano-3-phenoxybenzyl group whereas Type II pyrethroids contain it.

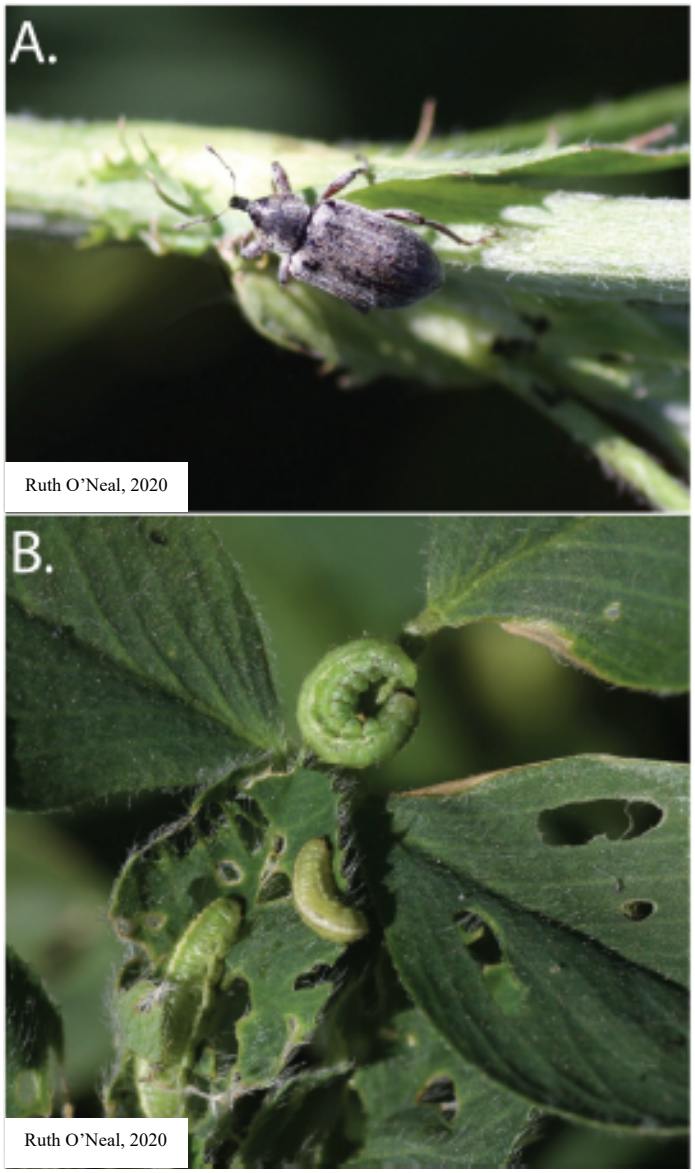


Figure 3. Alfalfa weevil adults (A) distinguished from other members of the family Curculionidae based on the dorsal dark strip. Larvae (B) look similar to clover leaf weevil and retain their characteristic dark head capsule across all instars.

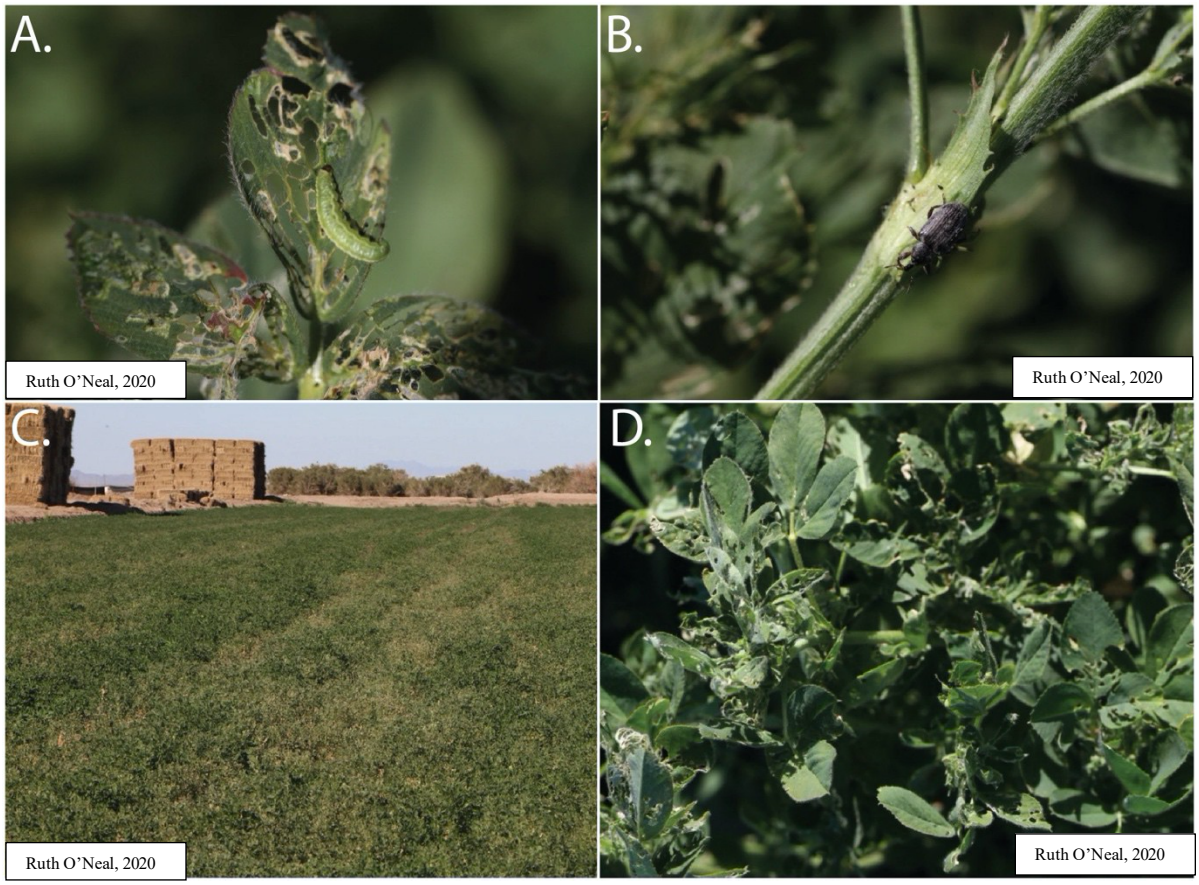


Figure 4. Photos of alfalfa weevil larvae (A) and adult (B) as well as the damage that they cause, mainly the skeletonization of alfalfa leaves by alfalfa weevil larvae (C and D).

# FIRST REPORT OF ALFALFA WEEVIL (COLEOPTERA: CURCULIONIDAE) RESISTANCE TO LAMBDA-CYHALOTHRIN IN MONTANA

## Contribution of Authors and Co-Authors

Manuscript in Chapter: 2

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Contributions: Project inception and design, data analysis of some laboratory bioassays, spray trial establishment, and manuscript writing.

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# FIRST REPORT OF ALFALFA WEEVIL (COLEOPTERA: CURCULIONIDAE) RESISTANCE TO LAMBDA-CYHALOTHRIN IN MONTANA

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**Abstract**

Forage alfalfa (*Medicago sativa* L) is a major agronomic crop grown nationally and Montana ranks highly in acres harvested. The alfalfa weevil (*Hypera postica* Gyllenhal (Coleoptera: Curculionidae)) is the primary defoliating pest that requires insecticide applications to prevent yield loss, particularly pyrethroid active ingredients that are both efficacious and cost effective. Reports from commercial alfalfa producers in Big Horn County Montana suggested local populations of alfalfa weevil had developed resistance to the pyrethroid active ingredient lambda-cyhalothrin (type II pyrethroid). Chemical control is an important component of integrated pest management of alfalfa weevil and the loss of pyrethroid active ingredients as an effective tool would result in additional production costs. Two locations in southern Big Horn County, and nine locations in four other Montana counties where resistance has not been reported, were sampled and assayed for resistance to lambda-cyhalothrin. Populations from three counties were susceptible, the concentration causing 50% mortality ($LC_{50}$) ranged from 0.02 to 0.10 µg/cm$^2$. In contrast, populations from Big Horn County did not reach 50% mortality at the highest concentration of lambda-cyhalothrin tested (3.30 µg/cm$^2$), indicating high levels of resistance have developed in these populations. A field trial in Big Horn County supported laboratory results of resistance; lambda-cyhalothrin at the highest label rate did not reduce alfalfa weevil populations. Additional bioassays suggest cross-resistance to zeta-cypermethrin (type II pyrethroid) but only partial cross-resistance to permethrin (type I pyrethroid).

**Key words:** Insecticide Resistance, Field Crop, Forage Crop, Crop Protection, Curculionidae

## Introduction

Alfalfa, *Medicago sativa* L., is the third most valuable row crop in the United States following corn and soybean (Nelson 2019). In 2018, Montana produced 5.6 million tons of alfalfa harvested from 2.1 million acres, making alfalfa production one of the key components of the Montanan economy (USDA-NASS 2019, Putnam et al. 2019). Alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), is one of the primary defoliators damaging alfalfa yields nationally (Peterson et al. 1992).

Alfalfa is the primary host of alfalfa weevil and feeding injury to the foliage is caused during the larval stage of their development (Cothran 1966). Adult alfalfa weevils can feed on alfalfa stems and foliage, but this damage is not considered to be economically damaging (Pellissier et al. 2017). The larvae cause economic injury to forage and seed alfalfa by reducing yield and harvest quality. Leaves appear skeletonized after larval feeding and the alfalfa canopy takes on a frosted appearance. This defoliation reduces the leaf to stem ratio and reduces forage quality in addition to yield since leaves contain more protein than stems (Hamlin et al. 1949, Berberet and McNew 1986).

Alfalfa weevils are native to Eurasia and North Africa and were introduced to the United States on at least three occasions, with each introduction being recognized as a distinct strain (Hsiao 1993, Bundy et al. 2005). Currently three strains are recognized, the eastern, western, and Egyptian strains. The western strain was first reported near Salt Lake City, Utah, in 1904 (Titus 1910), the Egyptian strain in 1939 near Yuma, Arizona (Wehrle 1940), and the eastern strain in 1952 in Maryland (Poos and Bissell 1953, Wood et al. 1978). Currently, only the western strain has been identified in Montana (Wanner, unpublished data).

Alfalfa weevil management practices, regardless of strain, are predominantly focused on chemical control. Chemical control is an important aspect of integrated pest management (IPM) of alfalfa weevil and in some situations is the only option available to producers to limit economic losses. Chemical control is recommended when economic thresholds are met. An average of 20 or more larvae (of any size) per standard sweep or 1.5-2.0 larvae per stem using the shake-bucket method is a commonly used economic threshold (Evans 1989, Blodgett 1996). The sweep net threshold was established more than 30 years ago and some regions are beginning to update and customize economic thresholds for their production system (Harrington et al. 2021). The new treatment threshold in low desert irrigated alfalfa is an average of 1 to 3 large larvae per sweep (Harrington et al. 2021). One alternative to chemical control is harvesting early (7-10 days before normal harvest) to salvage yield if weather permits, however, in the southwestern US harvesting early is not considered to be effective in many, but not all, circumstances (Summers 1998, Rethwisch personal communication).

Producers predominantly rely on insecticides with pyrethroid active ingredients (a.i.), mode of action (MoA) group 3A, to control alfalfa weevil populations since they are effective and inexpensive. Pyrethroids block the sodium ion channel in insects, resulting in prostration, paralysis, and eventual death (Davies et al. 2007). Recently, several field reports suggest reduced efficacy or complete failure of pyrethroids to control alfalfa weevil populations (Glen 2015, Orloff et al. 2016). In 2015, producers reported repeated failures of deltamethrin and lambda-cyhalothrin applied to control alfalfa weevil in seed alfalfa crops in southern Alberta, Canada (Glen 2015). Similarly, repeated applications of beta-cyfluthrin and lambda-cyhalothrin failed to provide effective control of alfalfa weevil in forage alfalfa fields in the Scott Valley, CA (Orloff

et al. 2016). These preliminary reports suggest that pyrethroid resistance may be developing or is established in western region alfalfa weevil populations.

During the 2019 growing season producers in Big Horn County, Montana, reported the failure of pyrethroid insecticides to reduce numbers of alfalfa weevil larvae in forage alfalfa fields. Descriptions of insecticide failure in Big Horn County matched those reported from southern Alberta, Canada and the Scott Valley in California; two-to-three applications of a pyrethroid insecticide failed to reduce the larval numbers which continued to increase. Currently there are few effective insecticides with different MoAs for managing alfalfa weevil, and due to cross-resistance within a MoA group, producers could lose all pyrethroid-based insecticides if resistance becomes widespread.

During 2020 we conducted concentration-response experiments in the laboratory using larvae collected from southern Big Horn County to test for resistance to lambda-cyhalothrin (limited assays of zeta-cypermethrin and permethrin were also completed for comparison). Alfalfa weevil larvae from Broadwater, Madison, Powder River and Richland Counties of Montana were assayed as reference populations where resistance has not yet been reported. Along with field trial data, the laboratory results confirm high levels of resistance to lambda-cyhalothrin in alfalfa weevils from southern Big Horn County, Montana.

## Materials and Methods

### *Insect Collections*

Third and fourth instar larvae were collected from two locations in Big Horn County (Supp. Fig. 1) and from commercial alfalfa fields in Broadwater (Supp. Fig. 2), Madison (Supp. Fig. 3), Powder River (Supp. Fig. 4), and Richland Counties (Supp. Fig. 5). All fields were

managed conventionally and included both dryland and irrigated fields. Field collections occurred on 25 May, 30 May, 1 June, 4 June, 6 June, and 8 June 2020.

Larvae were collected by sweep netting in a zig-zag pattern through each sampled field, placed in labeled paper bags with cut alfalfa from the sampled field, and transported to the laboratory for testing. Prior to bioassay testing, larvae were placed in 19-liter buckets (covered with no-see-um number 20 mesh, Quest Outfitters, Sarasota, FL) with fresh alfalfa (grown in Montana State University's greenhouse) for 24-48 hours at 21°C.

*Active Ingredients*

Lambda-cyhalothrin (type II pyrethroid), zeta-cypermethrin (type II pyrethroid) and permethrin (type I pyrethroid) were assayed as technical grade material. Lambda-cyhalothrin (alpha-cyano-3-phenoxybenzyl (Z)-(1RS)-cis 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethylcyclopropanecarboxylate) was provided by Syngenta (Basel, Switzerland), batch number 1071218, 89% purity. Zeta-cypermethrin ([Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) was provided by FMC Corporation (Newark, NJ), batch number 122279866, 92% purity. Permethrin (3-phenoxybenzyl (1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate) was purchased from Sigma-Aldrich (St. Louis, MO), batch number 45614, 92% purity.

*Treating Glass Vials*

Stock solutions of each technical active ingredient, accounting for their purity, were prepared in 95% acetone and 5% deionized water. Serial dilutions of stock solutions in 95% acetone produced seven concentrations for testing. One milliliter (mL) of each concentration was pipetted into sterile continuous thread vials (Discount Vials, Madison, WI, 27mm diameter x

95mm length), with five vials per concentration (n=5). The concentrations for lambda-cyhalothrin and zeta-cypermethrin were calibrated so that one mL delivered 3.3 micrograms per centimeter squared (µg/cm$^2$) to 0.0033µg /cm$^2$ to the surface of the vial in contact with the solution, and 33µg/cm$^2$ to 0.033µg/cm$^2$ for permethrin. Treated vials were placed on hotdog rollers at 21°C to evaporate the 95% acetone solution and stored at 21°C in a dark cabinet prior to testing. Fresh stock solutions and dilutions were prepared for each experiment and stored in brown PYREX reusable media storage bottles (Thermo Fisher Scientific, Pittsburgh, PA).

*Bioassay*

When populations were large enough, each bioassay consisted of seven concentrations and a 95% acetone control for each of the active ingredients. Ten 3$^{rd}$ to 4$^{th}$ instar larvae (selected visually based on size) were placed in each treated vial (n = 5). The lid of each vial had a 11mm diameter hole to allow for air circulation with perforated plastic below to prevent larvae from escaping. Vials were then stored in the dark for 24-hours at 21°C. After 24 hours the bioassays were evaluated for larval mortality. Larvae were classified as dead if they could not crawl more than one body length after exposure to heat (placed on a glass Petri dish set on a hotplate heated to 43-50°C).

Probit analysis was conducted to quantify the lethal concentration that generates 50% mortality ($LC_{50}$), using Probit Or LOgit analysis (POLO) software (LeOra Software, Parma, MO). POLO software corrects for control mortality to calculate $LC_{50}$ values. Chi-square ($\chi^2$) goodness of fit and the t-ratio of the slope values were used to evaluate each data set analyzed (Yu 2015). Datasets were excluded from this study if they had control mortality greater than 20% and/or the t-ratio of the slope was less than 1.96 (Robertson et al. 2017). Datasets whose fit with

the Probit model was rejected ($\chi^2$ goodness of fit p-values < 0.05) were inspected for an outlier value. If the removal of a single outlier resulted in the failure to reject the goodness of fit (p>0.05) the data set was included. In this study, a single outlier was removed from four lambda-cyhalothrin datasets: Big Horn County field site $2_c$ (omitted concentration 4); Broadwater County field site 3 (omitted concentration 6); and Powder River County field sites 1 (omitted concentration 6) and 3 (omitted concentration 5). A single outlier was removed from two permethrin datasets: Broadwater County field site 1 (omitted concentration 5); and Powder River County field site 2 (omitted concentration 6).

*Field Trial*

To corroborate our bioassay results we conducted a field trial organized in a randomized complete block design with four blocks (n = 4) where each plot measured 3.1 meters (m) by 30.5 m in field site 1, in Big Horn County (Table 1), which was sampled two-days prior for the bioassay component of this study. Four treatments were compared: 1) Lambda-cyhalothrin (Grizzly Too, WinField United, Arden Hills, MN) at 0.14 liters per hectare (L/ha) (1.92 ounces (oz) per acre); 2) Permethrin (Permethrin, Loveland Products, Inc., Loveland, CO) at 0.58 L/ha (8 oz per acre); 3) Indoxacarb (Steward® EC, FMC, Philadelphia, PA) at 0.85 L/ha (11.7 oz per acre) with Preference® (non-ionic surfactant plus antifoaming agent) at 0.06% by volume; and 4) untreated check. Treatments were applied on 6 June 2020 using a FIMCO (North Sioux City, SD) 94.63 Liter (25 Gallon) Sprayer (4.5 GPM Pump) with a 2.13m (7 foot) boom (3.66 m spray coverage) mounted to an ATV and calibrated to deliver 10 gallons of spray volume per acre.

Alfalfa weevil larval populations were recorded 13-days post application (19 June 2020) by taking ten 180° sweeps with a sweep net (BioQuip, Rancho Dominguez, CA) from each plot.

Samples were placed in labeled paper bags with fresh alfalfa and transported to the laboratory where they were sorted and larval numbers counted. To determine the distribution of larval stages during the field trial, a 0.093 square meter section of alfalfa (stems cut at ground level) was sample from control plots. Samples were collected one day prior to insecticide application (5 June 2020), one week after insecticide application (12 June 2020), and two weeks after insecticide application (19 June 2020). Samples were carefully sorted, including the vegetative buds, and the larvae stored in 95% ethanol. Larval instar stage was determined based on head capsule width measured using a stereo microscope fitted with an ocular micrometer (Bartell and Roberts 1974).

Larval counts were analyzed using Minitab Statistical Software (Minitab LLC, State College, PA). A one-way ANOVA was used to test for significant differences among treatments followed by a Tukey pairwise comparison ($p = 0.05$) to determine significant differences between treatments.

## Results

### *Concentration Response Experiments*

Alfalfa weevil larvae from eleven field sites in five different Montana counties were assayed with lambda-cyhalothrin, and when possible zeta-cypermethrin and permethrin, using concentration response experiments. Larvae from most sites, when placed in treated vials for 24 hours, responded to increasing concentrations of lambda-cyhalothrin with increasing mortality that fit a sigmoidal relationship when plotted against the log [concentration] (Fig. 1, Powder River County sites 1 and 2 as examples). However, this was not the case for larvae from Big Horn County, where a clear concentration dependent and sigmoidal response of mortality was

not observed (Fig. 1, Big Horn County sites 1 & 2). Furthermore, corrected mortality never exceeded 50% even at the highest concentration of 3.3 µg/cm$^2$. In these cases, we conservatively interpreted the $LC_{50}$ values to be greater than the highest concentration tested since mortality never exceeded 50%. The range of label rates for each a.i. are: lambda-cyhalothrin, 0.22 – 0.34 µg a.i. per cm$^2$; zeta cypermethrin, 0.16 – 0.28 µg a.i. per cm$^2$; and permethrin, 1.12 – 2.24 µg a.i. per cm$^2$, based on pounds a.i. per acre.

When sufficient larvae were available zeta-cypermethrin and permethrin were also assayed. Zeta-cypermethrin and lambda-cyhalothrin are classified as type II pyrethroids while permethrin is a type I pyrethroid. Larvae from Big Horn County sites 1 & 2 evidenced some concentration-responsiveness to zeta-cypermethrin, but similar to lambda-cyhalothrin mortality never exceeded 50% even at the highest concentration tested (Fig. 1, Big Horn County sites 1 & 2). Zeta-cypermethrin was not tested in other Montana counties. Permethrin was assayed against larvae from all four sites in Figure 1 and provided a distinctly different response pattern. Permethrin was the only a.i. tested against the Big Horn County larvae that elicited typical concentration responses with mortality reaching 100%, with similar results using Powder River County site 1 larvae (Fig. 1).

To provide a comparison to Big Horn County where producers reported pyrethroid resistance, larvae from ten sites in four additional counties, where resistance has not yet been reported, were assayed and the $LC_{50}$ values calculated. The $LC_{50}$ values for lambda-cyhalothrin tested against larvae collected from the four additional counties ranged from 0.02 to 0.77 µg/cm$^2$: Broadwater County, 0.26 to 0.77 µg/cm$^2$ (3 sites); Madison County, 0.02 and 0.06 µg/cm$^2$ (2 sites); Powder River County, 0.03 to 0.1 µg/cm$^2$ (3 sites); and, Richland County, 0.09 µg/cm$^2$ (1

site) (Table 1). Unlike larvae from Big Horn County, larvae from Broadwater, Madison, Powder River and Richland Counties all approached or reached 100% mortality at the highest concentration tested. The $LC_{50}$ for Big Horn County larvae was simply considered as >3.3 µg/cm$^2$ for lambda-cyhalothrin and zeta-cypermethrin since mortality did not exceed 50%; even this conservative estimate is 165 times higher than the lowest $LC_{50}$ value recorded from counties where resistance has not yet been reported.

In addition to the two Big Horn County sites, permethrin was assayed from six sites in three Montana counties. The $LC_{50}$ values for permethrin tested against larvae collected from counties that have not reported pyrethroid resistance ranged from 0.10 to 1.28 µg/cm$^2$; Broadwater County, 1.28 µg/cm$^2$ (1 site); Madison County, 0.17 µg/cm$^2$ (1 site); and Powder River County, 0.1 to 0.55 µg/cm$^2$ (3 sites) (Table 1). The $LC_{50}$ values for both lambda-cyhalothrin and permethrin were lowest for larvae sampled from Madison, Powder River and Richland Counties suggesting these are susceptible populations that have not developed resistance to pyrethroids. $LC_{50}$ values from Broadwater County larvae, however, were approximately ten times higher in comparison, suggesting resistance may be developing in these populations.

*Field Trial*

Lambda-cyhalothrin and permethrin were compared to indoxacarb (MoA22A) for alfalfa weevil control under field conditions, at Big Horn County site 1 where resistant larvae were observed (Table 1, Fig. 1). The average number of alfalfa weevil larvae sampled from field plots was significantly affected by insecticide treatment ($F=5.67$; $df= 3, 15$; $P= 0.012$) (Fig. 2). Compared to untreated control plots, indoxacarb (0.85 L/ha) significantly reduced larval

populations by 94% (Fig. 2, $p < 0.05$) thirteen days after treatment. In comparison, treatment with lambda-cyhalothrin at the higher label rate (0.14 L/ha; equivalent to 0.34 $\mu g/cm^2$ in the vial bioassay) had no effect on average larval numbers compared to untreated control plots (Fig. 2, $p > 0.05$). Bioassay mortality of larvae sampled from this field two days earlier did not exceed 50% at a concentration of 3.3 $\mu g/cm^2$, a rate 10 times greater than the equivalent higher label rate of 0.34 $\mu g/cm^2$. However, treatment with permethrin (0.585 L/ha; equivalent to 2.24 $\mu g/cm^2$ in the vial bioassay) appeared to have an intermediary effect, reducing the average number of alfalfa weevil larvae by 52% compared to the untreated plots. Overall, permethrin treated plots did not significantly differ from control or indoxacarb plots but were significantly different from those treated with lambda-cyhalothrin (Fig. 2, $p < 0.05$).

During the trial period larvae from control plots were staged into the four different instar stages. One day before treatment (5 June 2020) 52.8% of larvae were in the 3$^{rd}$ instar stage (Supp. Fig. 6). Throughout the trail period, 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ instar larvae infested the plots with 3$^{rd}$ instars being the most abundant stage.

**Discussion**

Laboratory bioassays and a field trial demonstrated that larvae from southern Big Horn County are highly resistant to lambda-cyhalothrin (type II pyrethroid), compared to three other Montana counties. The lambda-cyhalothrin $LC_{50}$ values for larvae sampled from Big Horn County (conservatively estimated as >3.3$\mu g/cm^2$, the highest concentration tested, since corrected mortality never exceeded 50%) was at least 33 to 165 times higher compared to susceptible populations from Madison, Powder River and Richland Counties (and 10 times greater than 0.34 $\mu g/cm^2$, equivalent to the higher label rate). Field trial data supported laboratory

results; lambda-cyhalothrin applied at the highest label rate had no effect on the number of larvae 13 days after treatment (Fig. 2). Field applications were timed well, when 52.8% of larvae were in the 3rd instar stage (Supp. Fig. 6). These results explain the lack of alfalfa weevil control observed by Big Horn County producers after two or three applications of pyrethroid insecticide to their commercial fields.

Different pyrethroid active ingredients target the same binding domain of the sodium ion channel and cross-resistance between MoA3A active ingredients is common (Soderlund and Bloomquist 1990, O'Reilly et al. 2006, Davies et al. 2007). Resistance to lambda-cyhalothrin could impart resistance to all pyrethroid active ingredients, a significant loss to the forage alfalfa industry. Although limited populations were assayed, data from southern Big Horn County suggest the larvae were also resistant to zeta-cypermethrin, another type II pyrethroid, supporting cross-resistance to MoA3A active ingredients in these populations. Similar to lambda-cyhalothrin, mortality of larvae from southern Big Horn County never exceeded 50% even at the highest concentration of zeta-cypermethrin tested (Fig. 1). Interestingly, permethrin, a type I pyrethroid, was more effective than lambda-cyhalothrin and zeta-cypermethrin in laboratory and field studies even though its potency is lower (the label rate of permethrin is seven-times higher than lambda-cyhalothrin). Type I pyrethroids were the first to be developed, and subsequent synthetic versions improved toxicity and persistence (Davies et al. 2007). This data suggests a lower level of cross-resistance between type I and type II pyrethroid active ingredients. One explanation might be the existence of unique binding sites for type I and type II pyrethroids within the binding domain of the sodium ion channel (Lawrence and Casida 1982, O'Reilly et al. 2006). The type II binding site results in a prolonged convulsive phase and better efficacy due to

the irreversible depolarization of the nerve axons and terminals when compared to type I pyrethroids (Davies et al. 2007, Breckenridge et al. 2009).

Mechanisms of insecticide resistance include enzymatic degradation, target site insensitivity, behavioral avoidance, and reduced cuticle penetration (Soderlund and Bloomquist 1990, Davies et al. 2007, Haddi et al. 2018). Behavioral avoidance based on contact and/or sensory detection results in reduced contact with the a.i. and reduced mortality (Haddi et al. 2018). Increased enzymatic activity, due to the upregulation of esterases (e.g., monooxygenases, carboxyl- esterase, and gluthione S-transferases) and P450 enzymes, degrades the insecticide reducing its concentration at the active site and reducing efficacy (Ramoutar et al. 2009). In this case, synergists such as piperonyl butoxide (PBO), that inhibit detoxification enzymes, may improve the efficacy of pyrethroid insecticides, particularly permethrin that demonstrated moderate activity against populations resistant to lambda-cyhalothrin in this study. Changes to the composition of cuticle lipids can reduce the amount of active ingredient that can penetrate into the insect by contact activity. Genetic mutations that reduce the sensitivity of the voltage-gated sodium ion channel target site often result in the highest levels of insecticide resistance (Farnham et al. 1987). Mutations to the voltage-gated sodium ion channel gene that reduce sensitivity to pyrethroid active ingredients has been well studied, different single nucleotide polymorphisms that confer resistance have been identified from several different insect orders (Soderlund 2005, Rinkevich et al. 2013). These mutations have been termed knockdown resistance (*kdr*) and *super-kdr*, both recessive resistant traits. Known mutation sites are located along the IIS4-S5 linker, IIS5 helix and IIIS6 helix of domain II, or the corresponding linker and/or helices of domains I and III (Davies et al. 2007). However, mechanisms of insecticide

resistance for the alfalfa weevil have not been reported. Further, in another weevil species, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), as many as three different resistance mechanisms can contribute to insecticide resistance (Haddi et al. 2018). Future research will focus on identifying the mechanism(s) of alfalfa weevil pyrethroid resistance.

Detailed economic analyses of the impacts of insecticide resistance in forage alfalfa have not been published. Nationally, the total annual economic costs of insecticide resistance in the US have been estimated at $1 billion; the added cost of replacement insecticides alone (not including crop losses) has been estimated at $40 million annually (Clark and Yamaguchi 2002). The economic cost of Colorado potato beetle insecticide resistance in Michigan is one well-characterized example. Additional management costs plus crop losses for the Michigan potato industry were $13.3 million in 1994 (13.7% of crop value). Economic costs continued to accrue because relatively inexpensive insecticides were no longer effective and their replacements were five times more costly. It is notable that the registration of chlorpyrifos (e.g., Lorsban®), used to control alfalfa weevil populations for several decades, is under Environmental Protection Agency (EPA) review and some states such as California are phasing out its use (EPA 2020). The continued annual cost to the Michigan potato industry caused by insecticide-resistant Colorado potato beetles was estimated to be $0.9 to $1.4 million/year (Grafius 1997). Insecticide resistance for a given crop-pest combination is an issue best addressed before resistance is widespread, at which point costs of mitigation will be much higher (IRAC 2021). Research and extension efforts are required to begin mitigating the economic impacts of pyrethroid-resistant alfalfa weevils.

Currently, at least five different pyrethroids are listed for the control of alfalfa weevil in forage alfalfa, including lambda-cyhalothrin, gamma-cyhalothrin, zeta-cypermethrin, beta-cyfluthrin and permethrin; active ingredients of commonly used commercial products such as Warrior II, Proaxis 0.5 EC, Mustang Maxx, Baythroid and Ambush, respectively (Townsend 2007, Long et al. 2017). The loss of all MoA3A insecticides, that are relatively inexpensive and effective, would significantly impact alfalfa producers if resistance becomes common. The limited number of effective alternative MoAs (i.e., indoxacarb) complicates resistance management strategies based on rotating MoA groups. In our experience, producers in the western region with pyrethroid resistance have begun using indoxacarb (MoA22A) to control alfalfa weevil. In addition to its higher cost, repeated use of indoxacarb may lead to resistance developing to this alternative MoA.

This study is the first to quantify pyrethroid resistance in alfalfa weevil populations and supports field reports of resistance in California, USA, and Alberta, Canada (Glen 2015, Orloff et al. 2016). Furthermore, based on $LC_{50}$ values, populations from Broadwater County appear to be developing pyrethroid resistance compared to populations from Madison, Powder River and Richland Counties. However, resistance has not been established outside of the two populations from Big Horn County and this study was not an extensive survey; the extent of resistance remains to be established. Bioassays using larvae collected from several alfalfa fields in the southwestern US failed to detect resistance to lambda-cyhalothrin in the low desert alfalfa production areas of Arizona and California where the Egyptian strain of the alfalfa weevil occurs (Mostafa and Harrington 2020). Mating between the Egyptian and western strains is limited by

the Wolbachia bacterial endosymbiont that infects the western, but not the Egyptian, strain (Hsiao 1993), and the potential effects on gene flow and insecticide resistance are unknown.

Future research will map the $LC_{50}$ values of alfalfa weevil populations from different regions in Montana and the western US. Mapping resistance will enable resistance management recommendations to be customized by region. Preliminary recommendations are based on reducing the selection pressure for resistance by employing the principles of IPM; spraying only when populations exceed the economic threshold; employing cultural methods such as early harvest; and rotating insecticide MoA groups. Future research also needs to confirm cross-resistance between pyrethroid active ingredients, determine mechanisms of resistance, and categorize resistance levels into classes such as low, moderate, and high.

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## Tables and Figures

**Table 1.** Lethal concentrations of three active ingredients that result in 50% mortality ($LC_{50}$, $\mu g/cm^2$) for alfalfa weevil larvae sampled from five Montana counties. Multiple sample collection dates within the same county are indicated by subscript letters that follow the field site number. Chi-square and p-values are provided as a measure of goodness of fit with the Probit analysis (the fit is not rejected if $p > 0.05$).

| Active ingredient | County | Field Site Date | T-ratio of the slope | $LC_{50}$ $\mu g/cm^2$ | $\chi^2_{df}$ | p-value | Number of concentrations |
|---|---|---|---|---|---|---|---|
| Lambda-cyhalothrin | Big Horn | $1_d$ | 4.12 | >3.3 | $9.78_5$ | 0.08 | 8 |
| | | $2_c$ | 2.51 | >1.0 | $3.09_2$ | 0.21 | 5 |
| | | $2_e$ | 4.08 | >3.3 | $7.62_4$ | 0.11 | 7 |
| | Broadwater | 1 | 5.1 | 0.26 | $7.39_5$ | 0.19 | 8 |
| | | 2 | 8.22 | 0.26 | $4.79_5$ | 0.44 | 8 |
| | | 3 | 7.82 | 0.77 | $2.68_2$ | 0.26 | 5 |
| | Madison | 1 | 8.33 | 0.02 | $2.47_5$ | 0.78 | 8 |
| | | 2 | 7.18 | 0.06 | $6.41_5$ | 0.27 | 8 |
| | Powder River | 1 | 4.58 | 0.03 | $2.73_2$ | 0.26 | 5 |
| | | 2 | 7.14 | 0.1 | $5.51_4$ | 0.24 | 7 |
| | | 3 | 7.83 | 0.09 | $6.58_4$ | 0.16 | 7 |
| | Richland | 1 | 6.58 | 0.09 | $5.13_5$ | 0.40 | 8 |
| Zeta-cypermethrin | Big Horn | 1 | 5.18 | >3.3 | $9.02_5$ | 0.11 | 8 |
| | | 2 | 5.57 | >3.3 | $3.12_5$ | 0.68 | 8 |

| Active ingredient | County | Field Site Date | T-ratio of the slope | $LC_{50}$ $\mu g/cm^2$ | $\chi^2_{df}$ | p-value | Number of concentrations |
|---|---|---|---|---|---|---|---|
| Permethrin | Big Horn | 1 a | 2.82 | 4.74 | 0.0041 1 | 0.95 | 4 |
| | | 1 b | 4.96 | 1.18 | 0.78 2 | 0.68 | 5 |
| | | 1 d | 7.9 | 1.54 | 3.22 5 | 0.67 | 8 |
| | | 2 e | 5.56 | 7.05 | 3.9 4 | 0.42 | 7 |
| | Broadwater | 1 | 6.12 | 1.28 | 5.19 3 | 0.16 | 6 |
| | Madison | 1 | 7.5 | 0.17 | 5.45 3 | 0.14 | 6 |
| | Powder River | 1 | 5.81 | 0.55 | 8.06 5 | 0.15 | 8 |
| | | 2 | 7.36 | 0.1 | 6.58 4 | 0.24 | 7 |
| | | 3 | 5.85 | 0.19 | 5.45 3 | 0.14 | 6 |

a- 5/25/2020 b- 5/30/2020 c- 6/1/2020 d- 6/4/2020 e- 6/6/2020

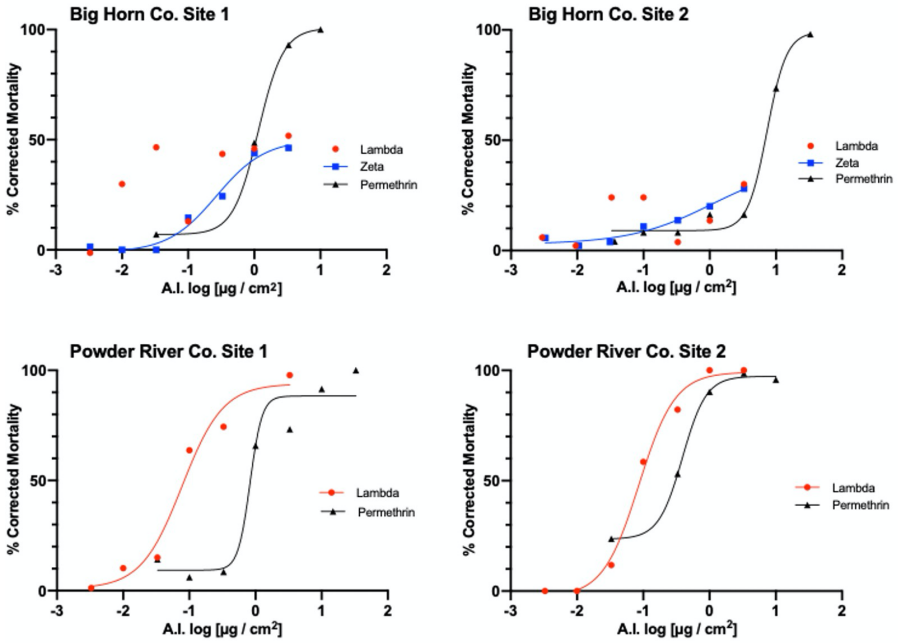



**Figure 1.** Concentration-responsive mortality by field site in Big Horn and Powder River Counties. Percent mortality plotted against the logarithm of active ingredient concentration: lambda-cyhalothrin (red symbols); zeta-cypermethrin (blue symbols); and permethrin (black symbols).

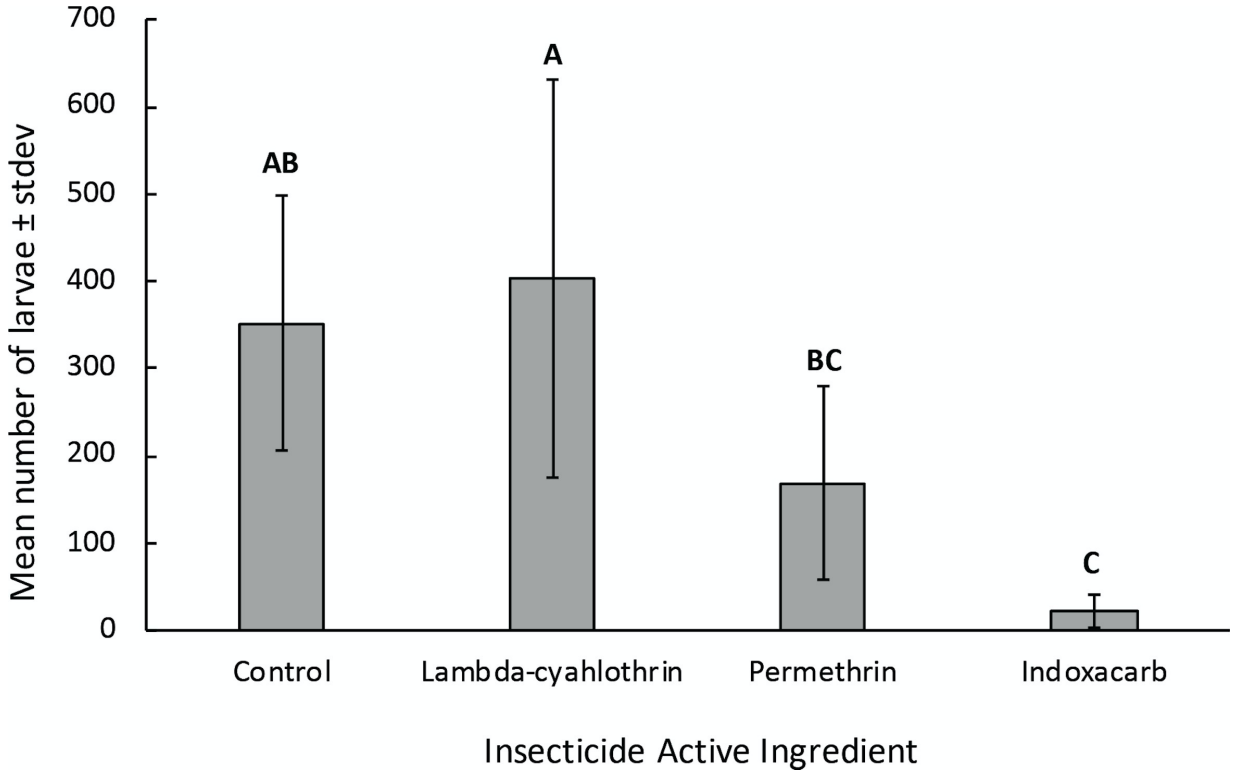
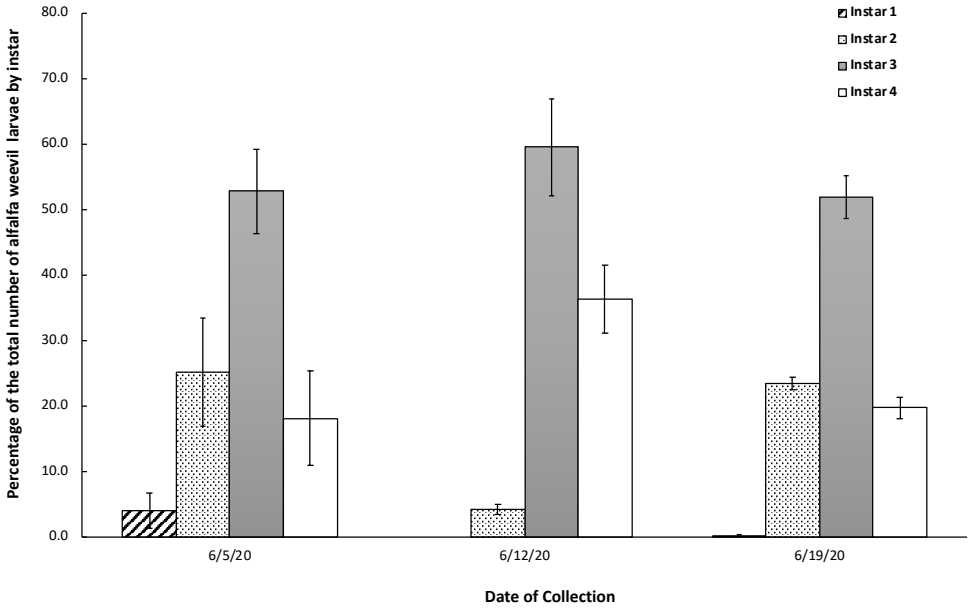
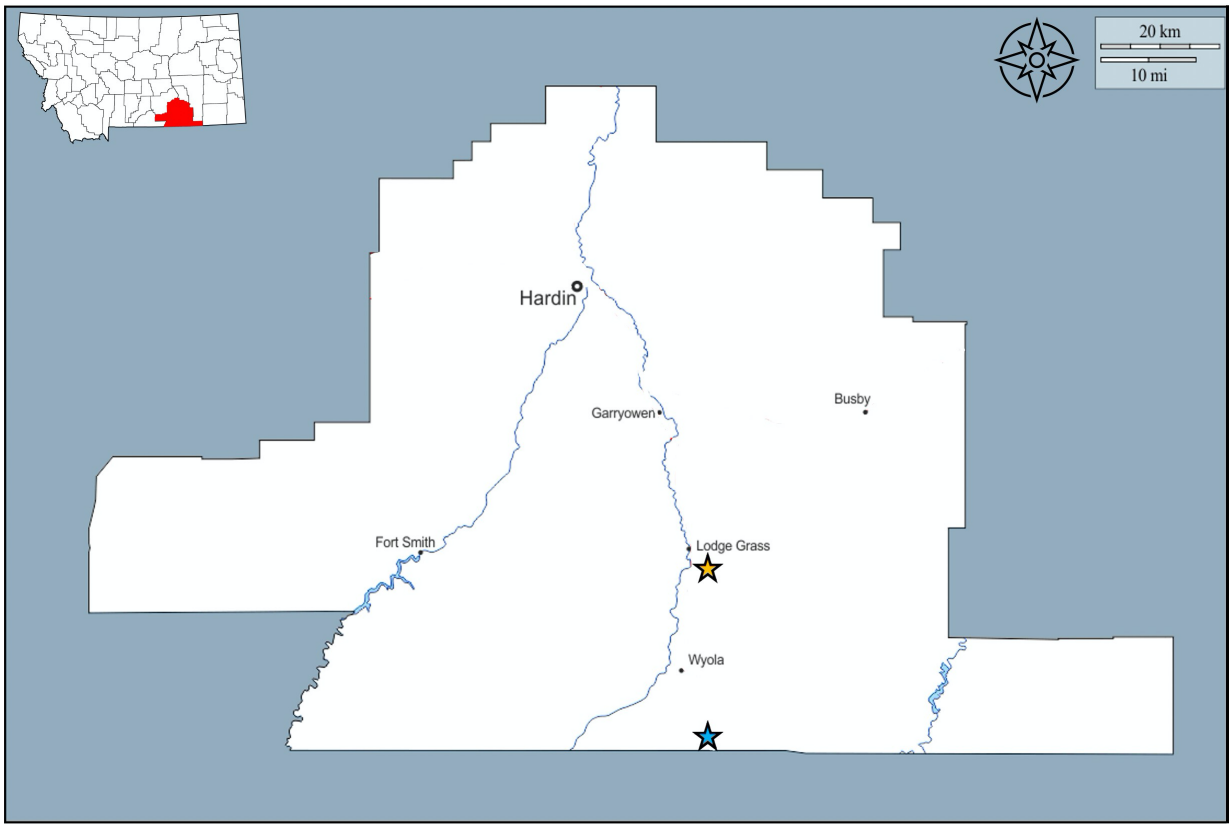


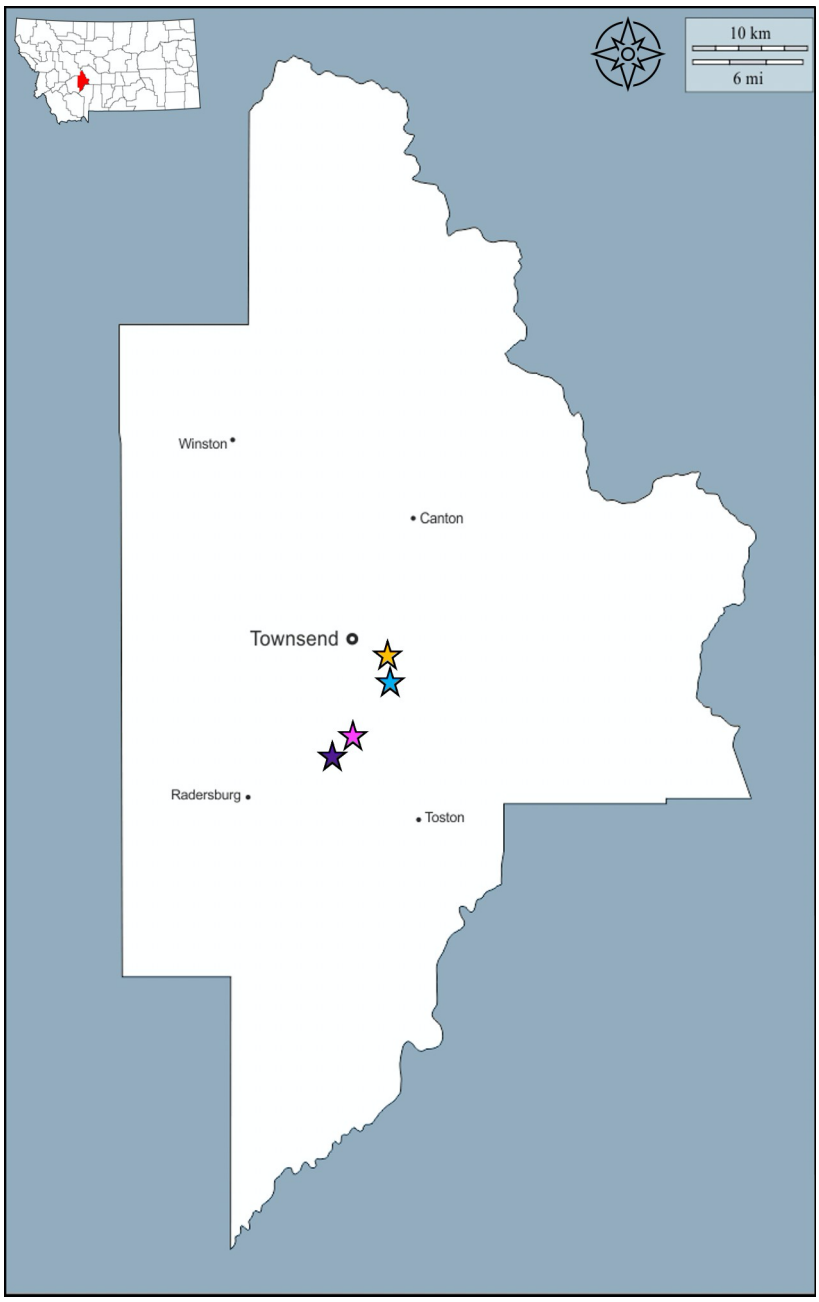

**Figure 2.** Mean number of alfalfa weevil larvae ($\bar{x}$ ± stdev) per ten sweeps 13-days after treating field plots located at site 1 Big Horn County, MT, with lambda-cyhalothrin, permethrin and indoxacarb at maximum label rates. Treatment effects are significant, ANOVA, (F=5.67; df= 3, 15; P= 0.012). Treatments with different letters are significantly different, p = 0.05, Tukey pairwise comparison.



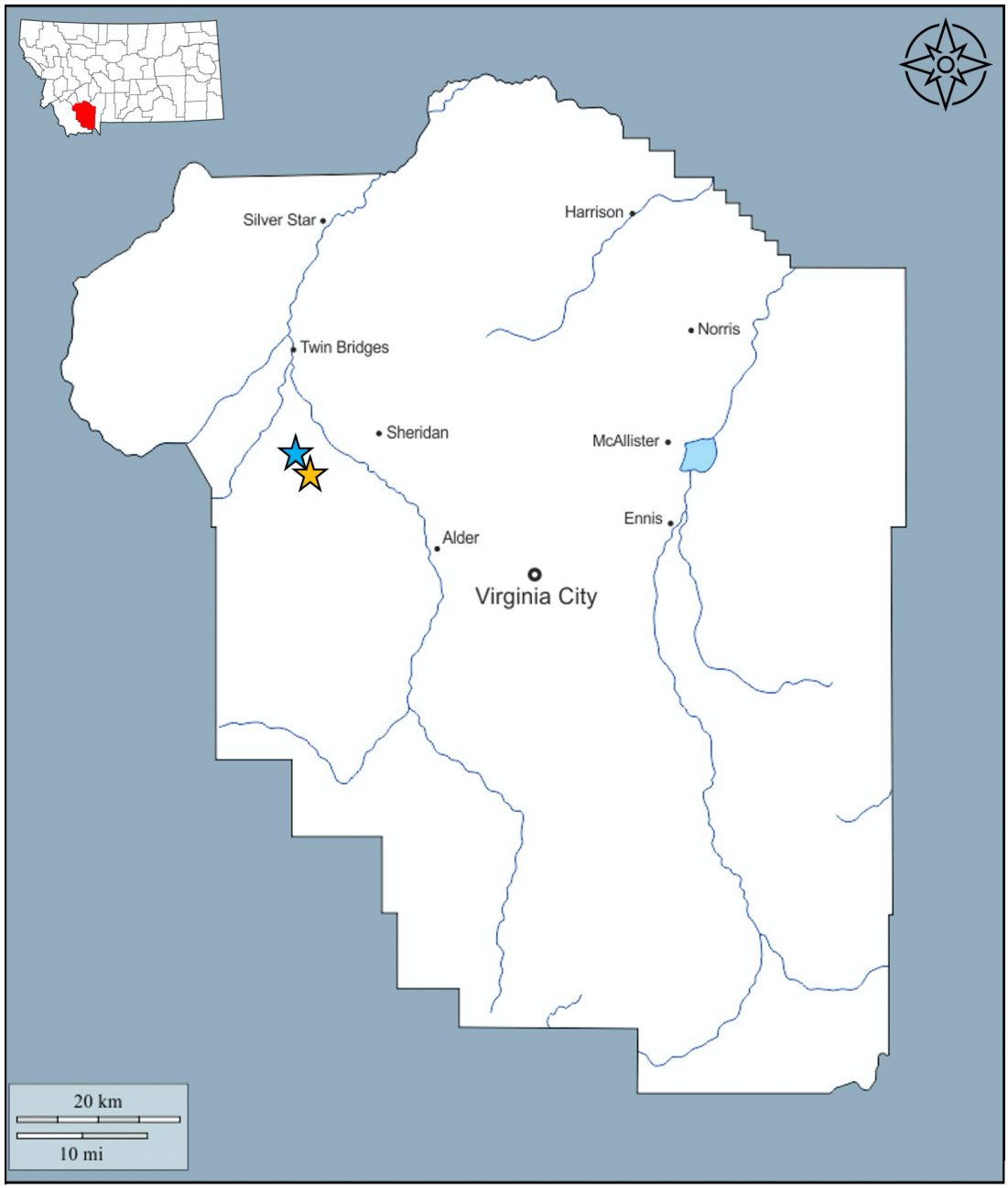

**Supplementary Figure 1.** Percentage of the total number of alfalfa weevil larvae collected from the control plots in the insecticide field trial by instar stage. Samples were collected one day before treatment, 6-days, and 13-days post insecticide application.



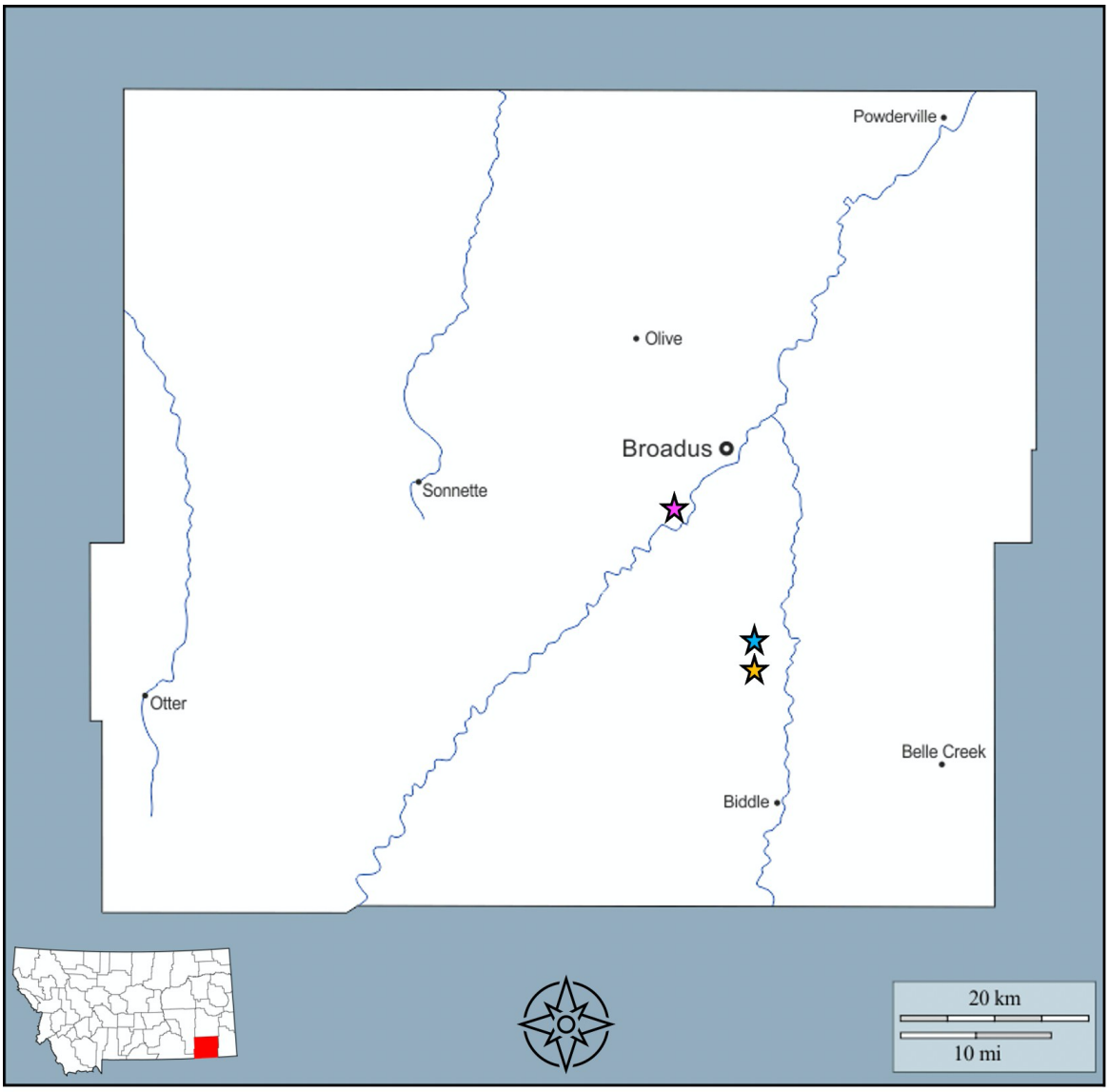

**Supplemental Figure 2.** Map of Big Horn County, MT 2020 field sites. The gold star represents field site 1 and the blue star represents field site two.



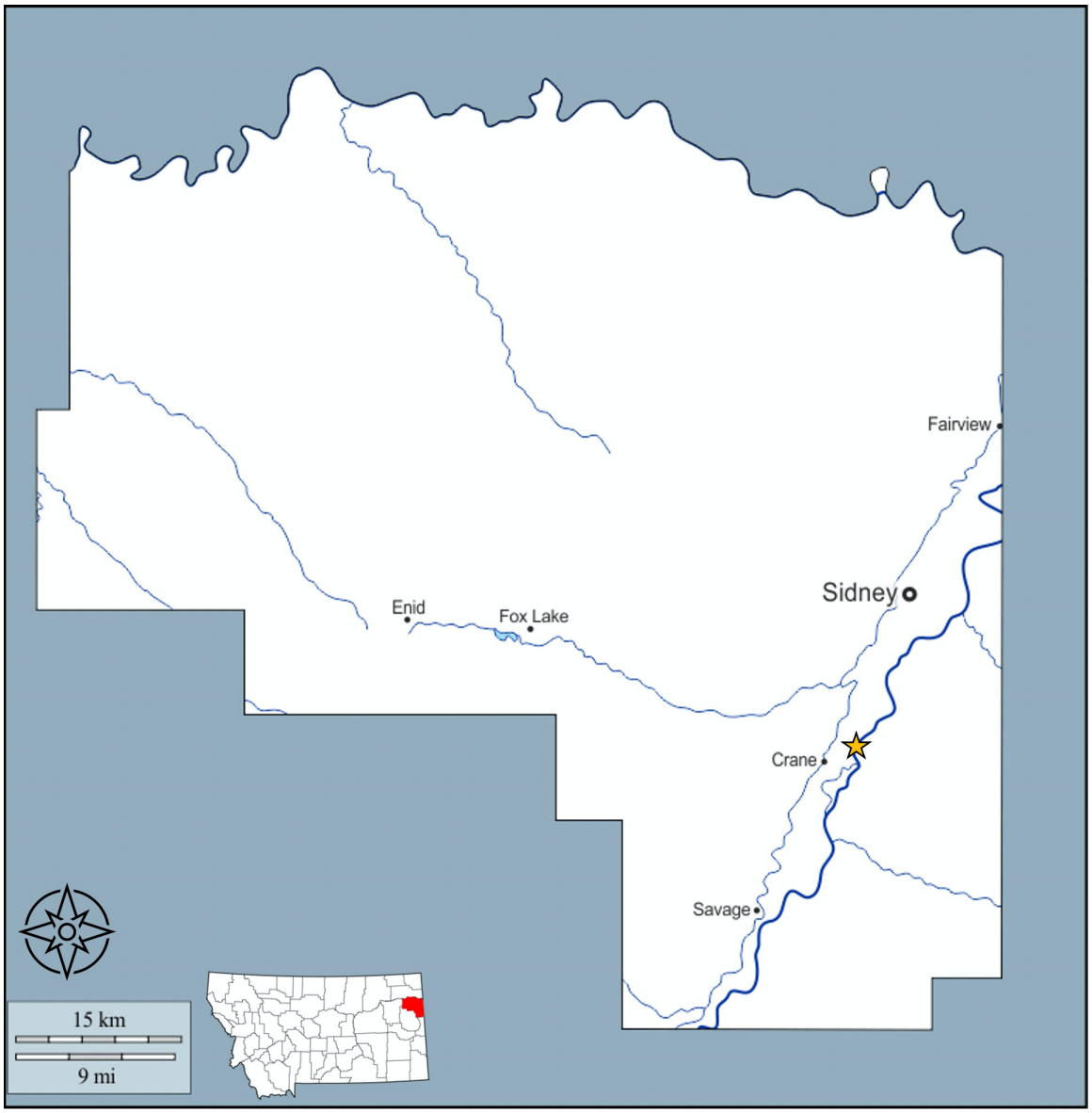

**Supplemental Figure 3.** Map of Broadwater County, MT field sites. The gold star represents field site 1, the blue star represents field site two, the pink star represents field site 3, and the purple star represents field site 4.




**Supplemental Figure 4.** Map of Madison County, MT field sites. The gold star represents field site 1 and the blue star represents field site two.




**Supplemental Figure 5.** Map of Powder River County, MT field sites. The gold star represents field site 1, the blue star represents field site two, and the pink star represents field site 3.




**Supplemental Figure 6.** Map of Richland County, MT field sites. The gold star represents field site 1.

# ALFALFA WEEVIL (COLEOPTERA: CURCULIONIDAE) RESISTANCE TO LAMBDA-CYHALOTHRIN IN THE WESTERN UNITED STATES

## Contribution of Authors and Co-Authors

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# ALFALFA WEEVIL (COLEOPTERA: CURCULIONIDAE) RESISTANCE TO LAMBDA-CYHALOTHRIN IN THE WESTERN UNITED STATES

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**Abstract**

Forage alfalfa (*Medicago sativa* L.) is a key agricultural commodity of the western region of the United States. The key insect pest of alfalfa, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), has developed resistance to the most common class of insecticide used to manage its damage. Alfalfa weevil samples from 71 commercial alfalfa fields located in Arizona, California, Montana, Oregon, Washington, and Wyoming were assayed for susceptibility to lambda-cyhalothrin during 2020 to 2022 using a laboratory concentration-response assay. Seventeen field sites representing all six states were highly resistant to lambda-cyhalothrin (resistance ratios > 100) and bioassay mortality often did not exceed 50% even at the highest concentration tested (3.30µg/cm$^2$ in 2020 and 10.00µg/cm$^2$ in 2021 - 2022). Field sites assayed with more than one pyrethroid active ingredient indicated likely cross-resistance between lambda-cyhalothrin and zeta-cypermethrin (type II pyrethroids) and variable and/or limited potential cross-resistance to permethrin (type I pyrethroid). Thirty-two field sites representing five states were susceptible to lambda-cyhalothrin (resistance ratios ranging from 1 to 20). While resistance is widespread, integrated resistance management strategies including rotating mode of action groups, applying chemical control tactics only when economic thresholds have been met, and utilizing cultural tactics can be employed to slow the further development of resistance.

**Key Words:** insecticide resistance, forage crop, Curculionidae, integrated resistance management

## Introduction

Alfalfa, *Medicago sativa* L. (Fabales: Fabaceae), is the third most valuable row crop in the United States (US), and in 2019, the western region of the US produced 15.4 million tons (Nelson 2019, Rankin 2020). *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), the alfalfa weevil, is the primary defoliating insect pest of alfalfa causing economic damage throughout the continental US (Peterson et al. 1992, Pellissier 2017). The larval stage of all alfalfa weevil strains can cause yield and quality loss by feeding on alfalfa leaves (Onstad and Shoemaker 1984, Hutchins et al. 1990). Alfalfa weevil is an invasive species native to Eurasia and North Africa and was introduced to the US on three known occasions. Each introduction is recognized as a distinct strain: the western (Salt Lake City UT, 1904), Egyptian (Yuma AZ, 1939), and eastern (Maryland, 1952) strains (Titus 1910, Wehrle 1940, Poos and Bissell 1953, Wood et al. 1978, Hsiao 1993, Bundy et al. 2005).

Due to the economic injury caused by alfalfa weevils, the application of effective and inexpensive pyrethroid insecticides (mode of action [MoA] group 3A) has been the primary method of management (Wright et al. 2015, IRAC 2022). The use of some older organophosphate (MoA 1B) insecticides has been restricted, limiting producer access to active ingredients with alternative MoA groups (Wright et al. 2015, Pellissier 2017, IRAC 2022). During the 1950s, organochlorine (MoA 2A) insecticides were used routinely for the control of alfalfa weevil, and by the early 1960s resistance to heptachlor had developed (Alder and Blickenstaff 1964, Bishop 1964). After several decades of pyrethroid use, alfalfa weevil resistance to lambda-cyhalothrin has been reported in Alberta Canada, California, and Montana

(Glen 2015, Orloff et al. 2016, Rodbell and Wanner 2021, Wanner et al. 2022). These repeated reports of pyrethroid insecticide failure highlight a growing concern for alfalfa producers.

One major challenge facing alfalfa weevil management is the lack of alternative MoA insecticides. Six pyrethroid (MoA 3A) active ingredients (five type II pyrethroids and one type I pyrethroid) are registered for the control of alfalfa weevil in forage alfalfa. Other than indoxacarb (MoA 22A), few effective alternative MoA insecticides are available. Spinosad (MoA 5) is recommended only for suppression, while the use of chlorpyrifos (MoA 1B) has recently been restricted (UC-ANR 2020, EPA 2021). With the potential for cross-resistance within MoA group 3A, alfalfa producers could lose the ability to rely upon all currently available pyrethroid-based insecticides, significantly limiting their options.

Integrated resistance management (IRM) strategies are urgently needed to slow the development of alfalfa weevil resistance to pyrethroids. Integrated resistance management strategies would not only prolong the efficacy of pyrethroid active ingredients. They would also slow the development of resistance to indoxacarb because of repeated use of this MoA in areas where pyrethroids are no longer effective. The first step of IRM is detection and monitoring of resistance, ideally early in resistance development (Croft 1990a). The goals of this research were to 1) determine the extent of lambda-cyhalothrin resistance in the western US and 2) assess the risk of cross-resistance between pyrethroid active ingredients. We used concentration-response bioassays to estimate lambda-cyhalothrin resistance ratios using larvae collected from Arizona, California, Montana, Oregon, Washington, and Wyoming. Commercial fields with highly resistant alfalfa weevils were identified from all six states surveyed in this study and preliminary evidence supports cross-resistance among type II pyrethroids.

## Materials and Methods

### *Insect Collections*

Field sampling in six western region states was partly targeted towards sites with suspected pyrethroid resistance, based on crop advisor, extension agent, and/or alfalfa producer reports, and included fields with no known history of insecticide resistance. Alfalfa weevil larvae from 71 commercial alfalfa fields were evaluated during a three-year period to assess the degree of lambda-cyhalothrin resistance in the western region.

Alfalfa weevil larvae were collected, and hand carried, or shipped overnight, to Montana State University or the University of California Davis where the bioassays were conducted. We made 34 weevil collections in 2020, including six counties in California, nine counties in Montana, one county in Washington, and one county in Wyoming (Table 2). Field collections extended from 21 February until 25 June 2020. In 2021, we made 32 collections from three counties in Arizona, five counties in California, three counties in Montana, one county in Oregon, one county in Washington, and two counties in Wyoming. Field collections extended from 9 February until 24 June 2021. In February 2022, alfalfa weevils were collected in two Arizona counties and Riverside County California.

Arizona field sites were generally located along the western border of the state with Pinal County the exception (9 sites total, an average of 4 per county). California field sites were generally located in the center of the state (24 sites total, an average of 3.7 per county). Distance between sites within the same county ranged from 0.40 to 88.5 kilometers (km). The commercial alfalfa fields were all irrigated and the majority conventionally managed.

Montana field sites were located across the southern region of the state with Richland County the exception (18 sites, an average of 2.3 per county). Wyoming field sites were generally located along the northern border with Montana and the south-eastern portion of the state (5 sites total, an average of 1.3 per county). Distance between sites within the same county ranged from 0.80 to 40 km. All field sites were conventionally managed, with a mixture of dryland and irrigated production systems.

Oregon and Washington sites were located along the border between the two states. Oregon field sites in 2021 were isolated to Umatilla County along the northern border (4 sites total). Washington field sites were located in two counties along the south-central portion of the state (3 sites total). Distance between sites within the same county ranged from 0.8 km to 34 km. All field sites were conventionally managed and were irrigated.

Larvae were collected with a sweep net while walking in a zig-zag pattern through each field. Once collected, samples were placed in plastic buckets or paper bags filled with cut alfalfa from the sampled field and transported in coolers to the laboratory for bioassay. In the laboratory, 19-liter plastic buckets filled with untreated fresh alfalfa and covered with No-see-um® number 20 mesh (Quest Outfitters, Sarasota, FL) were used to maintain larvae for 24 - 48 hours at room temperature (20 - 25°C). In 2020, visually estimated third to fourth instar larvae were sorted and used in the bioassay; 2021-2022 visually estimated second to third instar larvae were used.

*Active Ingredients*

Lambda-cyhalothrin, zeta-cypermethrin (type II pyrethroids), and permethrin (type I pyrethroid) were assayed using technical grade material. Lambda-cyhalothrin ([(R)-cyano-(3-

phenoxyphenyl)methyl] (1S, 3S)-3-[(Z)-2-chloro-3,3,3,-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate) was provided by Syngenta (Basel, Switzerland), 89% purity. Zeta-cypermethrin ([cyano-(3-phenoxyphenyl) methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) was provided by FMC Corporation (Newark, NJ), 92% purity. Permethrin ((3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate)) was purchased from Sigma-Aldrich (St. Louis, MO).

*Concentration Response Bioassays*

Detailed bioassay methods are reported in Rodbell and Wanner (2021). In brief, stock solutions of each active ingredient were prepared in 95% acetone. Using serial dilutions with 95% acetone, we generated solutions of seven concentrations. One milliliter (mL) of each concentration was added to the glass vials (Brindley 1975; Discount Vials, Madison, WI) and the acetone evaporated without heat on a commercial food roller at room temperature (21°C), yielding 0.0033-10.0 micrograms per centimeter squared ($\mu g/cm^2$) of vial surface area for lambda-cyhalothrin and zeta-cypermethrin, and 0.033-100.0 $\mu g/cm^2$ for permethrin. Treated vials were stored at room temperature in a dark cabinet and used during a two-week period for bioassay experiments. Each bioassay typically consisted of seven concentrations and a 95% acetone control (n = 5 replicated vials, 10 larvae per vial). After 24 hours in the dark at room temperature, the larvae were scored as dead or alive after exposure to a heated hot plate (43 - 50°C).

Probit analysis was used to quantify the lethal concentration of an active ingredient that generates 50% mortality ($LC_{50}$) using Probit Or LOgit analysis (POLO) software (LeOra Software, Parma, MO) (Robertson et al. 2017). Bioassays with control mortality >20% were

omitted from analysis (Robertson et al. 2017). If mortality was observed in the control group, correction for natural response was estimated by the POLO Probit analysis (Robertson et al. 1980). Analyses with a t-ratio of the slope less than 1.96 were excluded from this study (Yu 2015; Robertson et al. 2017). Data sets that did not fit the probit model were omitted based on the chi-square ($\chi^2$) goodness of fit test ($p < 0.05$) unless a single outlier was identified as the cause for the lack of fit. Outliers were identified through the graphical method of the residual values (Sarkar et al. 2011, Robertson et al. 2017). In this study, single outliers were removed from 22 lambda-cyhalothrin, 4 zeta-cypermethrin and 7 permethrin bioassays (Supp. Table S1). Larval mortality from some highly resistant sites failed to reach 50% and did not fit the requirements of probit analysis. In these cases, the $LC_{50}$ value was conservatively listed as greater than (>) the highest concentration tested (Brindley 1975, Haddi et al. 2018, Rodbell and Wanner 2021).

*Assessing Cross-Resistance Between Active Ingredients*

To assess cross-resistance between active ingredient types, alfalfa weevil larvae from individual fields in all sampled states were assayed with multiple active ingredients. We assayed weevils 14 fields with lambda-cyhalothrin, zeta-cypermethrin and permethrin and 10 additional fields with lambda-cyhalothrin and permethrin. The $LC_{50}$ resistance ratios for lambda-cyhalothrin were calculated using the average of the nine most susceptible location samples as the common denominator (0.013 $\mu g/cm^2$) (Forrester and Cahill 1987, ffrench-Constant and Roush 1990, Burton Jr. et al. 1996). Due to limited bioassays, all resistance ratios for permethrin and zeta-cypermethrin were calculated using the most susceptible $LC_{50}$ value for each active ingredient generated to descriptively illustrate patterns of cross-resistance (Tabashnik et al. 2011).

Evidence for cross-resistance was further tested by dividing the fields into two conservative categories based on their lambda-cyhalothrin resistance ratios (RR), fields with a RR ≤ 76.9 (considered susceptible or moderately resistant) and fields with a RR > 76.9 (considered highly resistant, Table 1). If cross-resistance exists between lambda-cyhalothrin and zeta-cypermethrin and/or permethrin, responses to these active ingredients (mortality at a diagnostic-concentration) should correspond to the resistance category defined by the lambda-cyhalothrin RR (Menger et al. 2020). For example, high resistance to lambda-cyhalothrin predicts low mortality to zeta-cypermethrin if cross-resistance exists. A concentration of 1.0 $\mu g/cm^2$ was analyzed as a diagnostic-concentration for zeta-cypermethrin and 3.3 $\mu g/cm^2$ for permethrin that is generally less potent. Percent mortality was log-transformed to meet the normality assumption of the one-way analysis of variance (ANOVA) that was performed on RStudio statistical software version 1.3.1093 (RStudio Team, Boston MA). The Tukey HSD test ($p = 0.05$) was used to identify significant differences between treatments.

**Results**

*Resistance Levels to Lambda-Cyhalothrin in the Western United States*

The 71 field sites were categorized into three general levels of resistance to lambda-cyhalothrin, to assess trends across the western US. Susceptible, moderately resistant, and highly resistant categories were defined relative to the higher label rate of lambda-cyhalothrin: <1x, 1-3x and >3x, respectively (Kostromytska et al. 2017, Table 1). The slope of the probit relationship was approximately 1.0 for susceptible and moderately resistant field sites and approximately 0.6 for highly resistant fields (Table 2).

Of the 71 fields tested with lambda-cyhalothrin, 32 were categorized as susceptible, 20 as moderately resistant, and 17 as highly resistant, to lambda-cyhalothrin (Table 2; resistance levels as defined in Table 1; Fig. 1). With this sampling intensity alfalfa fields with highly resistant larvae as well as susceptible larvae were identified in every surveyed state but Wyoming (limited sites tested in WY yielded moderate and high resistance ratings, Fig. 1).

Concentration-dependent mortality caused by lambda-cyhalothrin varied greatly among the 71 fields tested, resulting in $LC_{50}$ values ranging from 0.002 to 8.4 µg/cm$^2$ (Table 2). Eight field sites in five of the sampled states, did not exceed 8-50% mortality even at the highest concentration of lambda-cyhalothrin tested (3.3 or 10 µg/cm$^2$) (>3.3 µg/cm$^2$: Riverside County CA, field site 9; Big Horn County MT, field sites 1 & 2; Klickitat County WA, field site 1; and >10.0 µg/cm$^2$: La Paz County AZ, field site 7; Umatilla County OR, field site 4; Yakima County WA, field site 1; Table 2). For context, the label rate for lambda-cyhalothrin is 22.7 to 33.6 grams of active ingredient per hectare (0.02 to 0.03 pounds of active ingredient per acre; Warrior II with Zeon Technology®, Syngenta 2021), corresponding to 0.22-0.34 µg/cm$^2$ in the treated vials.

Seventeen of the 71 location samples tested were categorized as highly resistant and local agriculturalists suspected insecticide resistance based on control failures in the field. The 17 highly resistant fields represent two counties each in Arizona, Washington, and Wyoming, and one county each in California, Montana, and Oregon (Fig. 1). The majority of the remaining 48 fields sites were selected arbitrarily, and not based on reports of suspected resistance. Thirty-two of these fields were categorized as susceptible (Tables 1 and 2), representing six counties in California, six counties in Montana, two counties in Arizona, and one county in Oregon and

Washington. It is important to note that sampling was not intended to estimate the frequency of resistance in each state, but rather to initially characterize its extent in the western region of the US, and sampling was more extensive in California and Montana. Based on lambda-cyhalothrin $LC_{50}$ values, 16 fields were categorized as moderately resistant, representing four counties in California and Montana, and one county in Arizona and Wyoming.

The $LC_{50}$ estimates from field-sampled larvae were considered consistent based on regional patterns (as opposed to resampling the same field repeatedly). For example, there were four field sites (Riverside County CA, field site 1; Big Horn County MT, field site 1; Big Horn County, field site 2; and Madison County MT, field site 2) that were sampled in 2020 and 2021. All sampled sites but Riverside County, field site 1, were of the same resistance level across both years. The Riverside County CA, field site 1, $LC_{50}$ value increased in 2021, indicating that the estimated resistance level changed from susceptible to moderately resistant (Table 2). This field site was managed organically in an area with alfalfa predominantly managed conventionally and may have been impacted by migrating resistant adults. Some field sites in Montana were evaluated multiple times within the same year, generating similar $LC_{50}$ values between bioassays, including Big Horn County field sites $1_{2020}$, $2_{2020}$, and $3_{2021}$ (Table 2).

*Cross-Resistance Between Type I and II Pyrethroid Active Ingredients*

Our findings indicate cross-resistance between lambda-cyhalothrin and zeta-cypermethrin, however, there was limited cross-resistance between lambda-cyhalothrin and permethrin. Sixteen of the 72 field sites collected from 2020 to 2022 were assayed with zeta-cypermethrin (Table 3). $LC_{50}$ values ranged from a low of 0.09 µg/cm$^2$ (Broadwater County MT, field site 4) to a high of 7.75 µg/cm$^2$ (Big Horn County Montana, field site 1). For context, the

label rate for zeta-cypermethrin is 15.7 - 28.0 grams of active ingredient per hectare (0.014 - 0.025 pounds of active ingredient per acre, Mustang® Maxx Insecticide, FMC 2021), equivalent to about 0.16 - 0.28 µg/cm$^2$. $LC_{50}$ values were not calculated for field sites where maximum larval morality failed to reach 50% and are conservatively reported as greater than the highest concentration tested (>3.3 µg/cm$^2$: Big Horn County MT, field sites 1, & 2; and, >10 µg/cm$^2$ La Paz County AZ, field site 1, Umatilla County OR, field site 4, and Yakima County WA, field site 1). Four of five field sites that failed to reach 50% larval mortality after exposure to the highest concentration of zeta-cypermethrin also failed to reach 50% mortality after exposure to the highest concentration of lambda-cyhalothrin (Table 2). Mortality at Umatilla County OR field site 4 never exceeded 8% for any concentration tested. Larvae from alfalfa fields highly resistant to lambda-cyhalothrin also tended to be highly resistant to zeta-cypermethrin, supporting cross-resistance between these two type II active ingredients.

Lambda-cyhalothrin and zeta-cypermethrin cross-resistance is supported by diagnostic-concentration mortality between field sites that were grouped based on their lambda-cyhalothrin resistance ratio. Sixteen field sites were assayed with both lambda-cyhalothrin and zeta-cypermethrin; larvae from 6 fields had lambda-cyhalothrin RR < 76.9 (susceptible - moderate resistance) and 10 had RR > 76.9 (highly resistant to lambda-cyhalothrin). Generally, susceptibility to zeta-cypermethrin corresponded to susceptibility to lambda-cyhalothrin. Consistent with cross-resistance, diagnostic-concentration mortality resulting from zeta-cypermethrin was lower in the field sites highly resistant to lambda-cyhalothrin (RR > 76.9) and conversely, higher in the field sites susceptible to lambda-cyhalothrin (susceptible - moderate resistance, RR < 76.9) (97.9% vs 48.2%mortality, Fig. 2, $F = 49.67$; $df = 5, 24$; $P < 0.001$).

Evidence for cross-resistance between lambda-cyhalothrin and permethrin is variable. Thirty-one of the 72 field sites were also assayed with permethrin. The permethrin $LC_{50}$ values ranged from a low of 0.1 $\mu g/cm^2$ (Powder River County MT, field site 2) to a high of 19.4 $\mu g/cm^2$ (Gallatin County MT, field site 2). The label rate for permethrin, 112.0 - 224.1 grams per hectare (0.1 - 0.2 pounds of active ingredient per acre, Loveland Products Inc. 2013), is equivalent to 1.12 - 2.24 $\mu g/cm^2$in the bioassay vials. Unlike zeta-cypermethrin, alfalfa fields highly resistant to lambda-cyhalothrin were not always associated with higher permethrin $LC_{50}$ values (for example, Big Horn County MT). Additionally, some field sites susceptible to lambda-cyhalothrin appeared to be resistant to permethrin (for example, Umatilla OR, Yuma AZ and Gallatin MT counties). These results suggest variable patterns of resistance to lambda-cyhalothrin and permethrin at these field sites, including partial (but not complete) cross-resistance (Supp. Table S2 and Supp. Fig. S1).

Results from diagnostic-concentration assays with permethrin also supports the lack of complete cross-resistance since it was high both in lambda-cyhalothrin susceptible and highly resistant field sites (Fig. 2; $F = 49.67$; $df = 5, 24$; $P < 0.001$). Unlike lambda-cyhalothrin and zeta-cypermethrin, permethrin maximum mortalities were always higher than 50%, enabling $LC_{50}$ values to be calculated. Collectively, the evidence for cross-resistance between lambda-cyhalothrin and permethrin is mixed (Figure 2, Spp. Fig. S1).

**Discussion**

Alfalfa weevil lambda-cyhalothrin resistance is established across the western United States and highly resistant locations were identified in all six surveyed states. Larval mortality from some surveyed alfalfa fields never reached 50% mortality at the highest concentration

tested. Susceptible field sites were identified in every state surveyed, except Wyoming, where only four sites were sampled. Field sites that were tested with multiple pyrethroid active ingredients provided some evidence for cross-resistance between lambda-cyhalothrin and zeta-cypermethrin (type II pyrethroid), and variable and/or limited cross-resistance between lambda-cyhalothrin and permethrin (type I pyrethroid). Based on historical distributions, the field sites in southern Arizona and southern California correspond to the range occupied by Egyptian strain alfalfa weevils, suggesting resistance to lambda-cyhalothrin has developed in both the Egyptian and western strains (Titus 1910, Wehrle 1940, Clancy 1969).

Our findings of alfalfa weevil field sites highly resistant to lambda-cyhalothrin suggest that the mechanism(s) of resistance is widespread in the western region of the United States. However, further studies are required to identify the mechanism(s) of alfalfa weevil resistance to lambda-cyhalothrin and their geographic variation. In other insect species, mutations to the voltage gated sodium ion channel gene target site that reduce sensitivity to pyrethroid active ingredients (i.e., knockdown resistance (*kdr*) and *super-kdr*) as well as the utilization of detoxification enzymes and/or behavioral resistance have been identified (Feyereisen 1999, Soderlund 2005, Davies et al. 2007, Després et al. 2007, Scott et al. 2008, Rinkevich et al. 2013, Pereira et al. 2017, Haddi et al. 2018, Fujii et al. 2020, Keïta et al. 2021).

Susceptibility to an insecticide can depend on the size, life stage and sex of an insect (e.g., instar stage). Thus, insecticide applications should coincide with the most susceptible life stage to maximize efficacy, one recommendation to slow the development of resistance (Yu 1983, Koehler et al. 1993, Glunt et al. 2011, Zhu et al. 2020). Future research is needed to characterize the susceptibility of adult alfalfa weevils and the four instar stages to determine

optimal timing of pyrethroid and indoxacarb insecticide sprays in the western region of the United States.

Within insecticide naïve populations, susceptibility to an insecticide is variable, producing a range of $LC_{50}$ values, prompting some studies to use an average $LC_{50}$ value derived from several 'susceptible' location samples (Brown and Brogdon 1987, Forrester and Cahill 1987, Sawicki 1987, ffrench-Constant and Roush 1990, Burton Jr. et al. 1996). An average of the lowest lambda-cyhalothrin $LC_{50}$ values (0.013µg/cm$^2$, n = 9 independent field sites, three states) was used to represent susceptible alfalfa weevils in the western region (Forrester and Cahill 1987, ffrench-Constant and Roush 1990, Burton Jr. et al. 1996). Lambda-cyhalothrin $LC_{50}$ resistance ratios in this study ranged from 1 to 770 (Table 3). When characterizing susceptibility in field populations, other studies have found similar broad ranges of RRs (Luttrell et al. 1999, Shelton et al. 2003, Snodgrass and Scott 2003, Wang et al. 2010, Kostromyska et al. 2017). Intense resistance, a natural range of toxicity and the method of calculating RRs can contribute to the observed broad range of values.

Interpreting the significance of RRs produced by laboratory bioassays to insecticide efficacy in the field can be challenging. Ideally, $LC_{50}$ values from bioassays are correlated with insect mortality after field application, but this approach is laborious and not commonly employed (Ball 1981, Denholm et al. 1984, ffrench-Constant and Roush 1990). Rather, the RRs are commonly divided into discrete categories with inferred levels of field resistance, such as susceptible, moderate resistance, and high resistance (Table 1, Kostromytska et al. 2017). Such categories provide a framework for IRM recommendations with supporting field trials to verify bioassay-based categories. In this study, the moderate resistance category represents field sites

transitioning from susceptible (effective larval control) to resistant (control failure) after field applications of lambda-cyhalothrin. Alfalfa weevil highly resistant to lambda-cyhalothrin, are associated with control failure in the alfalfa fields where these larvae were sampled (Table 2, Big Horn County MT, field site 1). Lambda-cyhalothrin applied at its highest label rate failed to reduce larval counts compared to untreated check plots in a field trial where bioassays recorded an $LC_{50} > 3.3$ μg/cm$^2$ (RR > 330; Rodbell and Wanner 2021, Wanner et al. 2022; Table 2, Big Horn County MT field site 1).

Cross-resistance between different active ingredients within the same MoA group is common among insect pests, and our preliminary data suggest alfalfa weevils are cross-resistant to lambda-cyhalothrin and zeta-cypermethrin, both type II pyrethroid active ingredients (Yu 2015; Fig. 2). A descriptive comparison of the RRs of several active ingredients that results in a parallel trend of increasing RRs suggests cross-resistance exists (DeVries and Georghiou 1981, Scott 1990, Kostromytska et al. 2017). To supplement this descriptive approach and gain preliminary insights into cross-resistance we simplified the analysis by dividing field sites into two categories based on their susceptibility to lambda-cyhalothrin (RR < 76.9 vs RR >76.9). Alfalfa weevils resistant to lambda-cyhalothrin averaged less than 50% mortality in response to a discriminating dose of zeta-cypermethrin, supporting cross-resistance of these two active ingredients. Average mortality of resistant larvae after exposure to a discriminating dose of permethrin resulted in high mortality, supporting a lack of complete cross-resistance between lambda-cyhalothrin and permethrin (Fig. 2, Supp. Figure S1).

In several cases, susceptible and highly resistant field sites were identified in the same county, suggesting lambda-cyhalothrin resistance may be localized. The relatively limited

dispersal of adult weevils and the density and isolation of some alfalfa production areas, likely contribute to the size and severity of resistance pockets (Prokopy et al. 1967). For example, alfalfa is a primary economic crop grown in the Palo Verde Valley of southern California, receiving irrigation from the lower Colorado River, and surrounded by the Sonoran Desert. In this contiguous production area with intensively managed forage alfalfa, resistance to lambda-cyhalothrin is relatively uniform (Table 2). A pattern not seen in other counties included in this study (e.g., Yuma County, AZ; Umatilla County, OR; Yakima County, WA).

Soon after the advent of the "insecticide era" in the 1950s, insecticide resistance became a serious and persistent problem associated with routine pesticide use (e.g., calendar spraying; Pedigo et al. 2021). In addition to the monetary impact of yield loss, economic impacts can include higher costs associated with alternative MoAs required for adequate pest control (Grafius 1997). In the case of alfalfa weevil, reports of cross-resistance to both heptachlor and dieldrin insecticides (MoA 2A) began to emerge in 1962 and were subsequently replaced by pyrethroids (MoA 3A) (Adler and Blickenstaff 1964). Alternative MoA groups currently available for the control of alfalfa weevil are limited, and several are considered ineffective and/or have increased restrictions. In some areas, indoxacarb (MoA 22A) appears to be the only effective active ingredient where alfalfa weevils are highly resistant to pyrethroids. Indoxacarb (i.e., Steward) provided 92-96% control of alfalfa weevil larvae in fields resistant to lambda-cyhalothrin (Rodbell and Wanner 2021, Wanner et al. 2022; Table 2, Big Horn County MT field site 1). Thus, the loss of efficacy of all MoA 3A pyrethroid active ingredients would significantly limit available insecticide options for alfalfa weevil control and impede IRM strategies based on rotating insecticide MoA groups.

Insecticide resistance management is an approach to proactively manage resistance. In the case of alfalfa weevil, insecticide resistance management aimed at pyrethroid resistance has variable potential for success and can achieve different goals. For example, alfalfa weevil populations highly resistant to lambda-cyhalothrin have been identified from every state included in this study. For these populations, the first two goals of IRM, 1) preventing resistance from developing and 2) slowing the rate of resistance development, have already been bypassed (Croft 1990a). IRM strategies in these locations should be designed to revert lambda-cyhalothrin resistance to more susceptible levels (Croft 1990a). However, once resistance genes have been established, their frequencies can increase quickly with the resumption of insecticide use (Yu 2015). After more than five decades of pyrethroid use in agriculture, pyrethroid-naïve alfalfa weevil populations are likely quite rare. However, the identification of numerous locations with lambda-cyhalothrin susceptible alfalfa weevils was a promising outcome of this study. In these areas, IRM strategies could prevent the development of pyrethroid resistance, at least locally, for some time.

Integrated pest management (IPM) strategies function as important components of IRM (Croft 1990b, Dara 2021, Gallagher 2021). Therefore, the first recommendation is to use IPM strategies that are currently available to reduce insecticide use. Monitoring alfalfa weevil populations and employing economic thresholds to guide insecticide applications is a founding principle of IPM (Peterson et al. 2018, Pedigo et al. 2021). When economic thresholds are exceeded, cultural control practices should be considered before opting for chemical control. Recommended cultural control tactics include maintaining healthy alfalfa stands and harvesting

early (seven to ten days before normal harvest time) to salvage yield and increase larval mortality (Summers 1998, Pellissier et al. 2017, Pedigo et al. 2021).

Successful IRM strategies include reducing the constant pressure to select resistance to specific active ingredients by rotating to different MoA groups (Tabashnik 1990, IRAC 2022). Currently, only two MoA groups are considered effective for alfalfa weevils for seasonal rotation, MoA 3A (pyrethroids) and MoA 22A (indoxacarb). Until new MoA groups become available, a current recommendation is to apply MoA groups 3A and 22A no more than once every three years, as well as including cultural control tactics to limit insecticide use (IRAC 2022). If multiple insecticide applications are required during the same season or generation, the same MoA should be used within a year (Roush and Daly 1990). Other goals include preserving the efficacy of indoxacarb (MoA 22A) and maximizing the effectiveness of insecticide applications by optimizing operational variables (e.g., application timing, spray coverage, and equipment calibration) (Roush and Daly 1990). Furthermore, insects heterozygous for resistance can be relatively more susceptible compared to homozygous individuals (Rawlings et al. 1981, Roush and Daly 1990). Higher efficacy, including the use of the higher rates listed on the pesticide label, result in greater mortality of susceptible and heterozygous individuals, thus delaying the gene(s) frequency from increasing (Rawlings et al. 1981, Roush and Daly 1990).

Further research efforts are needed to establish patterns of cross-resistance between the different pyrethroid active ingredients registered for alfalfa weevil management, including field efficacy, to provide IRM recommendations to alfalfa stakeholders. A better understanding of the physiological mechanisms of pyrethroid resistance may lead to the development of molecular markers to better monitor the spread of pyrethroid resistance and the effectiveness of IRM

strategies. Emphasis should be placed on early efforts to monitor alfalfa weevil resistance to MoA 22A (i.e., indoxacarb), and to any new MoA group registered for the control of alfalfa weevil. Here, population genomics and next generation sequencing technology to detect early changes in genotype(s), coupled with laboratory bioassays that detect insecticidal activity patterns with greater sensitively, may represent promising approaches.

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## Tables and Figures

**Table 1.** To inform integrated resistance management (IRM) recommendations three categories of lambda-cyhalothrin resistance were defined based on lethal concentrations causing 50% mortality ($LC_{50}$) and the equivalent pesticide label rate for commercial products registered for alfalfa weevil control: 1) Susceptible, 2) Moderate resistance, and 3) High resistance. For each category, the equivalent resistance ratio (RR) range is listed using the average $LC_{50}$ value of the nine most susceptible location samples assayed in this study (Table 2).

| Resistance Category | $LC_{50}$ ($\mu g/cm^2$) | Resistance Ratio ($LC_{50}$/0.013) | Times (X) Higher Label Rate 0f 0.34 $\mu g/cm^2$ |
|---|---|---|---|
| Susceptible | <0.30 | <23.08x | < 0.9X |
| Moderate | 0.30 – 1.0 | 23.08x – 76.9x | 0.9X - 2.9X |
| High | > 1.0 | >76.9x | > 2.9X |

**Table 2.** Alfalfa weevil larval resistance (R) level to lambda-cyhalothrin in the western region of the United States based on assayed $LC_{50}$ values from 71 location samples: S is susceptible; M is moderate resistance; and H is high resistance. Probit analysis statistics are listed for the t-ratio of the slope, the chi-square ($\chi^2$) goodness of fit, and the degrees of freedom (df). Resistance ratios (RR) were calculated using the $LC_{50}$ value of the average of the nine most susceptible location samples, 0.013µg/cm$^2$.

| State | County | Site $_{year}$ | $LC_{50}$ µg/cm$^2$ | t-ratio $_{slope}$ | $\chi^2{}_{df}$ | p-value | RR | R Level |
|---|---|---|---|---|---|---|---|---|
| AZ | La Paz | $1_{2021}$ | 1.55 | 6.09 | $8.39_5$ | 0.14 | 119 | H |
| | | $2_{2021}$ | 0.65 | 8.27 | $7.9_5$ | 0.16 | 50 | M |
| | | $3_{2021}$ | 2.78 | 9.00 | $5.04_5$ | 0.41 | 214 | H |
| | | $4_{2021}$ | 0.53 | 7.00 | $0.04_2$ | 0.98 | 41 | M |
| | | $6_{2022}$ | 0.62 | 3.88 | $3.43_2$ | 0.18 | 49 | M |
| | | $7_{2022}$ | >10.0 | 3.25 | $2.62_2$ | 0.27 | >770 | H |
| | | $8_{2022}$ | 4.67 | 4.32 | $0.052_2$ | 0.97 | 360 | H |
| | Pinal | $1_{2021}$ | 0.15 | 8.52 | $6.08_4$ | 0.19 | 12 | S |
| | Yuma | $1_{2020}$ | 0.18 | 5.77 | $2.05_1$ | 0.15 | 14 | S |
| | | $2_{2021}$ | 0.12 | 6.64 | $3.30_4$ | 0.51 | 9 | S |
| | | $3_{2021}$ | 1.35 | 8.27 | $7.90_5$ | 0.16 | 104 | H |
| CA | Kern | $1_{2020}$ | 0.02 | 4.82 | $8.37_5$ | 0.14 | 1 | S |
| | | $2_{2021}$ | 0.004 | 3.75 | $4.37_4$ | 0.36 | 1 | S |
| | Kings | $1_{2020}$ | 0.34 | 7.35 | $6.78_4$ | 0.15 | 26 | M |
| | | $2_{2020}$ | 0.44 | 5.91 | $7.22_4$ | 0.12 | 34 | M |
| | | $3_{2020}$ | 0.002 | 10.4 | $3.95_5$ | 0.56 | 1 | S |
| | | $4_{2020}$ | 0.01 | 8.46 | $2.52_5$ | 0.77 | 1 | S |
| | Madera | $1_{2020}$ | 0.05 | 8.68 | $8.11_4$ | 0.09 | 4 | S |
| | Merced | $1_{2021}$ | 0.17 | 6.96 | $3.99_4$ | 0.41 | 13 | S |
| | | $2_{2020}$ | 0.04 | 7.34 | $3.72_5$ | 0.59 | 3 | S |
| | | $3_{2021}$ | 0.04 | 7.76 | $5.70_4$ | 0.22 | 3 | S |
| | | $4_{2021}$ | 0.02 | 10.10 | $6.99_5$ | 0.22 | 1 | S |
| | Riverside | $1_{2020}$ | 0.26 | 7.95 | $6.58_5$ | 0.25 | 20 | S |
| | | $1_{2021}$ | 0.92 | 6.54 | $6.07_5$ | 0.30 | 71 | M |
| | | $2_{2020}$ | 0.53 | 5.59 | $0.12_1$ | 0.73 | 41 | M |
| | | $3_{2020}$ | 0.51 | 4.51 | $0.38_1$ | 0.53 | 40 | M |
| | | $4_{2020}$ | 0.12 | 4.64 | $1.19_3$ | 0.76 | 9 | S |
| | | $5_{2021}$ | 0.01 | 8.63 | $3.09_4$ | 0.54 | 1 | S |
| | | $6_{2020}$ | 0.63 | 4.35 | $0.25_1$ | 0.62 | 48 | M |
| | | $7_{2020}$ | 0.22 | 4.51 | $0.39_1$ | 0.53 | 17 | S |
| | | $8_{2022}$ | 1.82 | 3.87 | $3.69_1$ | 0.055 | 140 | H |
| | | $9_{2022}$ | >3.33 | - | - | - | >256 | H |
| | Yolo | $1_{2021}$ | 0.30 | 7.32 | $5.86_4$ | 0.21 | 23 | M |
| | | $2_{2021}$ | 0.08 | 6.60 | $5.75_4$ | 0.22 | 6 | S |
| | | $3_{2020}$ | 0.13 | 5.30 | $6.88_5$ | 0.23 | 10 | S |
| | | $4_{2020}$ | 0.01 | 2.66 | $5.97_5$ | 0.31 | 1 | S |
| | | $5_{2021}$ | 0.51 | 7.66 | $4.38_5$ | 0.50 | 39 | M |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| MT | Beaverhead | $1_{2020}$ | 0.05 | 8.26 | $7.05_5$ | 0.22 | 4 | S |
| | Big Horn | $1_{2020*}$ | >3.33 | 4.12 | $9.78_5$ | 0.08 | >256 | H |
| | | $1_{2021}$ | 4.50 | 6.48 | $4.63_4$ | 0.33 | 346 | H |
| | | $2_{2020*}$ | >3.33 | 4.08 | $7.62_4$ | 0.11 | >256 | H |
| | | $2_{2020*}$ | >1.00 | 2.51 | $3.09_2$ | 0.21 | >77 | H |
| | | $2_{2021}$ | 1.78 | 4.86 | $6.50_4$ | 0.16 | 137 | H |
| | | $3_{2021}$ | 0.32 | 3.54 | $3.11_1$ | 0.08 | 25 | M |
| | | $3_{2021}$ | 0.57 | 6.76 | $3.61_5$ | 0.61 | 44 | M |
| | | $4_{2021}$ | 8.40 | 4.65 | $6.95_5$ | 0.22 | 646 | H |
| | Broadwater | $1_{2020*}$ | 0.26 | 5.1 | $7.39_5$ | 0.19 | 20 | S |
| | | $2_{2020*}$ | 0.26 | 8.22 | $4.79_5$ | 0.44 | 20 | S |
| | | $3_{2020*}$ | 0.77 | 7.82 | $2.68_2$ | 0.26 | 59 | M |
| | | $4_{2021}$ | 0.43 | 5.27 | $3.45_2$ | 0.18 | 33 | M |
| | Gallatin | $1_{2020}$ | 0.16 | 6.95 | $0.92_5$ | 0.97 | 12 | S |
| | | $2_{2020}$ | 0.31 | 7.47 | $1.87_4$ | 0.76 | 24 | M |
| | Madison | $1_{2020*}$ | 0.02 | 8.33 | $2.47_5$ | 0.78 | 1 | S |
| | | $2_{2020*}$ | 0.06 | 7.18 | $6.41_5$ | 0.27 | 5 | S |
| | | $2_{2021}$ | 0.26 | 7.01 | $3.67_3$ | 0.30 | 20 | S |
| | Powder River | $1_{2020*}$ | 0.03 | 4.58 | $2.73_2$ | 0.26 | 2 | S |
| | | $2_{2020*}$ | 0.1 | 7.14 | $5.51_4$ | 0.24 | 8 | S |
| | | $3_{2020*}$ | 0.09 | 7.83 | $6.58_4$ | 0.16 | 7 | S |
| | Ravalli | $1_{2020}$ | 0.42 | 3.92 | $4.65_5$ | 0.46 | 32 | M |
| | Richland | $1_{2020*}$ | 0.09 | 6.58 | $5.13_5$ | 0.40 | 7 | S |
| OR | Umatilla | $1_{2021}$ | 0.06 | 5.48 | $5.89_4$ | 0.21 | 5 | S |
| | | $2_{2021}$ | 0.17 | 5.88 | $7.9_4$ | 0.10 | 13 | S |
| | | $3_{2021}$ | 0.01 | 3.38 | $3.45_5$ | 0.63 | 1 | S |
| | | $4_{2021}$ | >10.0 | - | - | - | >770 | H |
| WA | Klickitat | $1_{2020}$ | >3.33 | 3.76 | $6.56_5$ | 0.26 | >256 | H |
| | Yakima | $1_{2021}$ | >10.0 | 3.76 | $6.56_5$ | 0.65 | >770 | H |
| | | $2_{2021}$ | 0.21 | 9.27 | $8.56_5$ | 0.13 | 16 | S |
| WY | Converse | $1_{2021}$ | 1.84 | 6.54 | $8.92_4$ | 0.06 | 142 | H |
| | | $1_{2021}$ | 6.92 | 4.2 | $9.60_5$ | 0.08 | 532 | H |
| | Platte | $1_{2021}$ | 0.52 | 7.97 | $3.92_4$ | 0.41 | 40 | M |
| | Sheridan | $1_{2020}$ | 2.23 | 5.05 | $11.09_5$ | 0.05 | 172 | H |

* Rodbell and Wanner 2021

**Table 3.** Lethal concentrations resulting in 50% mortality ($LC_{50}$ values) from 30 location samples assayed with permethrin (type I pyrethroid) and 16 location samples assayed with zeta-cypermethrin (type II pyrethroid). Probit analysis statistics are listed for the t-ratio of the slope, the chi-square ($\chi^2$) goodness of fit, and the degrees of freedom (df).

| Active ingredient | State | County | Field site $_{date}$ | $LC_{50}$ µg/cm$^2$ | T-ratio $_{slope}$ | $\chi^2{}_{df}$ | P value | # Concentrations |
|---|---|---|---|---|---|---|---|---|
| Zeta-cypermethrin | AZ | La Paz | $3_{2021}$ | >10.00 | 3.45 | $1.03_4$ | 0.90 | 6 |
| | | | $5_{2021}$ | 4.12 | 4.67 | $1.63_4$ | 0.80 | 6 |
| | | Pinal | $1_{2021}$ | 0.1 | 10.92 | $7.31_5$ | 0.20 | 7 |
| | MT | Big Horn | $1_{2020}$ | >3.33 | 5.18 | $9.02_5$ | 0.11 | 7 |
| | | | $1_{2021}$ | 7.75 | 6.32 | $10.95_5$ | 0.052 | 7 |
| | | | $2_{2020}$ | >3.33 | 5.57 | $3.12_5$ | 0.68 | 7 |
| | | | $2_{2021}$ | 5.82 | 5.2 | $10.62_5$ | 0.06 | 7 |
| | | | $3_{2021}$ | 0.53 | 5.13 | $0.0002_1$ | 0.96 | 3 |
| | | | $4_{2021}$ | 1.23 | 6.89 | $8.75_5$ | 0.12 | 7 |
| | | Broadwater | $4_{2021}$ | 0.09 | 5.2 | $1.03_1$ | 0.31 | 3 |
| | | Madison | $1_{2021}$ | 0.32 | 7.3 | $1.58_4$ | 0.31 | 6 |
| | | | $2_{2021}$ | 0.28 | 6.52 | $4.79_4$ | 0.31 | 6 |
| | OR | Umatilla | $3_{2021}$ | 0.32 | 7.35 | $3.80_5$ | 0.58 | 7 |
| | | | $4_{2021}$ | >10.00 | - | - | - | 7 |
| | WA | Yakima | $1_{2021}$ | >10.00 | 4.3 | $6.23_5$ | 0.27 | 7 |
| | WY | Platte | $1_{2021}$ | 0.48 | 6.94 | $0.35_2$ | 0.84 | 4 |
| Permethrin | AZ | La Paz | $3_{2021}$ | 12.92 | 10.36 | $10.14_5$ | 0.07 | 7 |
| | | | $5_{2021}$ | 13.29 | 7.51 | $4.66_3$ | 0.20 | 5 |
| | | Pinal | $1_{2021}$ | 0.74 | 6.81 | $3.29_4$ | 0.51 | 6 |
| | | Yuma | $2_{2021}$ | 13.33 | 6.55 | $8.92_4$ | 0.06 | 6 |
| | CA | Riverside | $1_{2021}$ | 6.76 | 2.75 | $9.38_4$ | 0.052 | 6 |
| | MT | Big Horn | $1_{2020}$ | 1.18 | 4.96 | $4.96_2$ | 0.08 | 4 |
| | | | $1_{2020}$ | 4.74 | 2.82 | $0.004_1$ | 0.95 | 3 |
| | | | $1_{2020}$ | 1.54 | 7.9 | $3.22_5$ | 0.67 | 7 |
| | | | $1_{2021}$ | 6.44 | 4.49 | $5.10_3$ | 0.16 | 5 |
| | | | $2_{2020}$ | 7.05 | 5.56 | $3.90_4$ | 0.42 | 6 |
| | | | $2_{2021}$ | 3.55 | 8.55 | $5.61_4$ | 0.23 | 6 |
| | | | $3_{2021}$ | 2.81 | 5.59 | $0.19_1$ | 0.67 | 3 |
| | | | $4_{2021}$ | 3.83 | 5.11 | $0.62_2$ | 0.73 | 4 |
| | | Broadwater | $1_{2020}$ | 1.28 | 6.12 | $5.19_3$ | 0.16 | 5 |
| | | | $3_{2021}$ | 2.67 | 6.63 | $8.90_4$ | 0.06 | 6 |
| | | | $4_{2021}$ | 0.47 | 5.80 | $0.26_1$ | 0.61 | 3 |
| | | Gallatin | $2_{2020}$ | 19.36 | 5.10 | $8.64_4$ | 0.07 | 6 |
| | | Madison | $1_{2020}$ | 0.17 | 7.5 | $5.45_3$ | 0.14 | 5 |
| | | | $2_{2021}$ | 2.08 | 7.23 | $3.12_4$ | 0.54 | 6 |
| | | Powder River | $1_{2020}$ | 0.55 | 5.81 | $8.06_5$ | 0.15 | 7 |
| | | | $2_{2020}$ | 0.10 | 7.36 | $6.58_4$ | 0.24 | 6 |
| | | | $3_{2020}$ | 0.19 | 5.85 | $5.45_3$ | 0.14 | 5 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | OR | Umatilla | $1_{2021}$ | 1.73 | 6.24 | $8.15_{4}$ | 0.09 | 6 |
| | | | $3_{2021}$ | 1.98 | 6.52 | $5.55_{3}$ | 0.14 | 5 |
| | WA | Yakima | $1_{2021}$ | 7.18 | 5.27 | $7.55_{3}$ | 0.06 | 5 |
| | | | $2_{2021}$ | 2.11 | 8.42 | $8.83_{4}$ | 0.07 | 6 |
| | WY | Converse | $1_{2021}$ | 2.70 | 8.47 | $4.00_{4}$ | 0.41 | 6 |
| | | | $2_{2021}$ | 2.17 | 6.94 | $7.89_{3}$ | 0.05 | 5 |
| | | Platte | $1_{2021}$ | 1.04 | 5.53 | $0.05_{1}$ | 0.82 | 3 |
| | | Sheridan | $1_{2020}$ | 0.70 | 5.84 | $5.22_{2}$ | 0.07 | 4 |
| | | | $2_{2020}$ | 5.54 | 5.27 | $3.40_{5}$ | 0.64 | 7 |

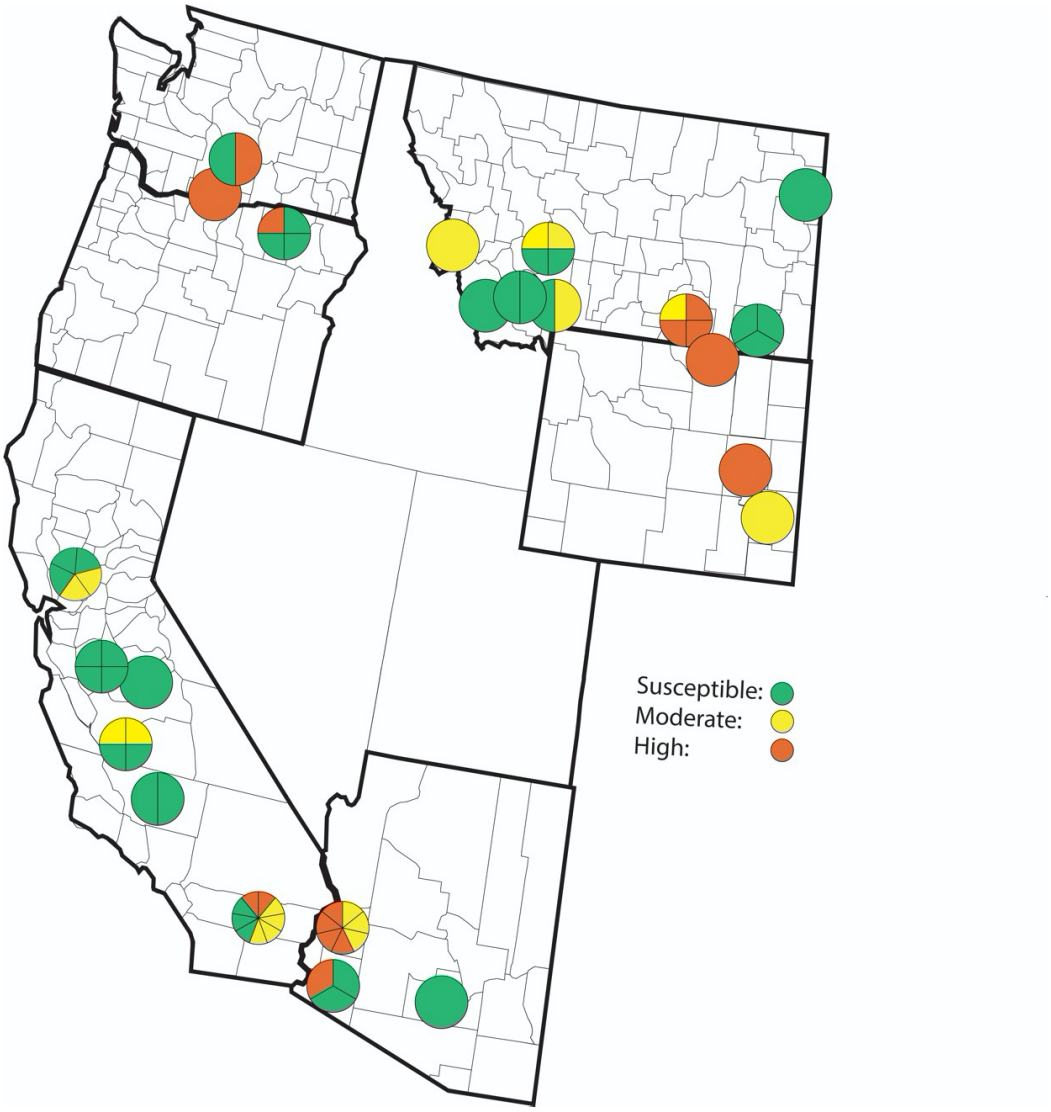



**Figure 1.** Map of lambda-cyhalothrin resistance level categories. Pie charts represent location samples within in a county (e.g., Big Horn County MT) or region (e.g., northern California). Each slice of the pie chart represents a location sample's lethal concentrations resulting in 50% mortality ($LC_{50}$) value. Magenta represents highly resistant location sample $LC_{50}$ values greater than > 1.0µg/cm$^2$, yellow markers represent moderately resistant location samples ($LC_{50}$ value 0.30 – 1.0 µg/cm$^2$), and green markers represent susceptible location samples ($LC_{50}$ value <0.30 µg/cm$^2$). For context, the label rate for lambda-cyhalothrin is 0.22-0.34 µg/cm$^2$.

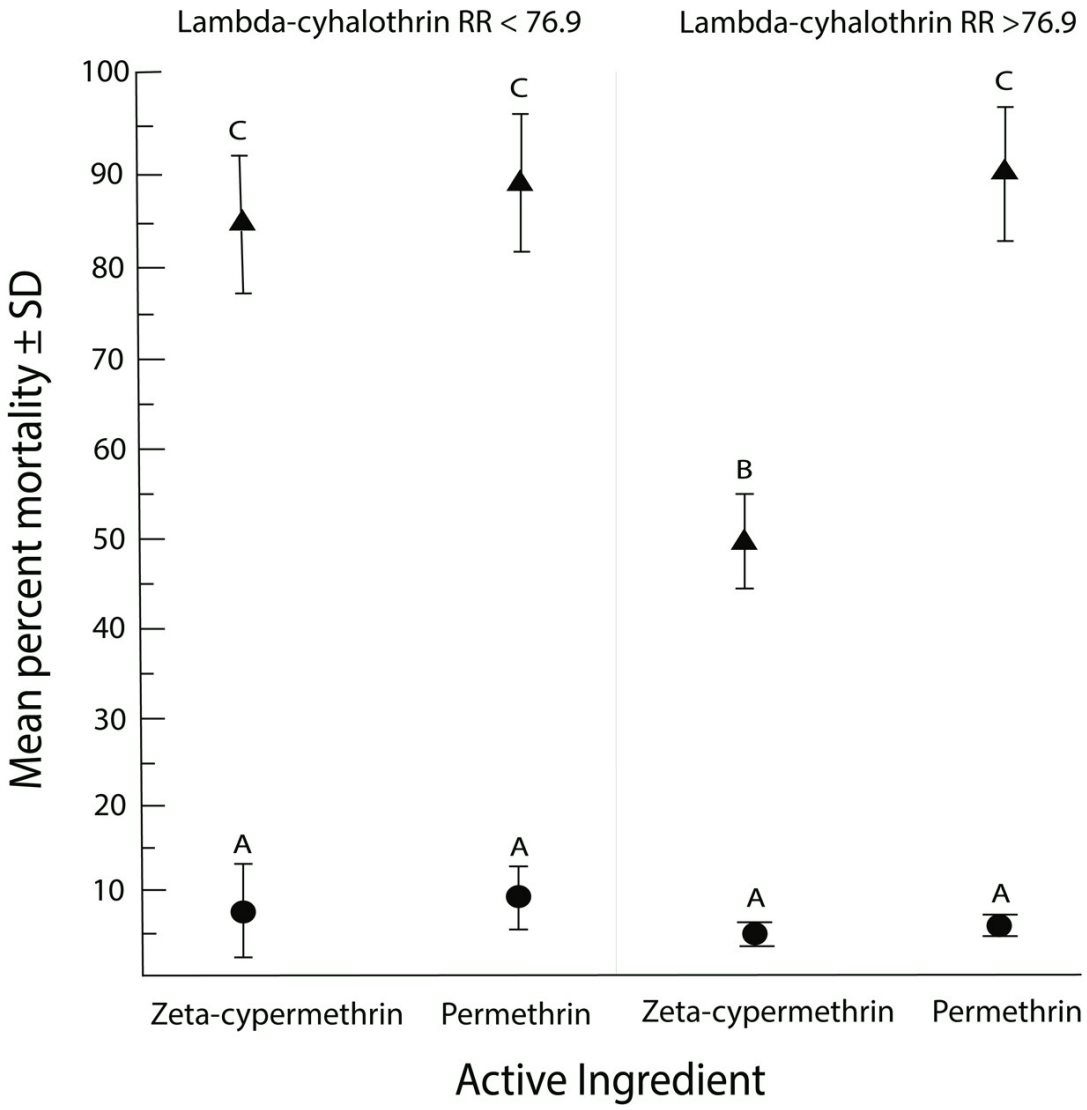
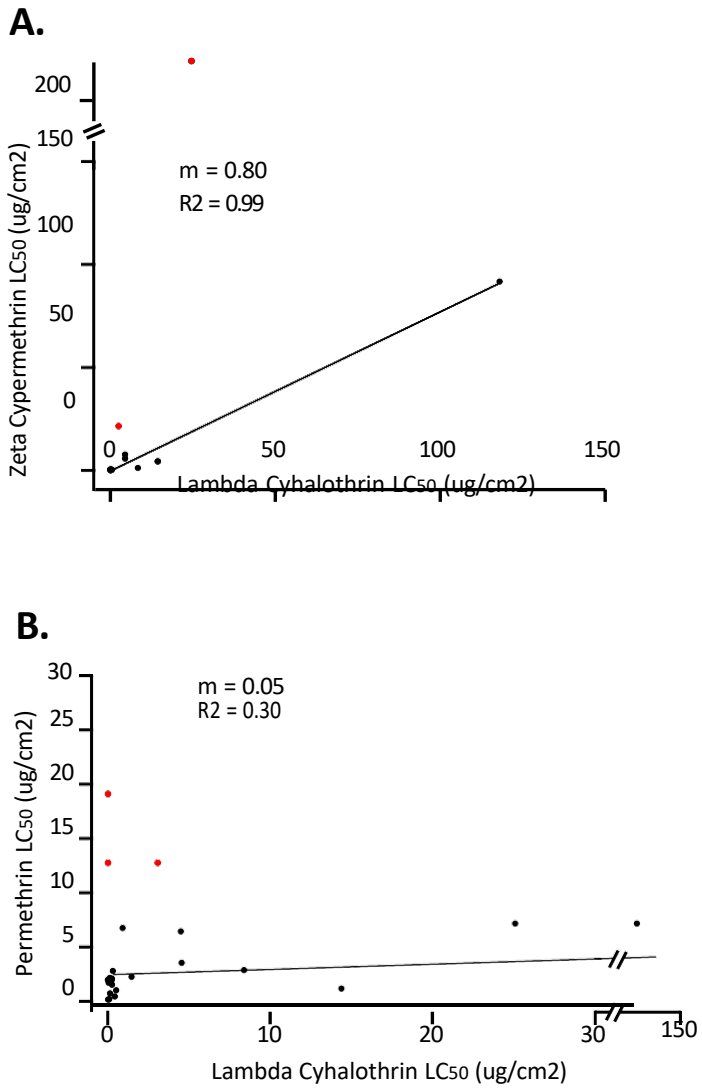



**Figure 2.** Mean percent larval mortality exposed to diagnostic concentrations of zeta-cypermethrin (1.0 μg/cm$^2$) and permethrin (3.3 μg/cm$^2$), between location samples grouped based on lambda-cyhalothrin resistance ratios (RR) <76.9 and >76.9. Triangles represent the diagnostic concentration and circles represent the acetone control groups. Different letters signify significant differences ($P < 0.05$) between treatments ($F = 49.67$; df = 5, 24; $P < 0.001$).




**Supplemental Figure S1.** Correlation between lambda-cyhalothrin lethal concentration causing 50% mortality ($LC_{50}$) values and permethrin (A) or zeta-cypermethrin (B) $LC_{50}$ values in µg/cm$^2$. $R^2$ values close to 1.0 are tightly correlated. Red dots illustrate $LC_{50}$ values that were removed as they were outliers of the limited dataset.

**Supplemental Table S1.** Active ingredient (AI) by state, county, and field site that had one outlier concentration (conc.) removed from the dataset. This table lists the original lethal concentration that causes 50% mortality ($LC_{50}$) and chi-square ($X^2$) value, as well as the $LC_{50}$ value and $X^2$ value produced with the outlier removed.

| AI | Year | State | County | Field Site | Original $LC_{50}$ | Original $X^2$ | Corrected $LC_{50}$ | Corrected $X^2$ | Conc. Removed |
|---|---|---|---|---|---|---|---|---|---|
| Lambda-cyhalothrin | 2020 | CA | Kings | $1_{2020}$ | 0.255 | 21.81 | 0.34 | 6.78 | 5 |
| | | | | $2_{2020}$ | 0.59 | 19.33 | 0.44 | 7.22 | 7 |
| | | | Riverside | $4_{2020}$ | 0.0 | 15.32 | 0.12 | 1.19 | 1 |
| | | | | $6_{2020}$ | 0.29 | 7.662 | 0.63 | 0.25 | 5 |
| | | | Madera | $1_{2020}$ | 0.00 | 37.21 | 0.05 | 8.11 | 2 |
| | | | Merced | $2_{2020}$ | 0.038 | 16.25 | 0.042 | 5.7 | 5 |
| | | MT | Big Horn | $2_{2020}$ | 117.85 | 19.66 | >1.0 | 3.09 | 4 |
| | | | Broadwater | $3_{2020}$ | 0.34 | 28.07 | 0.77 | 2.68 | 6 |
| | | | Powder River | $1_{2020}$ | 0.03 | 12.337 | 0.03 | 2.73 | 6 |
| | | | | $3_{2020}$ | 0.09 | 12.74 | 0.09 | 6.58 | 5 |
| | 2021 | AZ | La Paz | $4_{2021}$ | 0.91 | 14.75 | 0.53 | 0.04 | 4 |
| | | | Pinal | $1_{2021}$ | 0.13 | 11.98 | 0.15 | 6.08 | 5 |
| | | CA | Merced | $1_{2021}$ | 0.0 | 13.5 | 0.17 | 3.99 | 2 |
| | | | Riverside | $3_{2021}$ | 0.04 | 16.25 | 0.04 | 5.7 | 5 |
| | | | | $5_{2021}$ | 0.00 | 17.93 | 0.02 | 3.09 | 4 |
| | | | Yolo | $1_{2021}$ | 0.0 | 13.1 | 0.078 | 5.86 | 2 |
| | | | | $2_{2021}$ | 0.022 | 13.85 | 0.02 | 5.75 | 4 |
| | | MT | Big Horn | $1_{2021}$ | - | - | 4.5 | 4.63 | 1 |
| | | | Madison | $2_{2021}$ | 0.48 | 34.42 | 0.26 | 3.67 | 6 |
| | | OR | Umatilla | $1_{2021}$ | 0.1 | 13.76 | 0.06 | 5.89 | 4 |
| | | | | $2_{2021}$ | 0.06 | 36.24 | 0.17 | 7.9 | 2 |
| | | WY | Converse | $1_{2021}$ | 1.47 | 16.89 | 1.84 | 8.94 | 4 |
| Zeta-cypermethrin | 2021 | AZ | La Paz | $3_{2021}$ | 14.69 | 51.99 | 21.85 | 1.03 | 4 |
| | | | | $5_{2021}$ | 5.93 | 27.09 | 4.12 | 1.63 | 1 |
| | | MT | Madison | $1_{2021}$ | 0.20 | 18.75 | 0.32 | 1.58 | 2 |
| | | OR | Umatilla | $4_{2021}$ | 0.44 | 16.98 | 0.60 | 11.97 | 5 |
| Permethrin | 2020 | MT | Broadwater | $1_{2020}$ | 1.54 | 8.4 | 1.28 | 5.19 | 1 |
| | | | Powder River | $2_{2020}$ | 1.21 | 15.85 | 0.1 | 6.56 | 6 |
| | 2021 | AZ | La Paz | $5_{2021}$ | 17.4 | 17.07 | 13.29 | 4.66 | 5 |
| | | CA | Riverside | $1_{2021}$ | 6.65 | 11.65 | 6.76 | 9.38 | 3 |
| | | OR | Umatilla | $3_{2021}$ | 2.4 | 12.71 | 1.98 | 5.55 | 2 |
| | | WA | Yakima | $1_{2021}$ | 5.7 | 18.09 | 7.18 | 7.551 | 3 |

**Supplemental Table S2.** Permethrin (type I pyrethroid) and zeta-cypermethrin (type II pyrethroid) $LC_{50}$ values grouped based on the associated lambda-cyhalothrin resistance ratios (RR). To assess general patterns of cross-resistance between lambda-cyhalothrin and permethrin/zeta-cypermethrin, the location samples were divided into two groups based on the lambda-cyhalothrin resistance ratio (RR), <76.9 and >76.9, to simplify the analysis. RR <76.9 includes the susceptible (S) and moderate (M) resistance levels defined in Table 1 while RR >100 corresponds to the high resistance (H) group.

| County- State $_{\text{Field site}}$ | Lambda-cyhalothrin RR | Zeta-cypermethrin $LC_{50}$ | Permethrin $LC_{50}$ |
|---|---|---|---|
| Umatilla-$OR_3$ | 1.00 | 3.56 | 19.80 |
| Kern-$CA_2$ | 1.00 | - | - |
| Kings-$CA_3$ | 1.00 | - | - |
| Yolo-$CA_4$ | 1.00 | - | - |
| Riverside- $CA_5$ | 1.00 | - | - |
| Madison-$MT_{1\text{-}2020}$ | 1.00 | - | 1.70 |
| Kings-$CA_4$ | 1.00 | - | - |
| Kern-$CA_1$ | 1.00 | - | - |
| Merced-$CA_4$ | 1.00 | - | - |
| Powder River-$MT_1$ | 2.00 | - | 5.50 |
| Merced-$CA_2$ | 3.00 | - | - |
| Merced-$CA_3$ | 3.00 | - | - |
| Madera-$CA_1$ | 4.00 | - | - |
| Beaverhead-$MT_1$ | 4.00 | - | - |
| Umatilla-$OR_1$ | 5.00 | - | 17.30 |
| Madison-$MT_{2\text{-}2020}$ | 5.00 | - | - |
| Yolo-$CA_2$ | 6.00 | - | - |
| Powder River-$MT_3$ | 7.00 | - | 1.90 |
| Richland-$MT_1$ | 7.00 | - | - |
| Powder River-$MT_2$ | 8.00 | - | 1.00 |
| Yuma-$AZ_2$ | 9.00 | - | 133.30 |
| Riverside-$CA_4$ | 9.00 | - | - |
| Yolo-$CA_3$ | 10.00 | - | - |
| Pinal-$AZ_1$ | 12.00 | 1.11 | 7.40 |
| Gallatin-$MT_1$ | 12.00 | - | - |
| Umatilla-$OR_2$ | 13.00 | - | - |
| Merced-$CA_1$ | 13.00 | - | - |
| Yuma-$AZ_1$ | 14.00 | - | - |
| Yakima-$WA_2$ | 16.00 | - | 21.10 |
| Riverside-$CA_7$ | 17.00 | | |
| Broadwater-$MT_1$ | 20.00 | - | 12.80 |
| Madison-$MT_{2\text{-}2021}$ | 20.00 | 3.11 | 20.80 |
| Broadwater-$MT_2$ | 20.00 | - | - |

| | | | |
|---|---|---|---|
| Riverside-$CA_{1-2020}$ | 20.00 | - | - |
| Yolo-$CA_1$ | 23.00 | - | - |
| Gallatin-$MT_2$ | 24.00 | - | 193.60 |
| Big Horn-$MT_{3-2021}$ | 25.00 | 5.89 | 28.10 |
| Kings-$CA_1$ | 26.00 | - | - |
| Ravalli-$MT_1$ | 32.00 | - | - |
| Broadwater-$MT_4$ | 33.00 | 1.00 | 4.70 |
| Kings-$CA_2$ | 34.00 | - | - |
| Yolo-$CA_5$ | 39.00 | - | - |
| Riverside-$CA_3$ | 40.00 | - | - |
| Platte-$WY_1$ | 40.00 | 5.33 | 10.40 |
| La Paz-$AZ_4$ | 41.00 | - | - |
| Riverside-$CA_2$ | 41.00 | - | - |
| Big Horn-$MT_{3-2021}$ | 44.00 | - | - |
| Riverside-$CA_6$ | 48.00 | - | - |
| La Paz- $AZ_6$ | 49 | - | - |
| La Paz-$AZ_2$ | 50.00 | - | - |
| Broadwater-$MT_3$ | 59.00 | - | 26.70 |
| Riverside-$CA_{1-2021}$ | 71.00 | - | - |
| Big Horn-$MT_{2-2020}$ | >77.00 | >37 | 70.50 |
| Riverside-$CA_1$ | 92.00 | - | 67.60 |
| Yuma-$AZ_3$ | 104.00 | - | - |
| La Paz-$AZ_1$ | 119.00 | - | - |
| Big Horn-$MT_{2-2021}$ | 137.00 | 64.67 | 35.50 |
| Riverside-$CA_8$ | 140.00 | - | - |
| Converse-$WY_1$ | 142.00 | - | 27.00 |
| Sheridan-$WY_1$ | 172.00 | - | 7.00 |
| La Paz-$AZ_3$ | 214.00 | >111.11 | 129.20 |
| Riverside- $CA_9$ | >256 | - | - |
| Big Horn-$MT_{1-2020}$ | >256.00 | >37 | 11.80 |
| Big Horn-$MT_{2-2020}$ | >256.00 | - | - |
| Klickitat-$WA_1$ | >256.00 | - | - |
| Big Horn-$MT_{1-2021}$ | 346.00 | 86.11 | 64.40 |
| La Paz-$AZ_8$ | 360 | - | - |
| Converse-$WY_1$ | 532.00 | - | - |
| Big Horn- $MT_4$ | 646 | 1.23 | 3.83 |
| Yakima-$WA_1$ | >770.00 | >111.11 | 71.80 |
| Umatilla-$OR_4$ | >770.00 | >111.11 | - |
| La Paz-$AZ_7$ | >770 | - | - |
| Big Horn-$MT_{1-2020}$ | - | - | 15.40 |
| Converse-$WY_2$ | - | - | 21.70 |
| Big Horn-$MT_{1-2020}$ | - | - | 47.40 |
| Sheridan-$WY_2$ | - | - | 55.40 |
| La Paz-$AZ_5$ | - | 45.78 | 132.90 |
| Madison-$MT_{1-2021}$ | - | 3.55 | - |

LAMBDA-CYHALOTHRIN RESISTANT ALFALFA WEEVILS IN THE WESTERN UNITED STATES ARE RESISTANT TO MULTIPLE TYPE II PYRETHROID INSECTICIDES.

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# LAMBDA-CYHALOTHRIN RESISTANT ALFALFA WEEVILS ([COLEOPTERA]: [CURCULIONIDAE]) IN THE WESTERN UNITED STATES ARE RESISTANT TO MULTIPLE TYPE II PYRETHROID INSECTICIDES.

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**Abstract**

The annual cost of insecticide resistance was $1 billion in 2002. Currently insecticide resistance threatens forage alfalfa (*Medicago sativa* L. [Fabales: Fabaceae]) production in the western United States, as alfalfa weevil (*Hypera postica* Gyllenhal [Coleoptera: Curculionidae]) has developed resistance lambda-cyhalothrin and zeta-cypermethrin (type II pyrethroids) in the region. 109 laboratory concentration- response assays were conducted with type I pyrethroids: bifenthrin and permethrin; type II pyrethroids: alpha-cypermethrin, beta-cyfluthrin, lambda-cyhalothrin, zeta-cypermethrin; and Indoxacarb (mode of action 22A). Alfalfa weevils exposed to these active ingredients were collected in Arizona, California, Montanan, Oregon, Washington, and Wyoming. Field trials in Arizona, Montana, and Washington were conducted to corroborate laboratory bioassays. In all, laboratory bioassays illustrated a significant positive correlation indicating multiple resistance among type II pyrethroid active ingredients and limited multiple resistance between pyrethroid types. All field trials corroborated this pattern of multiple resistance. With resistance to lambda-cyhalothrin, other type II pyrethroids and permethrin failed to control these populations, while bifenthrin and indoxacarb were highly efficacious. Alfalfa weevil genomic DNA was collected from the field trials to identify if alfalfa weevil strain influenced patterns of resistance. Our data indicates that regardless of alfalfa weevil strain, type II pyrethroids and permethrin fail to control lambda-cyhalothrin resistant location samples. This pattern of multiple resistance challenges alfalfa production in the western United States, however, integrated resistance management and integrated pest management strategies can be utilized to slow further development of resistance in the region.

## Introduction

Insecticides are a critical component of integrated pest management (IPM), and insecticide resistance challenges IPM strategies (Pedigo et al. 2021, IRAC 2023). In 2002, the annual costs of insecticide resistance in the United States (US) was estimated at $1 billion (Clark and Yamaguchi 2002). Insecticide resistance poses a risk to forage alfalfa (*Medicago sativa* L. (Fabales: Fabaceae)) production in the US, with evidence of alfalfa weevil (*Hypera postica* Gyllenhal (Coleoptera: Curculionidae)) resistance to pyrethroid active ingredients (mode of action group (MoA) 3A) in the western US (Rodbell and Wanner 2021, Wanner et al. 2022, Rodbell et al. 2022).

The alfalfa weevil, an invasive insect, is an economically damaging pest of alfalfa in North America (Peterson et al. 1992, Pellissier 2017, Rankin 2020, Levi-Mourao et al. 2022). Larval feeding causes economic damage through defoliation, reducing forage yield, quality, and value (Onstad and Shoemaker 1984, Hutchins et al. 1990). Alfalfa weevil, native to Eurasia, was detected in the US on three occasions, with each introduction recognized as an individual strain. The first introduction occurred in Salt Lake City Utah in 1904 (western strain), the second in Yuma Arizona in 1939 (Egyptian strain), and the third in Maryland in 1952 (eastern strain) (Titus 1910, Wehrle 1940, Poos and Bissell 1953, Hsiao 1993, Bundy et al. 2005). Today, the Egyptian strain is in the southwestern US, the western strain is distributed in central California and across the northwestern US, and the eastern strain is distributed across the central and eastern regions of the US (Böttger et al. 2013).

Since the establishment of alfalfa weevils in the US, IPM tools were developed to manage its damage. Soon after its invasion, classical biological control was implemented by the

United States Department of Agriculture Agricultural Research Service (USDA-ARS) along with the Animal and Plant Health Inspection Service (APHIS), through the introduction of parasitoid wasps (Bryan et al. 1993). These agencies introduced eight parasitoid wasp species in the western region of the US, the most successful being ichneumonid wasps: *Bathyplectes curculionis* (Thomson), *B. anurus* (Thomson), and *B. stenostigma* (Thomson) (Bryan et al. 1993, Pellissier et al. 2017). Cultural IPM tactics are limited to grazing and maintaining a healthy alfalfa stand, while mechanical control tactics are limited to harvesting early to salvage yield (Summers 1998, Pellissier et al. 2017, Pedigo et al. 2021). In the 1950s, insecticides became the primary method to reduce alfalfa weevil damage, and by 1961 the routine application of organochlorine insecticides led to resistance to heptachlor and dieldrin (Bishop 1964, Pedigo et al. 2021).

Due to the loss of heptachlor and dieldrin, pyrethroids (MoA 3A) became the predominant insecticide used to control alfalfa weevil populations (Vardiman et al. 2022). Pyrethroids are divided into two pyrethroid types based on their chemical structure. Type I pyrethroids lack an α-cyano group whereas type II pyrethroids have an α-cyano group (Yu 2015). However, after decades of routine pyrethroid use, alfalfa weevils in the western US have evolved resistance to the type II pyrethroids, lambda-cyhalothrin and zeta-cypermethrin (Rodbell et al. 2022).

Currently, there are limited alternative MoA groups available for alfalfa weevil control in forage alfalfa systems, resulting in an increased reliance on indoxacarb (MoA 22A), in areas where pyrethroid resistance has established (Rodbell et al. 2022, Wanner et al. 2022, DPR-PUR 2023). Furthermore, with limited management options, the loss of pyrethroids poses a challenge

to alfalfa production. Pyrethroids represent 6 of the 13 active ingredients registered for alfalfa weevil management in forage alfalfa systems (Vardiman et al. 2022). A seventh pyrethroid, bifenthrin (type I), is registered in the US for alfalfa seed production (Hanson 2022).

Insecticide resistance management (IRM) recommendations for alfalfa weevils are needed to address this agricultural challenge. A critical step in developing an IRM strategy is to determine if resistance to lambda-cyhalothrin includes multiple resistance to all available pyrethroid active ingredients. Across three distinct alfalfa production areas of the western US, we aimed to 1) Test for resistance to multiple pyrethroid active ingredients using field collected alfalfa weevil larvae in laboratory contact bioassays; 2) Corroborate laboratory results using commercially formulated pyrethroid insecticides applied at field sites with quantified resistance to lambda-cyhalothrin; 3) Determine the alfalfa weevil strain(s) present at each field trial location.

**Materials and Methods**

Cross-resistance occurs when a single resistance mechanism causes resistance to multiple active ingredients. Multiple resistance, however, is when a population is resistant to multiple insecticides derived from multiple mechanisms of resistance (Yu 2015). As the mechanism(s) of resistance have not been identified, we will conservatively label populations presenting with resistance to multiple active ingredients as multiple resistance.

To assess multiple resistance, alfalfa weevil larvae were collected from six western states and larvae from each collection were assayed in the laboratory using six pyrethroid active ingredients. Indoxacarb was also tested when populations were large enough. These assays were used to generate estimates of the lethal concentrations that yield 50% mortality ($LC_{50}$). At three

geographically distinct sites where $LC_{50}$ values were estimated in the lab, the effectiveness of the corresponding commercial formulations of each active ingredient were also tested in field trials. The strain(s) inhabiting each of the three field trial sites were determined to help interpret potential patterns of resistance to the different active ingredients.

*Laboratory bioassay: insect collections*

To collect weevils for laboratory bioassays, we sampled with sweep nets (BioQuip, Rancho Dominguez, CA) in six western states (i.e., Arizona, California, Montana, Oregon, Washington, and Wyoming) from 3 June to 26 June 2021 and 14 February to 21 June 2022, covering 19 field locations. Field sampling was partially focused on fields with informed management history from crop advisor, extension agent, and/or producer reports.

Once collected, alfalfa weevils were placed in a 19-liter bucket with fresh alfalfa collected from the same field and covered with no-see-um number 20 mesh (Quest Outfitters, Sarasota, FL). In the lab, additional alfalfa (grown in Montana State University's greenhouse) was added to the buckets these were maintained 24-48 hours at 21°C.

*Laboratory bioassay: active ingredients*

All insecticides used for laboratory bioassays were technical grade material. Type I pyrethroid active ingredients were: bifenthrin and permethrin; Type II pyrethroid active ingredients included: alpha-cypermethrin, beta-cyfluthrin, lambda-cyhalothrin, and zeta-cypermethrin. When populations were large enough, indoxacarb bioassays, MoA 22A, were also conducted.

Bifenthrin (2-methyl-3-phenylphenyl)methyl (1*R*,3*R*)-3-[(*Z*)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate) was purchased from Sigma-Aldrich (St. Louis, MO), batch number BCBX0667, 98% purity. Permethrin (3-phenoxybenzyl

(1RS,3RS;1RS,3SR)-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate) was purchased from Sigma-Aldrich (St. Louis, MO), batch number 45614, 92% purity. Alpha-cypermethrin ([cyano-(3-phenoxyphenyl)methyl] 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) was purchased from Sigma-Aldrich (St. Louis, MO), batch number BCBT3763, 98% purity. Beta-cyfluthrin ([(*R*)-cyano-(4-fluoro-3-phenoxyphenyl)methyl] (1*S*)-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate), was purchased from Sigma-Aldrich (St. Louis, MO), batch number BCBX0283, 98% purity. Lambda-cyhalothrin (alpha-cyano-3-phenoxybenzyl (Z)-(1RS)-*cis* 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2- dimethylcyclopropanecarboxylate) was provided by Syngenta (Basel, Switzerland), batch number 1071218, 89% purity. Zeta-cypermethrin ([Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) was provided by FMC Corporation (Newark, NJ), batch number 122279866, 92% purity. Indoxacarb, (methyl (4*aS*)-7-chloro-2-[methoxycarbonyl-[4-(trifluoromethoxy)phenyl]carbamoyl]-3,5-dihydroindeno[1,2-e][1,3,4]oxadiazine-4*a*-carboxylate) purchased from FMC Corporation (Newark, NJ), 98**%** purity.

*Laboratory bioassay: concentration response bioassays*

Detailed methodologies are outlined in Rodbell and Wanner (2021) and Rodbell et al. (2022). Following these methods, stock solution for each of the active ingredients were prepared with 95% acetone (acetone and deionized water). Serial dilutions from the stock solution were generated with 95% acetone to provide seven concentrations.

Glass vials (Discount Vials, Madison, WI) were treated with 1 mL of an insecticide concentration and diluent 95% acetone was evaporated without heat on a commercial food roller

(WebstaurantStore, Lititz, PA) at room temperature (21°C) (Brindley 1975). Resulting in insecticide concentrations of 0.0033-10.0 µg/cm$^2$ for type II pyrethroids (e.g., lambda-cyhalothrin), 0.033-100.0 µg/cm$^2$ for type I pyrethroids (e.g., bifenthrin), and 0.0001-0.1 µg/cm$^2$ for indoxacarb (MoA 22A). Once treated, glass vials were stored in a refrigerator (4°C) and used within a two-week period. Each bioassay typically included seven concentrations and a 95% acetone control (n = 5 replicated vials, 10 larvae per vial). After 24-hours in the dark at room temperature, the larvae were scored as dead or alive after exposure on a heated hot plate (43-50 °C) (Rodbell and Wanner 2021, Rodbell et al. 2022).

Probit analysis was used to quantify the lethal concentration of an active ingredient that generated 50% mortality ($LC_{50}$) using Probit Or LOgit analysis (POLO) software (LeOra Software, Parma, MO) (Robertson et al. 2017). Bioassays with control mortality >20%, t-ratio of the slope less than 1.96, and/or chi-square ($\chi^2$) goodness of fit test $p < 0.05$ were omitted (Robertson et al. 2017). If mortality was observed in the control group, correction for natural response was estimated by the POLO probit analysis (Robertson et al. 1980). Data that did not fit the probit model and $\chi^2$ goodness of fit test were omitted unless a single outlier was identified as the cause for the lack of fit. Outliers were identified through the graphical visualization of the residual values (Sarkar et al. 2011, Robertson et al. 2017). Single outliers were removed from one alpha-cypermethrin, four lambda-cyhalothrin, one zeta-cypermethrin, three bifenthrin, four permethrin, and two indoxacarb bioassays (Supp. Table S1). Larval mortality from some highly resistant sites failed to reach 50% and did not fit the requirements of probit analysis, these $LC_{50}$ values was conservatively listed as greater than (>) the highest concentration tested (Brindley 1975, Haddi et al. 2018, Rodbell and Wanner 2021, Rodbell et al. 2022).

*Assessing multiple resistance between active ingredients*

To assess multiple resistance between pyrethroid active ingredients, larvae from the same field (23 field sites total) were assayed with multiple active ingredients that generated $LC_{50}$ values. Five field sites in Arizona, California, Montana, and Washington were assayed with type I pyrethroids: bifenthrin and permethrin; Type II pyrethroids: alpha-cypermethrin, beta-cyfluthrin, lambda-cyhalothrin, and zeta-cypermethrin; and indoxacarb (MoA 22A). Two field sites in Montana were assayed with type I pyrethroids: bifenthrin and permethrin; and Type II pyrethroids: alpha-cypermethrin, beta-cyfluthrin, lambda-cyhalothrin, zeta-cypermethrin. The remaining 16 field sites were assayed and generated $LC_{50}$ values for at least three pyrethroid active ingredients, of both pyrethroid types. Based on the $LC_{50}$ value, a location sample was conservatively labeled as susceptible, moderate, or highly resistant (Table 1).

All resistance ratios for type I pyrethroids: bifenthrin and permethrin; type II pyrethroids: alpha-cypermethrin, beta-cyfluthrin, and zeta-cypermethrin; and indoxacarb (MoA 22A), were calculated using the most susceptible $LC_{50}$ value for each active ingredient (Tabashnik et al. 2011). However, lambda-cyhalothrin resistance ratios were calculated using the average $LC_{50}$ value of the nine most susceptible locations identified by Rodbell et al. (2022), a denominator of 0.013 $\mu g/cm^2$.

To assess multiple resistance with lambda-cyhalothrin, we tested for a correlation between the $LC_{50}$ values of each active ingredient and lambda-cyhalothrin (Vassiliou et al. 2010). A Pearson Product Moment Correlation two-tailed test, conducted with GraphPad Prism Statistical Software (San Diego, CA), was used to determine significance in the correlation between two active ingredients. If multiple resistance exists between active ingredients, one can

expect to see a positive slope of the line of best-fit, a high $R^2$ value, and a p-value less than 0.05 (Smirle et al. 2002, Vassiliou et al. 2010, Min et al. 2014).

To further explore the degree of multiple resistance among pyrethroid active ingredients, we divided fields into two conservative categories defined by their lambda-cyhalothrin resistance ratios determined by Rodbell et al. (2022). If multiple resistance exists between pyrethroid active ingredients, the response to these active ingredients at the diagnostic concentration (i.e., percentage mortality), should reflect the lambda-cyhalothrin resistance category (Menger et al. 2020, Rodbell et al. 2022). Fields with resistance ratios <76.9 were considered susceptible or moderately resistant, and fields with resistance ratios >76.9 were considered highly resistant (Rodbell et al. 2022). An acetone control group and a diagnostic concentration of 3.3 $\mu g/cm^2$ was used for each active ingredient tested. Percentage mortality was log-transformed to meet the normality assumptions of ANOVA performed in RStudio statistical software version 1.3.1093 (RStudio Team, Boston MA). The Tukey HSD test ($P = 0.05$) was used to identify significant differences among treatments.

*Field trials*

In 2022, three field trials were conducted in Arizona (La Paz County), Montana (Big Horn County), and Washington (Yakima County). Each field trial was organized in a randomized complete block design containing five blocks (n = 5; 3.05 m by 9.14 m). Treatments consisted of six active ingredients applied at their maximum label rates: 1) Beta-cyfluthrin (Baythroid XL®, Bayer, Leverkusen Germany) at a rate of 0.20 L/ha (2.8 oz/ac); 2) Lambda-cyhalothrin (Warrior II®, Syngenta, Greensboro, NC) at a rate of 0.14 L/ha (1.92 oz/ac); 3) Zeta-cypermethrin (Mustang Maxx®, FMC, Philadelphia, PA) at a rate of 0.29 L/ha (4.0 oz/ac); 4)

Bifenthrin (Brigade®, FMC Corporation, Philadelphia, PA) at a rate of 0.47 L/ha (6.4 oz/ac); 5) Permethrin (Permethrin, Loveland Products, Inc., Loveland, CO) at a rate of 0.58 L/ha (8.0 oz/ac); 6) Indoxacarb (Steward EC®, FMC, Philadelphia, PA) at a rate of 0.83 L/ha (11.3 oz/ac) with Preference® (non-ionic surfactant plus antifoaming agent) at 0.95 liters per 378.54 liters of indoxacarb (2 pints per 100 gallons of indoxacarb); and 7) Untreated control. All insecticides were applied using a standard backpack sprayer (Chapin 63924®: 4-gallon 24v Rechargeable Battery Powered Backpack Sprayer; Chapin International Inc. Batavia, NY).

Field-trial evaluations occurred six days after insecticide application for La Paz County Arizona and Yakima County Washington. The Big Horn County Montana field trial evaluation occurred seven days after insecticide application. To evaluate the efficacy of each treatment ten 180° sweeps were taken with a standard 38.1 cm diameter sweep net (BioQuip, Rancho Dominguez, CA) from each plot. All samples collected from every plot and field trial were frozen for transport back to the research lab located at Montana State University. In the lab, we evaluated the 10-sweep samples by counting the number of alfalfa weevil larvae and adults.

*Field trial statistical analysis*

Alfalfa weevil counts for each field trial were analyzed separately in RStudio statistical software version 1.3.1093 (RStudio Team, Boston MA). Data were log-transformed to meet normality assumptions, and Tukey's HSD test ($P = 0.05$) was used to further identify significant differences between treatments.

*Identifying alfalfa weevil strain*

Larvae from each of the three field trial sites were preserved in 95% ethanol and stored at -20°C. Six individual larvae were randomly pulled from each vial, rinsed individually with 100% ethanol, and the genomic DNA extracted from each using a Qiagen DNeasy kit (Qiagen,

Germantown, MD). Polymerase chain reaction (PCR) methods described by Erney et al. (1996) and Kuwata et al. (2005) were used. Forward and reverse PCR primers C1-J-2797 and C2-N-3686 were used to amplify a 575 bp product of the mitochondrial COI/COII gene that was subsequently digested using the AluI restriction enzyme (Qiagen, Germantown, MD) (Kuwata et al. 2005). After electrophoresis on a 1.2% agarose gel, the western strain can be distinguished from the Egyptian/Eastern strains based on the number and size of the resulting restriction enzyme products: 486 and 357 base-pair (bp) products for the Egyptian/Eastern strains and 357, 284, 202 bp products for the western strain (Erney et al. 1996, Bundy 2005). To distinguish between eastern and Egyptian strain individuals, primers CB-J-11545 and N1-N-11841 were used to amplify and sequence a 300 bp region of the mitochondrial Cytochrome b / ND1 genes (Erney et al. 1996). This segment of mitochondrial DNA contains two nucleotides that differ by strain (eastern= AC; Egyptian= TT; and western= TC) (Erney et al. 1996). The 300 bp Cytochrome b/ND1 gene PCR product was sequenced in both directions (McLab, San Francisco, CA).

**Results**

*Resistance to multiple type II pyrethroid active ingredients in the Western United States*

The $LC_{50}$ values for all tested type II pyrethroids exceeded their corresponding maximum application rate when tested on lambda-cyhalothrin resistant alfalfa weevils (alpha-cypermethrin: 3.38 to >10.00 µg/cm$^2$; beta-cyfluthrin: 1.70 to >10.00 µg/cm$^2$; lambda-cyhalothrin: 1.32 to >10.00 µg/cm$^2$; zeta-cypermethrin: 3.75 to 7.75 µg/cm$^2$). In comparison, the $LC_{50}$ values for larvae susceptible to lambda-cyhalothrin generally did not exceed their corresponding maximum label rates (Tables 2 & 3). Overall, larvae from all three field trial sites were resistant to all type

II pyrethroid active ingredients but were susceptible to the type I pyrethroid, bifenthrin ($LC_{50}$ bifenthrin: 0.30 to 0.51 µg/cm$^2$, Table 4). However, permethrin, a type I pyrethroid, was not efficacious at controlling these lambda-cyhalothrin resistant locations (permethrin $LC_{50}$: 6.44 to 100.00 µg/cm$^2$, Table 4). Indoxacarb, of MoA 22A, also yielded very low $LC_{50}$ values at the three field trial sites where it was tested ($LC_{50}$: 0.01 µg/cm$^2$, Table 4). This pattern of multiple resistance to type II pyrethroid active ingredients was consistent at all three field trial sites that included both western and Egyptian strain alfalfa weevils. All six larvae analyzed from the MT and WA field sites were identified as western strain, while all six from the AZ site were Egyptian (Supp Fig. 1). The results from these three sites suggests a consistent pattern of resistance to multiple type II pyrethroid active ingredients regardless of strain.

*Type II pyrethroid $LC_{50}$ values and multiple resistance in the Western United States*

Lambda-cyhalothrin $LC_{50}$ values ranged from 0.06 µg/cm$^2$ to >10.00 µg/cm$^2$, generating RRs from 4.61 to 769.23, representing locations susceptible, moderately resistant, and highly resistant to lambda-cyhalothrin (n=22; Table 2). For context, the label rate for lambda-cyhalothrin is 0.22 to 0.34 µg/cm$^2$ (0.02-0.03 lb AI/ acre; 22.41-33.63 g AI/ha) (Buntin 2020).

Alpha-cypermethrin $LC_{50}$ values ranged from 0.35 to >10.00 µg/cm$^2$ (n=14; Table 2). For context, the label rate for alpha-cypermethrin is 0.13 to 0.28 µg/cm$^2$ (0.012-0.025 lb AI/ acre; 13.44-28.02 g AI/ha) (Buntin 2020). The linear correlation between alpha-cypermethrin and lambda-cyhalothrin produced a linear slope of 0.66 ($R^2 = 0.62$; $P = 0.0008$), indicating, that as resistance to one active ingredient increases so too does resistance to the other (Fig. 1A).

Beta-cyfluthrin $LC_{50}$ values ranged from 0.13 to >10.00 µg/cm$^2$ (n=17; Table 2). For context, the label rate for beta-cyfluthrin is 0.14 to 0.25 µg/cm$^2$ (0.0125-0.022 lb AI/ acre; 14.01-

24.66 g AI/ha) (Buntin 2020). The linear correlation between beta-cyfluthrin and lambda-cyhalothrin produced a linear slope of 0.64 ($R^2 = 0.55$; $P = 0.0004$), indicating, that as resistance to one active ingredient increases so too does resistance to the other (Fig. 1B).

Zeta-cypermethrin $LC_{50}$ values ranged from 0.09 µg/cm$^2$ to >10.00 µg/cm$^2$ (n=14; Table 2). For context, the label rate for zeta-cypermethrin is 0.16 to 0.28 µg/cm$^2$ (0.014-0.025 lb AI/acre; 15.69-28.05 g AI/ha) (Buntin 2020). The linear correlation between zeta-cypermethrin and lambda-cyhalothrin was generated using data collected from 2020 to 2022 (n=28). The linear correlation generated a slope of 0.61 ($R^2 = 0.41$; $P = 0.0005$), indicating, that as resistance to one active ingredient increases so too does resistance to the other (Fig. 1C).

*Type I pyrethroid $LC_{50}$ values and multiple resistance in the Western United States*

Bifenthrin $LC_{50}$ values ranged from 0.01 µg/cm$^2$ to 0.86 µg/cm$^2$ (n=15; Table 3); for context, the label rate for bifenthrin is 0.67 to 1.12 µg/cm$^2$. The linear correlation between bifenthrin and lambda-cyhalothrin generated a slope of 0.0045 ($R^2 = 0.006$; $P = 0.77$), indicating that as resistance to lambda-cyhalothrin increases, bifenthrin $LC_{50}$ values did not similarly increase (Fig. 1D).

Permethrin, $LC_{50}$ values ranged from 0.47 µg/cm$^2$ to >100.00 µg/cm$^2$; for context, the label rate for permethrin is 1.12 to 2.24 µg/cm$^2$ (n=21; Table 3). The linear correlation between permethrin and lambda-cyhalothrin was generated using data collected from 2020 to 2022 (n=39) and generated slope of 1.72 ($R^2 = 0.29$; $P = 0.006$), representing a moderate degree of multiple resistance between these active ingredients (Fig. 1E; Vassiliou et al. 2010).

Overall, these data indicated lack of multiple resistance between type I pyrethroid, bifenthrin, and type II pyrethroid lambda-cyhalothrin. These correlations further indicated

moderate multiple resistance between type I pyrethroid, permethrin, and type II pyrethroid lambda-cyhalothrin ($R^2$ = 0.29; $P$< 0.05; Fig. 1E). Finally, correlations between type II pyrethroids indicate multiple resistance between active ingredients ($R^2$ = 0.41- 0.62; $P$: <0.05) (Fig. 1A-C) (Vassiliou et al. 2010).

*Pyrethroid multiple resistance in the Western United States*

Percentage mortality at a diagnostic-concertation further described multiple resistance among type II pyrethroids. Larval cohorts exposed to the acetone control never exceeded 20% mortality regardless of active ingredient or resistance level (RR < 76.9 or RR > 76.9). Average control mortality (acetone control) remained at or below 10% for all active ingredients tested. At the diagnostic concentration, average mortality of larvae with RR < 76.9 was greater than 80-90% for both type I and II active ingredients (Fig. 2) (except for alpha-cypermethrin). The pattern of mortality for type I and II active ingredients differed in sites where lambda-cyhalothrin resistance was high. Percentage mortality for type I pyrethroids bifenthrin and permethrin exceeded 90% while type II pyrethroids produced significantly lower average mortalities, 25-60% (Fig. 2) ($F_{20, 192}$ = 98; $P$ < 0.001). However, alpha-cypermethrin was the least efficacious active ingredient tested regardless of the degree of resistance.

*Multiple resistance to commercial Insecticides formulated with type II pyrethroids*

Commercial insecticides with type I and II pyrethroid active ingredients were tested in commercial forage alfalfa fields with alfalfa weevils highly resistant to lambda-cyhalothrin (sites from AZ, MT and WA, Table 1). In all three locations, type II pyrethroid insecticides failed to provide adequate control of the lambda-cyhalothrin resistant alfalfa weevil larvae. Consistent with the laboratory bioassay results, bifenthrin and indoxacarb were highly efficacious.

Significant differences between treatments ($P < 0.05$) were observed at all three field trial sites. Generally, all type II pyrethroids and the type I pyrethroid, permethrin, did not significantly differ from each other, with Brigade® (active ingredient: bifenthrin) providing significant reduction in alfalfa weevil population from all type II pyrethroids and the control plots (e.g., La Paz Co. Arizona: $F_{6, 28} = 13.32$; $P < 0.001$; Figs. 3A-C, Table 4). Steward® (active ingredient: indoxacarb, MoA 22A) was applied in the Big Horn County, Montana, La Paz County, Arizona, and Yakima County, Washington field trials. Regardless of location, Steward® reduced alfalfa weevil populations compared to all type II pyrethroids, the type I pyrethroid permethrin, and the control plots (Big Horn Co. Montana: $F_{6,28}=25.48$; $P < 0.001$; Yakima Co. Washington: $F_{6,28} = 10.56$; $P < 0.001$) (Figs. 3A-C, Table 4).The Lodge Grass MT site had the largest alfalfa weevil population with control plots yielding 1,300 larvae per 10 sweeps. While type II pyrethroids reduced these numbers by approximately 70%, the total numbers remained above the economic threshold of 20/sweep, unlike the results obtained from Brigade® (i.e., bifenthrin) and Steward® (i.e., indoxacarb) (Fig. 3B) (Evans 1989, Blodgett 1996).

*Alfalfa Weevil Strain Identification*

Alfalfa weevils collected from the Arizona field trial site were the Egyptian strain, and alfalfa weevils collected from the Montana and Washington filed trial sites were both western strain (Supp Fig. 1). Thus, alfalfa weevil strain had no impact on the pattern of multiple resistance.

**Discussion**

Regardless of alfalfa weevil strain, laboratory bioassays illustrated a pattern of multiple resistance among type II pyrethroids and limited multiple resistance between pyrethroid types, a

pattern seen from location samples collected in Arizona, California, Montana, Oregon, Washington, and Wyoming. Our laboratory findings were corroborated by field trials in which alfalfa weevil populations with resistance to lambda-cyhalothrin, were not controlled by type II pyrethroids and permethrin. Bifenthrin was the only pyrethroid efficacious against lambda-cyhalothrin resistant alfalfa weevils. Indoxacarb, MoA 22A, was also effective at controlling alfalfa weevils regardless of their resistance to lambda-cyhalothrin, a pattern observed in all three field trials.

Patterns of multiple resistance among type II pyrethroids and moderate degree of resistance to permethrin has occurred for other insect species and may indicate which mechanism(s) of resistance are present in the evaluated systems. For example, *Anopheles funestus* in Cameroon were identified as being resistant to type II pyrethroids lambda-cyhalothrin and deltamethrin as well as resistant to the type I pyrethroid permethrin. However, the degree of resistance was higher among the type II pyrethroids than permethrin (Menze et al. 2016). Higher degrees of resistance among type II pyrethroids than type I pyrethroids (e.g., bifenthrin) is in line with a *super-kdr* mutation. Although unidentified for the alfalfa weevil, *super-kdr* is sensitive to chemical structure, resulting in a higher degree of resistance to type II pyrethroids (e.g., lambda-cyhalothrin) than type I pyrethroids (e.g., bifenthrin) (Khambay et al. 1994, Davies et al. 2007). This pattern is in line with our experimental findings as our laboratory bioassays identified significant positive correlations for all type II pyrethroids ($R^2$: 0.41-0.62; $P< 0.05$; Fig. 1). Similar observations were documented when evaluating percentage mortality at diagnostic concentrations, as lambda-cyhalothrin resistant alfalfa weevils exposed to other type II pyrethroids ranged from 25% to 60% mortality whereas bifenthrin and permethrin provided 98%

and 93% mortality (Fig. 2). Finally, field trials further confirmed this pattern of multiple resistance as type II pyrethroids generally provided limited efficacy at controlling lambda-cyhalothrin resistant alfalfa weevils, as did permethrin (32.60% to 72.00% mortality), whereas bifenthrin and indoxacarb were highly efficacious in these systems (96.30% to 99.98% mortality) (Fig.3). Thus, our results indicate that *super-kdr* may be a mechanism of resistance present in the tested forage alfalfa systems. In comparison, *kdr* has the same degree of resistance to all MoA 3A active ingredients, a pattern not seen in our study (Khambay et al. 1994, Davies et al. 2007). In line with our findings of lack of multiple resistance to indoxacarb, a similar pattern was observed by Yu and McCord (2007) with a strain of diamondback moth (*Plutella xylostella*), as their strain, highly resistant to permethrin, was also susceptible to indoxacarb.

Multiple mechanisms of resistance often occur simultaneously within an individual, resulting in the enhanced efficacy of each resistance mechanism (Yu 2015). Additional mechanisms include behavioral avoidance, target site insensitivity, enhanced detoxification, and reduced penetration (Soderlund and Bloomquist 1990, Roberts et al. 1997, Yu 2015, Balabanidou et al. 2018, Haddi et al. 2018). For example, pyrethroid resistant rice weevil (*Sitophilus oryzae*) strains, expressed target site insensitivity (*kdr*), behavioral avoidance, and enhanced detoxification (PBO, TPP, and DEM) resistance mechanisms (Haddi et al. 2018). Additionally, differing mechanisms of resistance were identified in populations of rice weevils in Latin America compared to Australia and Turkey (Haddi et al. 2018). Thus, it is possible that the mechanisms of resistance may differ among pyrethroid resistant alfalfa weevils in the western US.

Interpreting laboratory bioassay results to field efficacy can be challenging, especially when there is a range of $LC_{50}$ values generated within a study. Field trials conducted on the same field where three location samples were collected aids in confirming the discrete resistance category of a location sample. Often, corroborating laboratory bioassays with field trial data is not employed because it is laborious (Ball 1981, Denholm et al. 1984, ffrench-Constant and Roush 1990). In this study, we confirmed the degree of resistance identified with laboratory bioassays for three location samples with field trials. All three trials produced the same pattern of multiple resistance, confirming our laboratory bioassays (Table 4; Fig. 3).

Broad ranges of $LC_{50}$ values and their corresponding RRs were generated for all pyrethroid active ingredients included in this study (Tables 2 & 3). Indoxacarb was the only active ingredient that did not similarly present with broad $LC_{50}$ and RR ranges (Table 3). Such broad RR ranges have been identified in numerous entomological studies when evaluating the susceptibility of field populations of insects (Luttrell et al. 1999, Shelton et al. 2003, Snodgrass and Scott 2003, Wang et al. 2010, Kostromytska et al. 2017, Rodbell and Wanner 2021, Rodbell et al. 2022). These broad ranges of $LC_{50}$ values and their corresponding RRs can be influenced by the intensity of resistance, the size of the insect, life stage and/or instar stage, and the sex of the insect (Yu 1983, Koehler et al. 1993, Glunt et al. 2011, Zhu et al. 2020). Thus, future research is needed to identify the most susceptible life stage of the alfalfa weevil so that the timing of an insecticide application coincides with the most susceptible life stage.

Cross-resistance and multiple resistance among active ingredients within the same MoA group is common for insect pests (Yu 2015). The three methods employed to identify multiple resistance are linear correlation, descriptive comparisons of RRs from a location sample tested

against multiple active ingredients, and mean percentage mortality at diagnostic concentrations (DeVries and Georghiou 1981, Scott 1990, Carriére et al. 1996, Vassiliou et al. 2010, Kostromytska et al. 2017, Menger et al. 2020). Having used these three methods as well as field trials, our data has documented multiple resistance among all type II pyrethroid active ingredients, a moderate degree of multiple resistance to permethrin, and a lack of multiple resistance to bifenthrin and indoxacarb.

In 2020, laboratory bioassays and a field trail identified resistance to lambda-cyhalothrin in two commercial alfalfa fields in Big Horn County Montana (Rodbell and Wanner 2021). Similar degrees of resistance were observed in highly localized field sites in six western states, with highly resistant (RR > 76.9) populations occurring within the same county as susceptible locations (RR < 76.9) (e.g., Yuma County AZ and Umatilla County OR) (Rodbell et al. 2022). Our findings, confirm results from Rodbell and Wanner (2021) and Rodbell et al. (2022) that with a high degree of resistance to lambda-cyhalothrin (RR >76.9) zeta-cypermethrin will also not be effective. Our data further illustrates that other type II pyrethroids (i.e., beta-cyfluthrin and alpha-cypermethrin) will also not control lambda-cyhalothrin resistant alfalfa weevils (Figs 1, 2, and 3). However, bifenthrin and indoxacarb were highly efficacious in these systems. Bifenthrin is registered for seed alfalfa and cannot be legally applied in forage alfalfa systems. Thus, indoxacarb is the only active ingredient efficacious in pyrethroid resistant forage alfalfa systems. As all type II pyrethroids and permethrin are not effective in lambda-cyhalothrin resistant forage alfalfa systems, the continued use of MoA 3A insecticides is no longer recommended for those locations. Thus, pyrethroid resistance threatens forage alfalfa production and the economic stability of this $8.7 billion a year industry (Gula 2023).

Alfalfa is grown in 48 states and the production systems and climate conditions can vary significantly. This research encompassed the diversity of alfalfa production in the western region of the US, where the number of harvests a year can range from 1 (e.g., dryland production in MT) to as high as 10 (e.g., irrigated low desert production, AZ; Ottman and Smith 2021). Field sites differed in their irrigation status from flood irrigated (AZ, CA), pivot irrigated (MT, OR, WA, and WY), to dryland (MT). Such diversities in production and climate impact the phenology of the alfalfa weevil and crop development, and its synchrony (Stilwell et al. 2010). Thus, requiring management strategies specific to these systems to better restrict the development of resistance and improve the efficacy of IRM tactics. For example, the effectiveness of a degree-day model that predicts alfalfa weevil development varies in different climates and specific models have been developed for specific alfalfa weevil strains (i.e., Egyptian strain vs. western strain) (UC-IPM 2014; Stilwell et al. 2010, Pellissier et al. 2017). Another key IPM tool, economic threshold, can be affected by irrigation status, the annual number of harvests, end-use of the alfalfa (e.g., used as feed of farm or sold), and the multi-pest complexes that differ greatly between the states included within this study. IRM programs will need to incorporate the diversity of alfalfa production, as one recommendation may not address all diversities within these production systems.

IPM strategies are important facets of IRM, and further research efforts are needed to improve IPM strategies addressing pyrethroid resistant alfalfa weevils (Croft 1990, Dara 2021, Gallagher 2021). As female alfalfa weevils oviposit as many as 2000 eggs over her lifetime, early season control tactics may prove to be a highly efficacious method of reducing larval

populations later in the season (KSU 2022). However, this will require further research efforts focused on determining effective survey methodologies and thresholds for adult alfalfa weevils.

Given limited chemical control tactics available for pyrethroid resistant alfalfa weevils, IRM coupled with IPM provide methods to limit the development of pyrethroid resistance in susceptible systems and to revert resistant populations to more susceptible levels. The IRM tactics recommended for the alfalfa weevil are 1. Survey fields and apply insecticides at the economic threshold; 2. Rotate control tactics, including the MoA of chemical control tactics; and 3. Ensure the optimum efficacy of the insecticide is being realized. Non-chemical control tactics include grazing of cattle and/or sheep in the winter or early spring and harvesting early (7-10 days before harvest) (Pellissier et al. 2017). However, the efficacy of these tactics will depend on the production system (Pellissier et al. 2017, Rodbell and Wanner 2021).

Further research is needed to identify the mechanism(s) of pyrethroid resistance to improve IRM recommendations. Emphasis should be on continued monitoring of pyrethroid resistance to determine the rate of improved pyrethroid efficacy in resistant systems, and early monitoring efforts to document early signs of resistance to MoA 22A (i.e., indoxacarb). A combination of populational genomics and gene sequencing technology, would be highly sensitive approaches in addressing these challenges. Extension efforts are needed to provide IRM and IPM tactics specific to local productions systems, to ensure optimal alfalfa weevil control is being realized while limiting the use of chemical control tactics.

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## Tables and Figures

**Table 1.** To identify the degree of resistance the lethal concentration causing 50% mortality ($LC_{50}$) value for lambda-cyhalothrin was divided into three conservative categories based on its associated maximum label rate. Location samples were divided into three conservative resistance categories defined in Rodbell et al. (2022), these categories represent susceptible, moderate, and high resistance.

| Resistance Category | Type II $LC_{50}$ ($\mu g/cm^2$) | Type II RR $LC_{50}/0.013 \mu g/cm^2$ | Approximate Times (X) Maximum Label Rate |
|---|---|---|---|
| Susceptible | <0.30 | <23.08 | < 0.9X |
| Moderate | 0.30 – 1.0 | 23.08 – 76.9 | 0.9X - 2.9X |
| High | >1.0 | >76.9 | > 2.9X |

**Table 2.** Lethal concentration of type II pyrethroids (MoA 3A) causing 50% mortality ($LC_{50}$) from location samples collected from Arizona (AZ), California (CA), Montana (MT), Oregon (OR), Washington (WA), and/or Wyoming (WY). Resistance ratios (RR) and probit analysis statistics are presented for the t-ratio of the slope, chi-square ($\chi^2$), and the degrees of freedom (df). The degree of a location samples resistance to an active ingredient was based on the $LC_{50}$ value as it pertains to the maximum label rate (Table 1). A location sample could be determined to be susceptible (<1x's maximum label rate), moderate (1-2.9x's maximum label rate), or highly (>2.9x's maximum label rate) resistant.

| Active ingredient | State | County | Field site $_{date}$ | $LC_{50}$ µg/cm$^2$ | T-ratio $_{slope}$ | $\chi^2{}_{df}$ | P value | RR | R Level |
|---|---|---|---|---|---|---|---|---|---|
| Alpha-cypermethrin | AZ | Pinal | $1_{2022}$ | 0.69 | 4.89 | $0.22_1$ | 0.64 | 1.97 | M |
| | MT | Big Horn | $1_{2021}$ | >10.00 | 3.93 | $6.51_4$ | 0.16 | >28.57 | H |
| | | | $1_{2022}$ | 3.23 | 3.51 | $0.53_1$ | 0.47 | 9.23 | H |
| | | | $2_{2022}$ | 2.16 | 2.05 | $2.05_1$ | 0.15 | 6.17 | H |
| | | Broadwater | $1_{2021}$ | 1.06 | 3.48 | $0.65_1$ | 0.42 | 3.03 | H |
| | | | $1_{2022}$ | >10.00 | 2.04 | $0.43_1$ | 0.51 | >28.57 | H |
| | | | $2_{2022}$ | 0.35 | 4.96 | $0.35_1$ | 0.55 | 1.00 | M |
| | | Madison | $1_{2021}$ | 2.47 | 2.30 | $0.96_1$ | 0.33 | 7.06 | H |
| | | | $1_{2022}$ | 1.09 | 3.55 | $0.02_1$ | 0.89 | 3.11 | H |
| | WA | Klickitat | $1_{2022}$ | 2.79 | 4.44 | $1.57_1$ | 0.21 | 7.97 | H |
| | WY | Sheridan | $1_{2021}$ | 3.37 | 4.36 | $9.00_5$ | 0.11 | 9.63 | H |
| | | | $1_{2022}$ | 2.16 | 2.05 | $2.05_1$ | 0.96 | 6.17 | H |
| Beta- | AZ | La Paz | $1_{2022}$ | >10.00 | 0.65 | $0.13_1$ | 0.72 | >76.92 | H |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| cyfluthrin | | Pinal | $1_{2022}$ | 0.32 | 5.51 | $0.87_{1}$ | 0.035 | 2.46 | M |
| | CA | Riverside | $1_{2022}$ | 1.48 | 3.57 | $0.20_{1}$ | 0.66 | 11.38 | H |
| | MT | Big Horn | $1_{2021}$ | 9.57 | 5.61 | $0.75_{5}$ | 0.98 | 73.62 | H |
| | | | $1_{2022}$ | 4.88 | 1.96 | $0.43_{1}$ | 0.51 | 37.54 | H |
| | | | $2_{2021}$ | 1.12 | 4.80 | $4.50_{5}$ | 0.48 | 8.62 | H |
| | | | $2_{2022}$ | 2.29 | 4.32 | $0.02_{1}$ | 0.88 | 17.62 | H |
| | | Broadwater | $1_{2022}$ | 0.72 | 3.54 | $1.29_{1}$ | 0.26 | 5.54 | M |
| | | | $2_{2022}$ | 0.13 | 4.61 | $0.06_{1}$ | 0.80 | 1.00 | S |
| | | | $3_{2021}$ | 0.87 | 5.91 | $0.004_{1}$ | 0.95 | 6.69 | H |
| | | Madison | $1_{2021}$ | 0.43 | 5.93 | $0.35_{1}$ | 0.55 | 3.31 | M |
| | | | $2_{2021}$ | 0.25 | 0.05 | $6.46_{1}$ | 0.83 | 1.92 | S |
| | OR | Umatilla | $1_{2022}$ | 1.90 | 4.32 | $0.50_{1}$ | 0.48 | 14.62 | H |
| | WA | Klickitat | $1_{2022}$ | 2.14 | 5.22 | $2.81_{1}$ | 0.09 | 16.46 | H |
| | | | $2_{2022}$ | 8.50 | 4.00 | $2.78_{1}$ | 0.09 | 65.38 | H |
| | | | $3_{2022}$ | 2.97 | 1.97 | $1.15_{1}$ | 0.28 | 22.85 | H |
| | | Yakima | $1_{2022}$ | 1.70 | 4.60 | $2.91_{1}$ | 0.09 | 13.08 | H |
| | WY | Sheridan | $1_{2022}$ | >10.00 | 2.21 | $0.65_{1}$ | 0.42 | >76.92 | H |
| Lambda-cyhalothrin | AZ | La Paz | $1_{2022}$ | >10.00 | 3.26 | $5.33_{2}$ | 0.07 | >769.2 | H |
| | | Pinal | $1_{2022}$ | 0.93 | 5.47 | $0.40_{2}$ | 0.82 | 71.54 | M |
| | CA | Riverside | $1_{2022}$ | 1.82 | 3.87 | $3.69_{1}$ | 0.54 | 140 | M |
| | | | $1_{2022}$ | 9.61 | 4.87 | $6.38_{4}$ | 0.17 | 739.23 | H |
| | MT | Big Horn | $1_{2021}$ | 4.50 | 6.48 | $4.63_{4}$ | 0.33 | 346.15 | H |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $1_{2022}$ | 7.40 | 3.09 | $10.31_5$ | 0.07 | 569.23 | H |
| | | | $1_{2022}$ | >10.00 | 2.64 | $6.62_5$ | 0.25 | >769.2 | H |
| | | | $2_{2021}$ | 8.40 | 4.65 | $6.95_5$ | 0.22 | 646.15 | H |
| | | | $2_{2022}$ | >10.00 | - | - | - | >769.2 | H |
| | | | $3_{2021}$ | 0.32 | 3.54 | $3.11_1$ | 0.08 | 24.61 | M |
| | | | $3_{2021}$ | 0.57 | 6.76 | $3.61_5$ | 0.61 | 43.85 | M |
| | | Broadwater | $2_{2022}$ | 0.39 | 8.54 | $7.04_4$ | 0.13 | 30.00 | M |
| | | | $2_{2022}$ | 0.74 | 6.09 | $10.21_5$ | 0.07 | 56.92 | M |
| | | | $3_{2021}$ | 0.43 | 5.27 | $3.45_2$ | 0.18 | 33.08 | M |
| | | Madison | $1_{2021}$ | 0.06 | 7.14 | $5.03_4$ | 0.29 | 4.61 | S |
| | | | $2_{2021}$ | 0.26 | 7.01 | $3.67_3$ | 0.30 | 20.00 | S |
| | OR | Umatilla | $1_{2022}$ | 0.20 | 5.84 | $8.16_4$ | 0.09 | 15.38 | S |
| | | | $1_{2022}$ | 0.59 | 4.34 | $2.38_2$ | 0.31 | 45.38 | M |
| | WA | Klickitat | $1_{2022}$ | 2.00 | 3.50 | $8.27_5$ | 0.14 | 153.85 | H |
| | | | $2_{2022}$ | 3.77 | 2.52 | $2.82_2$ | 0.24 | 290.00 | H |
| | | Yakima | $1_{2022}$ | 1.32 | 5.82 | $3.26_4$ | 0.51 | 101.54 | H |
| | WY | Sheridan | $1_{2022}$ | >10.00 | - | - | - | >769.2 | H |
| Zeta-cypermethrin | AZ | La Paz | $1_{2022}$ | 3.75 | 1.98 | $1.82_1$ | 0.18 | 41.67 | H |
| | | Pinal | $1_{2022}$ | 0.65 | 5.86 | $0.50_1$ | 0.48 | 7.22 | M |
| | CA | Riverside | $1_{2022}$ | >10.00 | 2.02 | $0.15_1$ | 0.70 | 111.11 | H |
| | MT | Big Horn | $1_{2021}$ | 7.75 | 6.32 | $10.95_5$ | 0.052 | 86.11 | H |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $1_{2022}$ | 4.48 | 2.27 | $0.30_{1}$ | 0.59 | 49.78 | H |
| | | | $2_{2021}$ | 1.23 | 6.90 | $8.75_{5}$ | 0.12 | 13.67 | H |
| | | | $3_{2021}$ | 0.53 | 5.13 | $0.00\ 02_{1}$ | 0.99 | 5.89 | M |
| | | Broadwater | $1_{2021}$ | 0.09 | 5.20 | $1.02_{1}$ | 0.31 | 1.00 | S |
| | | Madison | $1_{2021}$ | 0.32 | 7.31 | $1.58_{4}$ | 0.81 | 3.56 | S |
| | | | $2_{2021}$ | 0.28 | 6.52 | $4.79_{4}$ | 0.31 | 3.11 | S |
| | WA | Klickitat | $2_{2022}$ | >10.00 | 2.23 | $1.06_{1}$ | 0.30 | 111.11 | H |
| | | | $3_{2022}$ | 1.44 | 2.15 | $3.20_{1}$ | 0.07 | 16.00 | H |
| | WY | Sheridan | $1_{2021}$ | 5.82 | 5.21 | $10.6\ 2_{5}$ | 0.06 | 64.67 | H |
| | | | $1_{2022}$ | 8.79 | 2.67 | $1.29_{1}$ | 0.26 | 97.67 | H |

**Table 3.** Lethal concentration of type I pyrethroids (MoA 3A) and indoxacarb (MoA22A) causing 50% mortality ($LC_{50}$) from location samples collected from Arizona (AZ), California (CA), Montana (MT), Oregon (OR), Washington (WA), and/or Wyoming (WY). Resistance ratios (RR) and probit analysis statistics are presented for the t-ratio of the slope, chi-square ($\chi^2$), and the degrees of freedom (df). The degree of a location samples resistance to an active ingredient was based on the $LC_{50}$ value as it pertains to the maximum label rate for the tested active ingredient (Table 1). A location sample could be determined to be susceptible (<1x's maximum label rate), moderate (1-2.9x's maximum label rate), or highly (>2.9x's maximum label rate) resistant.

| Active ingredient | State | County | Field site$_{date}$ | $LC_{50}$µg/cm$^2$ | T-ratio $_{slope}$ | $\chi^2{}_{df}$ | P value | RR | R Level |
|---|---|---|---|---|---|---|---|---|---|
| Bifenthrin | AZ | Pinal | $1_{2022}$ | 0.05 | 4.66 | $1.09_5$ | 0.95 | 5 | S |
| | CA | Riverside | $1_{2022}$ | 0.27 | 5.13 | $1.10_1$ | 0.29 | 27 | S |
| | MT | Big Horn | $1_{2022}$ | 0.11 | 4.94 | $0.71_1$ | 0.40 | 11 | S |
| | | | $2_{2021}$ | 0.14 | 3.24 | $0.47_4$ | 0.98 | 14 | S |
| | | | $2_{2022}$ | 0.15 | 3.24 | $0.64_1$ | 0.42 | 15 | S |
| | | Broadwater | $1_{2021}$ | 0.01 | 5.00 | $0.01_1$ | 0.99 | 1 | S |
| | | | $2_{2022}$ | 0.73 | 5.94 | $0.32_1$ | 0.57 | 73 | S |
| | | Madison | $1_{2021}$ | 0.11 | 3.75 | $1.59_3$ | 0.66 | 11 | S |
| | | | $2_{2021}$ | 0.01 | 6.01 | $0.08_1$ | 0.77 | 1 | S |
| | OR | Umatilla | $1_{2022}$ | 0.09 | 6.10 | $4.39_2$ | 0.11 | 9 | S |
| | WA | Klickitat | $1_{2022}$ | 0.39 | 7.74 | $0.76_2$ | 0.68 | 39 | S |
| | | | $2_{2022}$ | 0.86 | 7.78 | $1.51_1$ | 0.22 | 86 | S |
| | | | $3_{2022}$ | 0.04 | 5.41 | $5.04_2$ | 0.08 | 4 | S |
| | | Yakima | $1_{2022}$ | 0.36 | 5.56 | $1.21_2$ | 0.54 | 36 | S |
| | WY | Sheridan | $1_{2022}$ | 0.19 | 6.70 | $1.57_2$ | 0.46 | 19 | S |
| Permethrin | AZ | La Paz | $1_{2022}$ | 16.37 | 3.89 | $2.78_1$ | 0.10 | 34.83 | H |
| | | Pinal | $1_{2022}$ | 17.04 | 4.68 | $0.03_1$ | 0.87 | 36.26 | H |
| | CA | Riverside | $1_{2022}$ | 16.37 | 2.78 | $2.78_1$ | 0.10 | 34.83 | H |
| | | | $2_{2022}$ | 1.99 | 3.61 | $0.01_1$ | 0.94 | 4.23 | S |
| | MT | Big Horn | $1_{2021}$ | 6.44 | 4.49 | $5.10_3$ | 0.16 | 13.70 | M |
| | | | $1_{2022}$ | 19.72 | 6.04 | $4.29_2$ | 0.12 | 41.96 | H |
| | | | $2_{2021}$ | 3.83 | 5.11 | $0.62_2$ | 0.73 | 8.15 | M |
| | | | $2_{2022}$ | 8.43 | 3.36 | $6.04_2$ | 0.05 | 17.94 | H |
| | | | $3_{2021}$ | 2.81 | 5.59 | $0.19_1$ | 0.67 | 5.98 | M |
| | | Broadwater | $1_{2021}$ | 2.67 | 6.63 | $8.92_4$ | 0.06 | 5.68 | M |
| | | | $1_{2022}$ | 24.18 | 3.41 | $3.29_2$ | 0.19 | 51.45 | H |
| | | | $3_{2021}$ | 0.47 | 5.80 | $0.26_1$ | 0.61 | 1.00 | S |
| | | Madison | $1_{2021}$ | 1.92 | 5.57 | $4.74_4$ | 0.31 | 4.08 | S |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | $2_{2021}$ | 2.08 | 7.23 | $3.12_{4}$ | 0.54 | 4.43 | S |
| | OR | Umatilla | $1_{2022}$ | 4.62 | 3.97 | $0.30_{1}$ | 0.58 | 9.83 | M |
| | WA | Klickitat | $1_{2022}$ | 9.43 | 5.83 | $0.52_{1}$ | 0.47 | 20.06 | H |
| | | | $2_{2022}$ | 12.67 | 5.17 | $0.81_{2}$ | 0.67 | 26.96 | H |
| | | Yakima | $1_{2022}$ | 100.00 | 2.94 | $0.76_{1}$ | 0.76 | 70.21 | H |
| | WY | Sheridan | $1_{2021}$ | 3.55 | 8.55 | $5.61_{4}$ | 0.23 | 7.55 | M |
| | | | $1_{2022}$ | 14.01 | 5.45 | $0.93_{1}$ | 0.33 | 29.81 | H |
| Indoxacarb MoA 22A | AZ | La Paz | $1_{2022}$ | 0.01 | 3.93 | $0.27_{1}$ | 0.61 | 10 | S |
| | | Pinal | $1_{2022}$ | 0.001 | 5.22 | $2.23_{1}$ | 0.13 | 1 | S |
| | CA | Riverside | $1_{2022}$ | 0.01 | 4.40 | $0.24_{1}$ | 0.62 | 10 | S |
| | MT | Big Horn | $1_{2022}$ | 0.01 | 3.15 | $8.03_{5}$ | 0.15 | 10 | S |
| | | | $2_{2022}$ | 0.02 | 6.08 | $8.00_{4}$ | 0.09 | 20 | S |
| | OR | Umatilla | $1_{2022}$ | 0.01 | 5.60 | $6.02_{4}$ | 0.20 | 10 | S |
| | WA | Klickitat | $1_{2022}$ | 0.01 | 8.64 | $1.56_{5}$ | 0.91 | 10 | S |
| | WY | Sheridan | $1_{2022}$ | 0.01 | 6.09 | $3.56_{5}$ | 0.61 | 10 | S |

**Table 4.** Lethal concentration of type II and type I pyrethroids (MoA 3A) and Indoxacarb (MoA22A) causing 50% mortality ($LC_{50}$) from location samples collected from the three lambda-cyhalothrin resistant field trial sites: La Paz County, Arizona (AZ), Big Horn County, Montana (MT), and Yakima County, Washington (WA). The Yakima Washington field trial site had low populations, and we were unable to include zeta-cypermethrin and indoxacarb in our laboratory bioassays (-).

| Active Ingredient ($\mu g/cm^2$) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Field Trial | A-cypermethrin | β-cyfluthrin | λ-cyhalothrin | Z-cypermethrin | Bifenthrin | Permethrin | Indoxacarb |
| AZ | >10.00 | >10.00 | >10.00 | 3.75 | 0.51 | 16.40 | 0.01 |
| MT | >10.00 | 9.57 | 4.50 | 7.75 | 0.30 | 6.44 | 0.01 |
| WA | 3.38 | 1.70 | 1.32 | - | 0.36 | 100.00 | - |

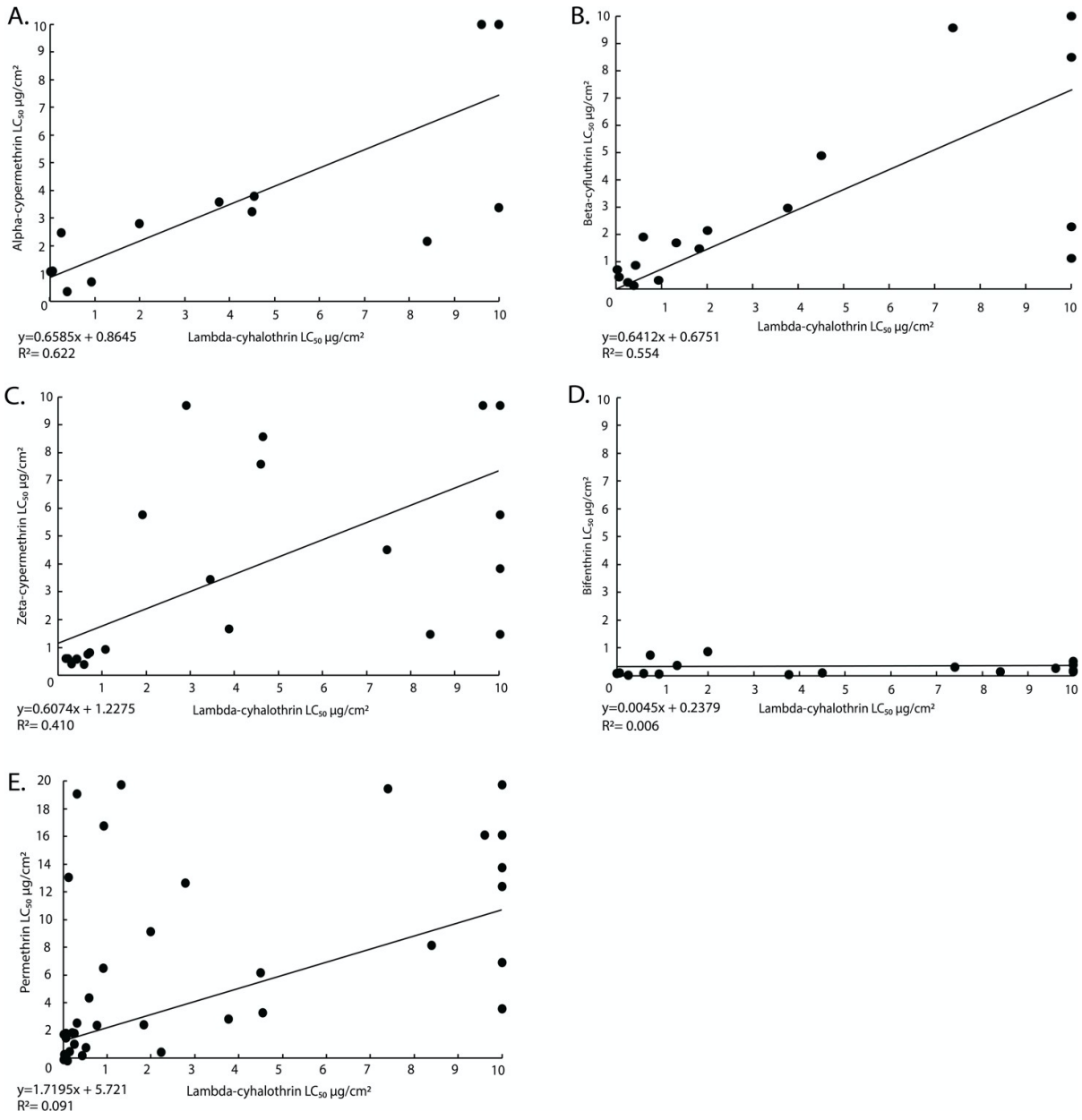



**Figure 1.** Correlation between pyrethroid active ingredients with the y-axis representing the lethal concentration causing 50% mortality ($LC_{50}$) of a pyrethroid active ingredient, and the X-axis representing lambda-cyhalothrin (type II pyrethroid, MoA 3A) $LC_{50}$ values. Correlations help determine the presence or absence of multiple resistance between lambda-cyhalothrin and other type II (e.g., alpha-cypermethrin) or type I (e.g., bifenthrin) active ingredients. Multiple

resistance can be identified via the $R^2$ value and the P-value of the slope of the line of best fit. Regarding cross-resistance and multiple resistance, the P-value signifies if the slope of the line of best fit is significantly different from zero. In this case, the line of best fit has identified multiple resistance among type II pyrethroids (i.e., alpha-cypermethrin, beta-cyfluthrin, and zeta-cypermethrin, A-C), lack of multiple resistance to the type I pyrethroid bifenthrin (D), and moderate multiple resistance to the type I pyrethroid permethrin (E), with increased resistance to the type II pyrethroid lambda-cyhalothrin.

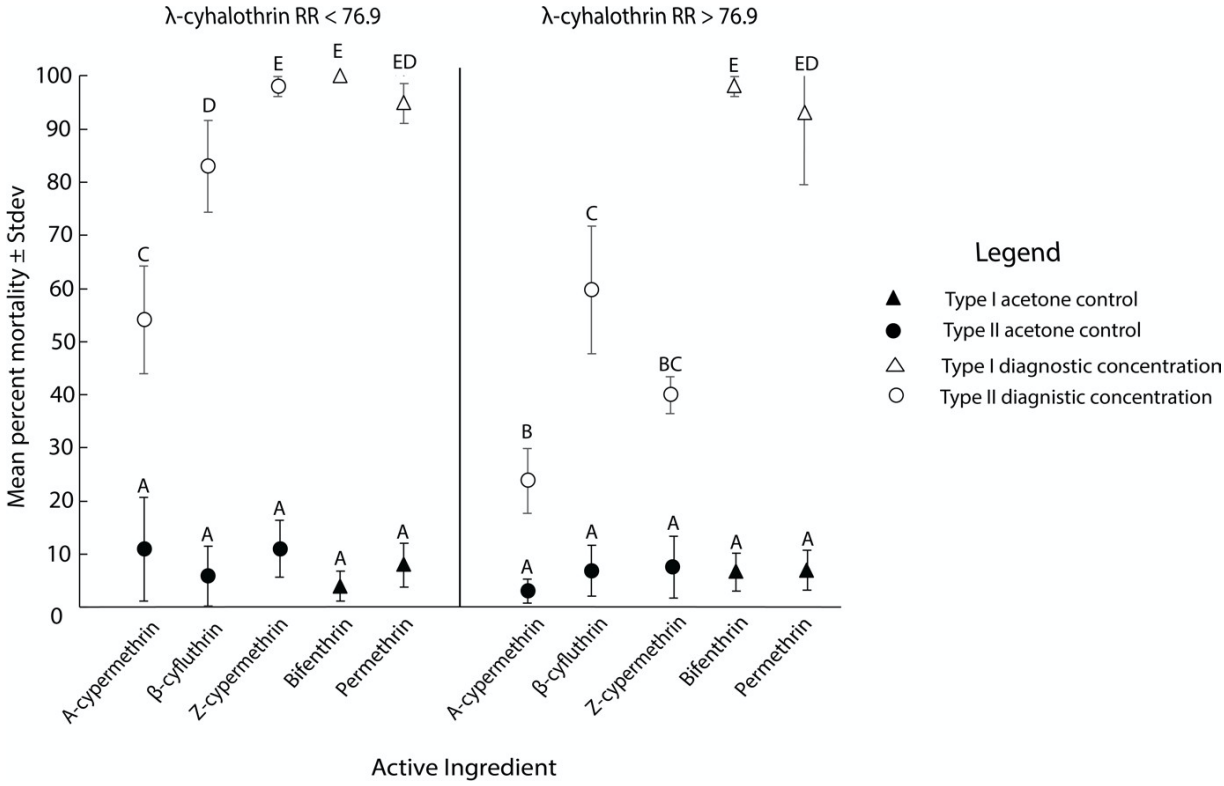



**Figure 2.** Mean percentage alfalfa weevil larval mortality with exposure to the diagnostic concentration number 6 of out bioassays: 3.3 µg/cm$^2$ for type II pyrethroids, 33µg/cm$^2$ for type I pyrethroids, and the acetone control for all active ingredients tested. Regardless of pyrethroid type, location samples were organized based on their resistance ratio (RR) to the type II pyrethroid, lambda-cyhalothrin (RR < 76.9 and RR >76.9). In this instance, significant difference between treatments (P < 0.05) are denoted by different letters ($F_{20, 192} = 98$; $P < 0.001$).

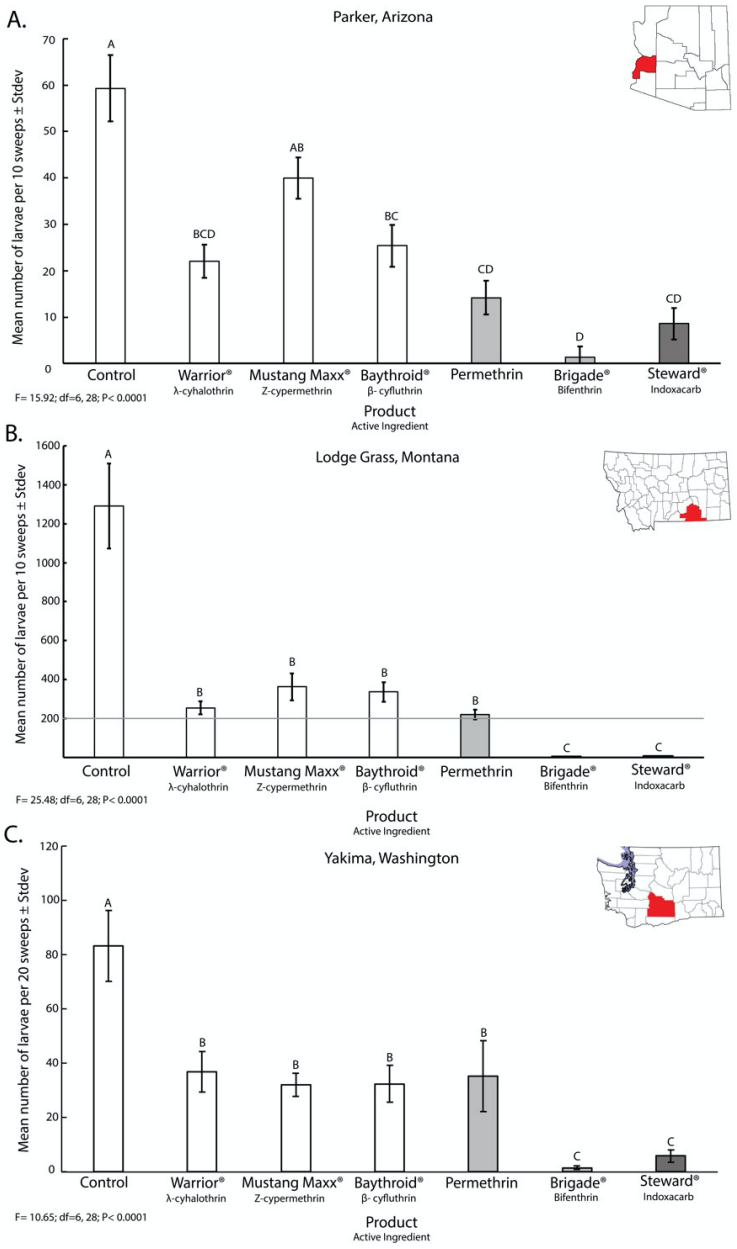
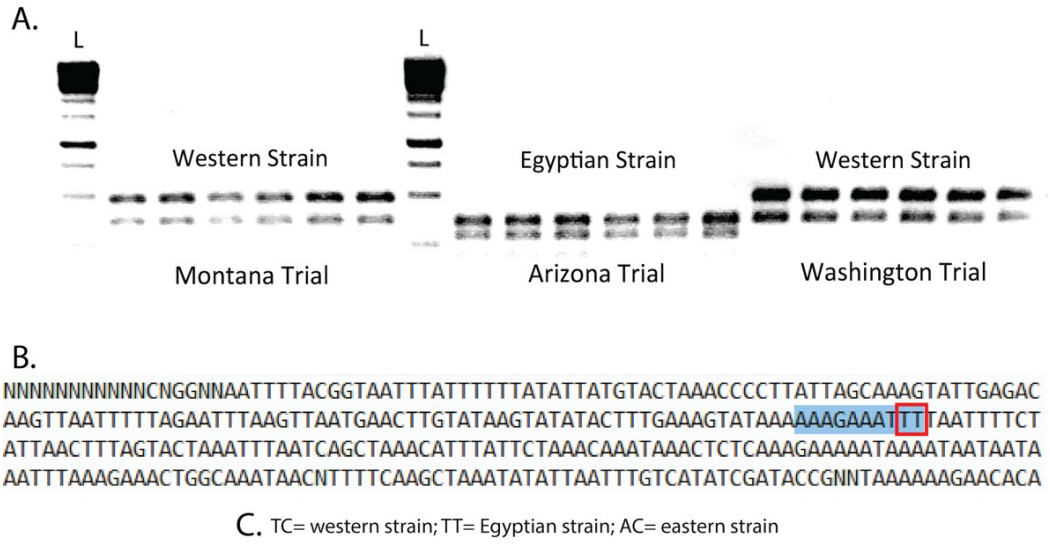



**Figure 3.** Mean number of alfalfa weevil larvae (x ± SD) per 10 sweeps (B & C or 20-sweeps A) collected one week after insecticide application. Field plots were located at Arizona La Paz County Site 1, Montana Big Horn County Site 1, and Washington Yakima County Site 1, with all insecticides applied at their maximum label rate. Warrior, Mustang-Maxx, and Baythroid active ingredients are type II pyrethroids, while permethrin and Brigade (i.e., bifenthrin) active

ingredients are type I pyrethroids, and Steward, active ingredient indoxacarb, representing an alternative MoA group (22A). Treatment effects are significantly different based on analysis of variance, (Arizona: $F_{6, 28} = 13.32$; $P < 0.001$; Montana: $F_{6, 28} = 25.48$; $P < 0.001$; Washington: $F_{6, 28} = 10.56$; $P < 0.001$). Different letters represent significant differences between treatments, $P < 0.05$, Tukey pairwise comparison.

**Supplemental Table S1**. Active ingredient by state, county, and field site that had one outlier concentration (conc.) removed from the dataset. This table lists the concentration that was removed.

| Active Ingredient | Year | State | County | Field Site | Conc. Removed |
|---|---|---|---|---|---|
| Alpha-cypermethrin | 2021 | MT | Big Horn | 1 | 1 |
| Lambda-cyhalothrin | 2022 | MT | Big Horn | 1 | 1 |
| | | | Madison | 1 | 5 |
| | | OR | Umatilla | 1 | 5 |
| | | WA | Yakima | 1 | 4 |
| Zeta-cypermethrin | 2021 | MT | Madison | 1 | 2 |
| Bifenthrin | 2022 | MT | Big Horn | 1 | 4 |
| | | | | 2 | 1 |
| | | WA | Klickitat | 2 | 2 |
| Permethrin | 2022 | OR | Umatilla | 1 | 4 |
| | | WA | Klickitat | 1 | 1 |
| | | | Yakima | 1 | 2 |
| | | WY | Sheridan | 1 | 4 |
| Indoxacarb- MoA 22A | 2022 | OR | Umatilla | 1 | 3 |




**Supplemental Figure 1.** Alfalfa weevil strain identification, via (A) PCR and restriction digest presented on an agarose gel. The presented location samples represent alfalfa weevils from the three field trials in Big Horn County, Montana, La Paz County, Arizona, and Yakima County, Washington. The base pair (bp) of the bands distinguishes alfalfa weevil strain, with the western strain individuals generating bands at the 486, 357, and 84 bp level, and Egyptian strained individuals generating bands at the 357, 284, 202, and 84 bp level. In this case, both the Montana and the Washington field trials consisted of western strain individuals, whereas the Arizona field trial consisted of Egyptian strain individuals. Alfalfa weevils from the Arizona field trial were subjected to genomic sequencing (B). The red box highlighting TT represents the genomic sequence output informing our identification of the Arizona being Egyptian strain (B and C).

# ALFALFA WEEVIL: A CHALLENGING INSECT PEST OF FORAGE ALFALFA IN THE WESTERN UNITED STATES

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## Abstract

Alfalfa weevil, *Hypera postica* Gyllenhal, native to Eurasia, is an invasive insect pest of alfalfa in North America. Alfalfa weevil causes economic damage in the central and western regions of the United States and is a minor pest of alfalfa in the northeastern region of the country. This pest species is currently managed through biological (e.g., introduced parasitoid wasps), mechanical (e.g., harvesting one week early), cultural (e.g., maintaining a healthy alfalfa stand), and chemical (e.g., insecticide applications) control tactics. However, over the last half-century there has been an over reliance on chemical control tactics, primarily pyrethroids (Mode of Action group (MoA) 3A), resulting in resistance developing within localized populations. Pyrethroid resistance can be addressed by following well established integrated resistance management (IRM) and integrated pest management (IPM) strategies, that could lead to long-term sustainable management of this pest species. This case study provides diverse recommendations that can be utilized to help reduce reliance on chemical control tactics. Furthermore, new research endeavors that may provide improved IRM and IPM recommendations for this pest species are presented.


## Learning Objectives

1. Become familiar with the alfalfa weevil life cycle, its invasion of North America, and associated crop damage.
2. Understand alfalfa weevil control tactics that are available, how to utilize them, and understand their limitations.

3. How to utilize a combination of methods to help prolong the efficacy of chemical control tactics currently on the market.
4. Review current research projects, their objectives, and how the data will inform and improve IRM and IPM recommendations of alfalfa weevil into the future.

## Introduction

The genus *Hypera* contains a minimum of 280 described species of weevil, many of which are agricultural pests. One species, the alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), is one of the most economically damaging agricultural pests of alfalfa in North America. Alfalfa weevil is native to Eurasia and North Africa and has become an invasive insect pest in North America as well as Japan and South Korea where it infests forage and seed alfalfa production systems (Sanaei et al. 2019). The alfalfa weevil was first discovered in the United States in 1904 in Salt Lake City, Utah, then in 1939 in Yuma, Arizona, and finally, in 1952 in Maryland. Each of these discoveries represent a new strain in the United States, the western (Utah), Egyptian (Arizona), and eastern (Maryland) (Pellissier et al. 2017). Since then, the alfalfa weevil has become one of the primary economically damaging insect pests of alfalfa in the United States and has become resistant to the primary mechanism of managing their populations, pyrethroid insecticides.

## Distribution in North America

Alfalfa weevil larvae feed on alfalfa terminals, foliage, and crown shoots. When infestation and feeding damage is high, larvae skeletonize the leaves resulting in dry leaves that are grey or white in color (Fig. 1). In extreme cases, larval feeding damage can result in

decreased growth and maturity of the alfalfa stand (Pellissier et al. 2017). Yield loss is no exception, as the alfalfa weevil reduces yield on average by 10 to 15% annually. Furthermore, alfalfa weevil larvae feed predominantly on alfalfa leaves, where most of the plant's protein can be found. This negatively impacts forage protein content, which reduces forage quality. Thus, both forage yield and quality are negatively associated with larval feeding (Hintz et al. 1976).

## Economic Impact in the US and Canada

Alfalfa weevil larvae feed on alfalfa terminals, foliage, and crown shoots. When infestation and feeding damage is high, larvae skeletonize the leaves resulting in dry leaves that are grey or white in color (Fig. 1). In extreme cases, larval feeding damage can result in decreased growth and maturity of the alfalfa stand (Pellissier et al. 2017). Yield loss is no exception, as the alfalfa weevil reduces yield on average by 10 to 15% annually. Furthermore, alfalfa weevil larvae feed predominantly on alfalfa leaves, where most of the plant's protein can be found. This negatively impacts forage protein content, which reduces forage quality. Thus, both forage yield and quality are negatively associated with larval feeding (Hintz et al. 1976).

## Life Cycle

Alfalfa weevil eggs are yellow in color and oval, approximately 0.5 mm in length. The egg color alters as it develops, as the dark head capsule of the developing larvae is notable through the casing, especially in the later stages of this life stage. Alfalfa weevil larvae are light green in color with a dorsal white strip along the length their abdomen and have a black head capsule that increases in width as they develop. Pupating alfalfa weevils are encased in small white silken cocoons for the duration of this life stage. Adults are brown in color with a dark

brown stripe along the middle of their oval abdomen and are approximately 1.6 to 3 cm in length. Like all members of the Curculionidae family, alfalfa weevil adults possess a very noticeable rostrum or snout protruding from its head containing their chewing mouth parts.

Seasonal phenology of the alfalfa weevil life cycle is dependent on the climate conditions that vary across North America. However, all alfalfa weevils have one generation per year (Fig. 2). In general, female alfalfa weevils oviposit clusters of 5-20 eggs (800-2,000 eggs total) within alfalfa stems, generally 15 cm (6-inches) above the soil surface. Generally, the egg stage of the alfalfa weevil lasts approximately 7 to 21 days after oviposition, depending on temperature (Pellissier et al. 2017). Larvae develop through four instar stages before entering pupation (Fig.3.). Larval development lasts from 2 to 4 weeks, during which the early instars feed within the stem of the plant and the terminal buds, and the larger 3$^{rd}$ and 4$^{th}$ instars feed openly on leaves, causing the majority of the economic damage. Pinholes in the developing leaves are an indication of early instar feeding within the buds. During pupation, mature 4$^{th}$ instar larvae encapsulate themselves in cocoons on the foliage or near the soil surface (Fig. 2). After 1 to 2 weeks the next generation of adult alfalfa weevils emerge and feed prior to a summer estivation period where they shelter in the leaf litter of the alfalfa fields or within the edge habitat of the field, to avoid desiccation due to high summer temperatures and alfalfa harvests, and then disperse to overwintering sites (Prokopy and Gyrisco 1965). After their dormancy, the adults will reemerge and return to alfalfa fields in the fall, however, depending on location (e.g., Arizona versus Montana) adults may overwinter and return to the fields in the spring (Pellissier et al. 2017).

## Scouting Methods

The best way to make informed decisions about when to apply control tactics is through monitoring or scouting alfalfa fields for adult emergence in the early season and a few weeks later for larval emergence. One method to determine when scouting should occur is based on degree day (DD) models. In general, scouting should begin once the accumulated DDs reach between 350-400, with a base of 48°F (180- 204 DD with a base of 8.89°C), coinciding with pique 1st and 2nd instar larval stage, generally occurring prior to economic thresholds are reached (PestWeb 2023).

There are three main scouting methods for alfalfa weevil. The first scouting method is sweep-net sampling using a 38-cm diameter sweep net swung in a 180° arc, with one swing per step. 10-20 sweeps are taken at 3 to 5 locations within the field to calculate an average number of larvae per sweep. Using this method, the economic threshold is 20 larvae per sweep (Pellissier et al. 2017). The second, is the shake-bucket method, where stems are randomly and gently collected from various locations within the field, placed into a bucket and then shaken vigorously to dislodge the larvae from the plant. The number of larvae in the bucket are counted, and the number per stem calculated (e.g., divide number of larvae by the number of stems collected). Using the shake bucket method, the economic threshold is 1.5-3 larvae per stem. This average value represents the number of alfalfa weevil larvae per stem, representing the alfalfa weevil density for that field site. The third method is looking at alfalfa weevil damage on the plant terminals. Randomly selected terminals, 50 in total, are evaluated for pinholes and defoliation, close monitoring efforts are recommended if 30-50% of terminals exhibit damage (Blodget 1996). However, each method has limitations and specific thresholds are being updated for

different production systems (local sources should be consulted). Sweep netting is the most efficient method but is biased towards sampling larger 3rd and 4th instar larvae, compared to the shake-bucket method that dislodges more of the 1st and 2nd instars.

## Economic Threshold

The general economic threshold for the alfalfa weevil is 1.5-3 weevils per stem or 20 larvae per sweep. However, economic thresholds are meant to be updated and adjusted depending on the overall cost of treatment and value of the product (seed or hay). Thus, the true economic threshold for an individual production system may be greater than or lower than the described values. For example, Harrington et al. (2021) updated the economic threshold for alfalfa weevil in irrigated alfalfa systems in the low desert of Arizona. They adjusted the threshold to 1-3 third and fourth instar larvae per sweep compared to the 20 larvae per sweep standard. Highlighting the importance of evaluating the economic threshold for each system, and to make management decisions based on that specific value. Note that this value should be updated every time a value within the equation changes (e.g., fuel or hay value). The first step is to identify the cost of management per acre. Then identify the economic injury level (1) and the economic threshold (2) using the following formulas:

1)

$$EIL = \frac{C}{V \times I \times D \times K}$$

2)

$$ET = EIL \times C^{-x}$$

EIL= Economic Injury Level

ET= Economic Threshold

C= Cost of management per area

V= Market value per unit of produce

I= Injury per insect per production unit (e.g., percent defoliation per acre, expressed as a proportion)

D= Damage per unit injury (e.g., bushels lost/acre/percent defoliation)

K= Proportionate reduction in potential injury or damage (e.g., 0.09 for 90% reduction in injury)

$C^{-x}$= Factor of increase in damage per unit of time (-x) expressed in weeks (e.g., 0.6/week; or 60% increase in damage per week)

(Pedigo et al. 2021)

However, simplified formulas can be found on North Dakota State University (2018) Extension's website.

## Management Options

Alfalfa weevil can be managed through biological, cultural, chemical, and mechanical control tactics. Biological control tactics can be a serious source of mortality, with the alfalfa weevil larval life stage being the most vulnerable to disease, parasitism, and predation. However,

when alfalfa weevils first established in North America, there was a lack of native obligate parasitoid species, allowing for unfettered population growth. Key predators of larvae are lady beetles (Coleoptera: Coccinellidae), damsel bugs (Hemiptera: Nabidae), and Lacewings (Neuroptera: Chrysopidae) (Table 1; Fig. 4; Pellissier et al. 2017). However, one of the most successful sources of biological control of alfalfa weevil is eight *Ichneumonidae* parasitoid wasps, which were released by United States Department of Agriculture – Agricultural Research Service (USDA-ARS) and Animal and Plant Health Inspection Service (APHIS) in the end of the 20$^{th}$ century (Pellissier et al. 2017). The most successful of these are of the *Bathyplectes* genus (Hymenoptera: Ichneumonidae) like *Bathyplectes curculionis* (Thomson), *B. anurus* (Thomson), and *B. stenostigma* (Thomson). Although parasitism rates are generally lower in the western US than other parts of the country, parasitism rates in MT and ND ranged from 4.1 to 85.1% by *Bathyplectes curculionis*, with parasitism greatly influence by irrigation status and location (Rand 2013).

Chemical control is commonly used by alfalfa producers for alfalfa weevil management. The most common active ingredient used are the pyrethroids (Mode of Action Group (MoA) 3A) and an oxadiazine insecticide (i.e., Indoxacarb, MoA 22A) (Rodbell et al. 2022). Insecticides should only be applied when the alfalfa weevil densities reach the economic threshold. Pyrethroids and indoxacarb negatively impact beneficial insects, therefor adjusting the timing of the insecticide application to reduce non-target insect exposure is important (e.g., do not apply if there is floral bloom). Spinosad, a biopesticide, is the only organic chemical control tactic available for alfalfa weevil, however, it only suppresses alfalfa weevil populations (Vardiman et al. 2022; ARBICO Organics 2023).

Cultural control and its efficacy can differ by geography and production system. Grazing of livestock (sheep or cattle) on alfalfa fields can effectively remove eggs, but the timing of grazing needs to correspond with insect phenology. In warmer climates with fall egg laying winter grazing can be effective, while in northerly climates grazing must occur after egg oviposition in the spring.

Early harvest, 7-10 days before normal harvest can salvage yield when economic levels of alfalfa weevil are present. This tactic is effective in areas where the larval population peaks at harvest time, typically first harvest in northerly latitudes. Early harvest can result in significant larval mortality, particularly during hot dry weather that desiccates the larvae (Prasad 1993, Pellissier et al. 2017). However, larvae can survive and continue to feed under the windrow, especially when the hay takes longer to dry (raking the windrows can speed drying and increase larval mortality), stunting the growth of the next stand.

Additional cultural control tactics have been suggested but their effectiveness needs further research, including intercropping, flaming during alfalfa dormancy, and rolling with weighted rollers (Pellissier et al. 2017). Using weighted rollers is an additional source of alfalfa weevil mortality in the fall (more southern latitudes) or early spring (more northern latitudes) by squashing eggs within the stems.

## Insecticidal Control and its Challenges

During the 1950s and 1960s the insecticides used to control alfalfa weevil in the US were primarily organochlorines (MoA 3B), heptachlor and dieldrin (Bishop 1964). In 1962, however, reports of resistance to either heptachlor or dieldrin occurred in Virginia and in Utah and indicated poor control of adults and larvae, with repeated applications of heptachlor failed to kill

resistant adults in laboratory bioassays (Bishop 1964). Cross-resistance to dieldrin was noted for populations never exposed to the insecticide, indicating cross-resistance to multiple active ingredients of the same MoA (Bishop 1964). Today, the main source of alfalfa weevil control is pyrethroid (MoA 3A) insecticides, however, reports of insecticide failures from concerned producers indicate an emerging issue in the United States and Canada (Glen 2015, Orloff et al. 2016).

13 active ingredients are registered for the control of alfalfa weevil in forage alfalfa systems in the United States (Vardiman et al. 2022). These active ingredients vary in their efficacy (e.g., suppression), with only two highly efficacious MoA registered for alfalfa weevil in forage alfalfa systems (i.e., Pyrethroids (MoA 3A) and Indoxacarb (MoA 22A)). Pyrethroids are classified as type I and type II, based on the presence of a cyano-group (type II) or absence (type I). In general, type II pyrethroids are more toxic than type I pyrethroids because of this structural distinction, as type II pyrethroids modify the sodium ion channel for a longer period (Davies et al. 2007). Currently there is 1 type I and 6 type II pyrethroid active ingredients registered for forage alfalfa in the US (Vardiman et al. 2022).

However, the efficacy of pyrethroids in North America is in question. The first reports of control failure in the field began in 2015, in Alberta, Canada and California (Glen 2015, Orloff et al. 2016). In 2021, reports of control failure in Montana were confirmed as resistance was identified using laboratory bioassays and field trials (Rodbell and Wanner 2021). This research was expanded to several western US states and lambda-cyhalothrin (i.e., active ingredient of Warrior®) resistance has now been confirmed in Arizona, California, Montana, Oregon, Washington, and Wyoming (Rodbell et al. 2022) (Fig. 5). Additionally, data from Rodbell et al.

(2022) suggests multiple resistance between lambda-cyhalothrin and zeta-cypermethrin (both type II pyrethroids), highlighting the risk of multiple resistances to other type II pyrethroids. Field trials in Big Horn County, Montana corroborate this pattern of resistance (Wanner et al. 2022A). These studies indicate resistance to pyrethroids is well established across the western region of the United States. However, susceptible populations remain, highlighting the importance of conserving them in the landscape (Wanner et al. 2022B).

## Integrated Resistance Management for Pyrethroid Resistant Alfalfa Weevil

Data indicates the loss in efficacy of all type II pyrethroids, like Baythroid® (a.i. Beta-cyfluthrin), Grizzly Too® (a.i. lambda-cyhalothrin), and Mustang-Maxx® (a.i. zeta-cypermethrin). The continued reliance of type II pyrethroids will reinforce resistance to these insecticides. Outbreak levels of alfalfa weevil larvae in resistant fields are challenging and expensive to control, threating forage production and its economic feasibility (Wanner et al. 2022B). Thus, IRM recommendations are urgently needed to mitigate production losses in these systems.

What alternative MoA groups are available for the control of alfalfa weevil larvae? Indoxacarb (MoA 22A, active ingredient of Steward EC®) is generally efficacious against pyrethroid resistant populations. One concern is that indoxacarb will be used routinely in place of pyrethroids, raising the risk of resistance developing to this MoA.

Three recommendations that can help limit the impact of pyrethroid resistant alfalfa weevil on forage alfalfa production systems. First, watch for slipping field efficacy of pyrethroid insecticide products and conduct in-field small plot treatments to compare effectiveness (Wanner et al. 2022B). Second, rotate the insecticide MoA group so that the same MoA group is not

applied more than once every three years. Given the lack of MoA choices, IPM tactics such as spraying only when economic thresholds have been met or employing early harvest will be necessary to maintain a 3 or more year MoA rotation. In doing so, producers limit the selection pressure placed on their alfalfa weevil population, thus conserving the efficacy of pyrethroids and indoxacarb in areas with low to moderate resistance. In these instances, it is recommended that producers should: 1. Survey their fields and apply insecticides at the economic threshold; 2. Rotate control tactics, especially the MoA group; 3. Ensure the optimum efficacy of the insecticide is being realized. This can be done by ensuring optimum insecticide application timing, applying higher label rates, and include adjuvants that optimize coverage and precision (Wanner et al. 2022B). Third, if a producer's production system is in an area with high pyrethroid resistance, it is highly unlikely that pyrethroids will still be effective. In these areas, producers will have to rely on indoxacarb (i.e., Steward, MoA 22A) and IPM strategies in rotation. After 3 or more years, test the efficacy of pyrethroids to determine whether they can return to the 3 or more year rotation that include pyrethroids.

## How to Monitor Fields for Pyrethroid Resistant Alfalfa Weevil?

The first step in integrated resistance management is monitoring for resistance within the field early in resistance development. The easiest ways to identify a loss in efficacy of an insecticide is through insecticide spray trials and glass vials. Strips of an alfalfa field can be treated on a small section of a commercial field. The commercial pyrethroid formulation(s) can be applied using an ATV sprayer with a 4m boom or a pressurized backpack sprayer, and applied at the maximum label rate, compared to an untreated strip (i.e., control strip). These strips should be replicated four times organized in a randomized complete block design; the replicates help

account for variation in the field. Before insecticide application, collect 10-20 sweep samples from each strip, and again one-week after insecticide application, placing collected material from each strip in its own labeled paper bag. The contents of each bag should be counted to identify the number of larvae collected from each strip which allows for the efficacy of each insecticide to be determined and management decisions to be made accordingly. Typically, strips treated with a pyrethroid active ingredient should have >90% fewer larvae compared to the untreated control plots 7-days after applications. The same degree in efficacy (90% fewer larvae) should also be observed in strips treated with indoxacarb (MoA 22A). If, pyrethroids fail to reduce the alfalfa weevil population in these trials, indoxacarb should still provide 90% control. If these trials are repeated annually over years, any loss in efficacy should be apparent, allowing for quick management decisions to better mitigate economic losses from resistance.

Alternatively, glass vials treated with an insecticide provide a method that does not require alfalfa acreage and can be done at any point of the season, barring that there are enough weevils to collect. Following the methodology outlined in Rodbell and Wanner (2021) and Rodbell et al. (2022), for each pyrethroid active ingredient tested, ten-glass vials (Discount Vials, 27 mm diameter × 95 mm length) would be required. Five vials as a control (1mL of acetone) and five vials each treated with one mL of the insecticide at a concentration of 18.9 µg/mL for type II pyrethroids and 189.0 µg/mL for type I pyrethroids. These concentrations, represent concentrations closest to the maximum label rate for the associated insecticides. Identifying alive and dead/moribund individuals can be done by following the Rodbell and Wanner (2021) and Rodbell et al. (2022) methods using a hotplate. If, at these conservative diagnostic concentrations

50% mortality is not achieved, it is likely that the location sample is resistant to the insecticide, and intensive IRM and IPM tactics should be employed.

## Future Research Areas to Improve Alfalfa Weevil Management

- Evaluating current economic thresholds: Since Harrington et al. (2021) published an updated economic threshold specific to the low desert region of the United States, it is important to test and update economic thresholds in other irrigated and non-irrigated alfalfa production zones in Canada, Mexico, and the United States.
- Evaluating pyrethroid efficacy in North America: Researchers in Canada are exploring the efficacy of pyrethroids in Canada. The results of this effort will greatly inform management decisions and integrated resistance management strategies in the country. Furthermore, other researchers are identifying pyrethroid resistant alfalfa weevils in Utah and Oklahoma, states not addressed by previous studies (i.e., Rodbell et al. 2022).
- Identifying molecular markers for resistance: A large collection of alfalfa weevils preserved in ethanol is currently being used for population genomics studies in the western United States and Canada. This collaborative study benefits from knowledge of the pyrethroid resistance status of each population and a first draft of the Hypera postica whole genome sequence generated recently by USDA-APHIS. The goal is to find molecular markers that associate with resistance, confirm their predictive value and develop highly sensitive molecular tests for early detection of resistance.
- Identify optimum timing of insecticide application: An academic and industry collaboration is underway to research the precise timing of insecticide applications for

optimal control of alfalfa weevil in Montana, Washington, and Oklahoma states. Typically, insecticide applications are recommend when threshold levels of 2nd and 3rd instar larvae are reached. However, recent field trials suggest earlier applications of indoxacarb may be more effective in some production systems, possibly through toxicity to ovipositing females and hatching first instars when this phenology occurs in more narrow time window. If this is the case, thresholds based on adult weevils will need to be developed and the current degree day model improved to predict the duration and peak period of oviposition.

## Conclusion

Alfalfa weevil is an invasive pest that was first reported in North America in 1904 (Salt Lake City, UT) and is now established throughout the continental United States and parts of Canada and Mexico as a primary pest of forage alfalfa. Alfalfa weevil management has predominantly relied on chemical control tactics, resulting in insecticide resistance. Over the last half-century pyrethroids have been widely used to control their populations, and recently, pyrethroid resistance has developed in the western United States. Integrated resistance management strategies provide an avenue to limit or avoid pyrethroid resistance. Further research and outreach is required to implement an effective IRM program to mitigate further economic loses. Together, these efforts will provide desperately needed information to ensure sustainable control of this key pest of forage alfalfa.

## Discussion Points

1. List reasons why or how alfalfa weevil has become a major pest of alfalfa in North America.
2. Using research and the equations provided, estimate the economic threshold for an alfalfa producer in your production system.
3. Discuss advantages and disadvantages of biological, chemical, cultural, and mechanical control strategies and how they can be best integrated for sustainable management.
4. Discuss current chemical control options for alfalfa producers, their challenges, and their best use.
5. Research and write an IPM plan for the alfalfa weevil including advantages and disadvantages of the different tactics. Include ideas from the suggested reading, including landscape approaches to manage this pest species.

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## Tables

**Table 1.** List of common predators and parasitoids of alfalfa weevil in the western United States. Figure 4 provides photos of some of the listed natural enemies. Pest and predator complexes may vary depending on location and production system.

| Name | Order | Family | Predator or Parasitoid |
|---|---|---|---|
| Lady Beetles | Coleoptera | Coccinellidae | Predator |
| Damsel Bugs | Hemiptera | Nabidae | Predator |
| Assassin Bugs | Hemiptera | Reduviidae | Predator |
| Green Lacewings | Neuroptera | Chrysopidae | Predator |
| Brown Lacewings | Neuroptera | Hemerobioidae | Predator |
| *Bathyplectes curculionis* (Thomson) | Hymenoptera | Ichneumonidae | Parasitoid |
| *Bathyplectes anurus* (Thomson) | Hymenoptera | Ichneumonidae | Parasitoid |
| *Bathyplectes stenostigma* (Thomson) | Hymenoptera | Ichneumonidae | Parasitoid |

## Figures

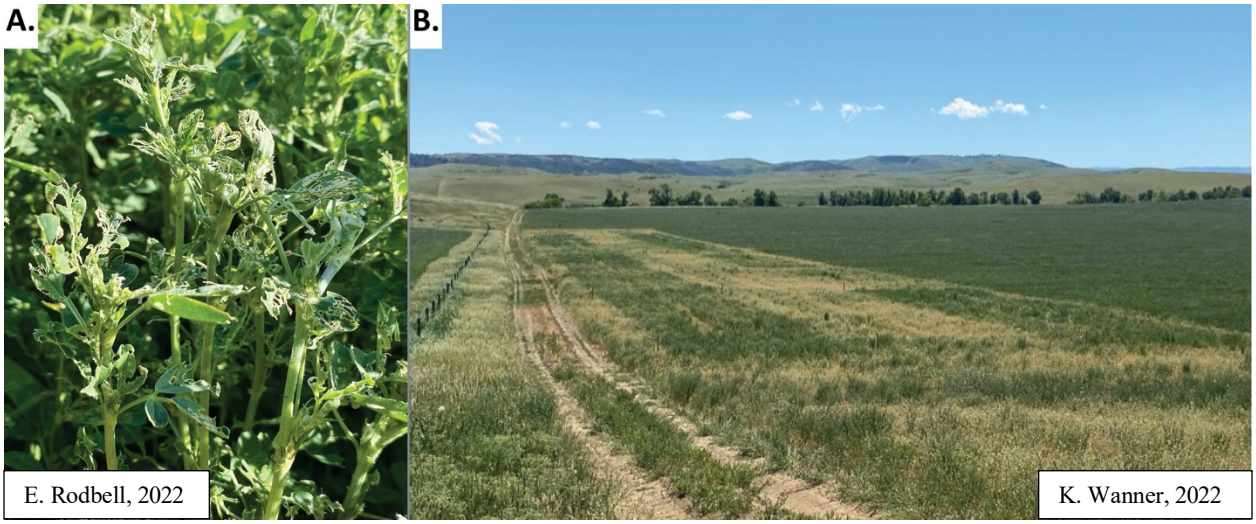


**Figure 1.** Defoliation derived from alfalfa weevil larval feeding. Figure A is a closeup photo of alfalfa damage. Figure B illustrates skeletonization of the alfalfa stand, damage that can be seen from the side of a road. Through defoliation, alfalfa weevil negatively impacts yield and quality of alfalfa.

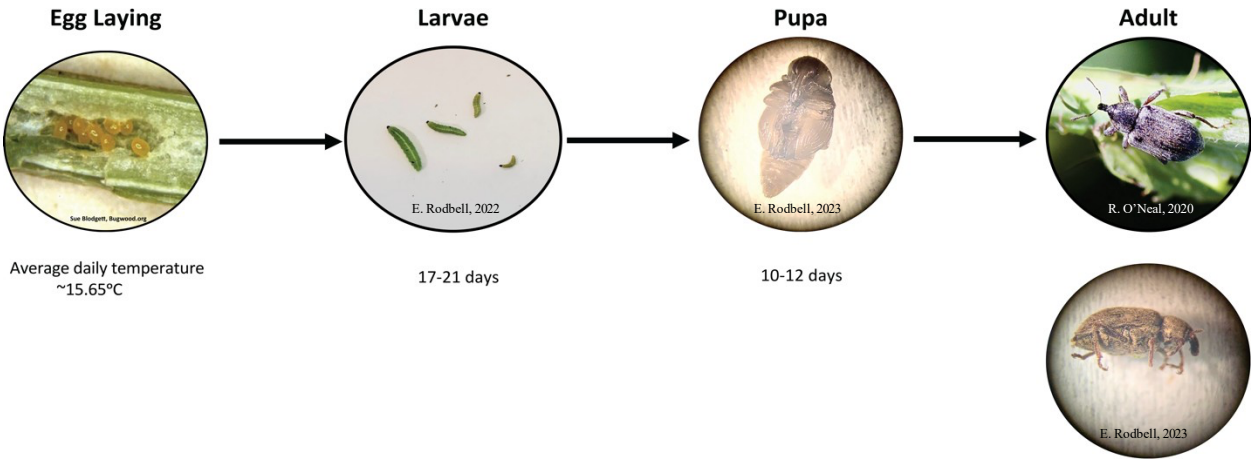


**Figure 2.** Alfalfa weevil life cycle, in the northern Rocky Mountains of the United States. Life cycle timing varies on where you are in the continent. This is a simplified version of the alfalfa weevil life cycle, as in some cases, more complex life cycles occur in the more southern regions of the United States and northern Mexico.

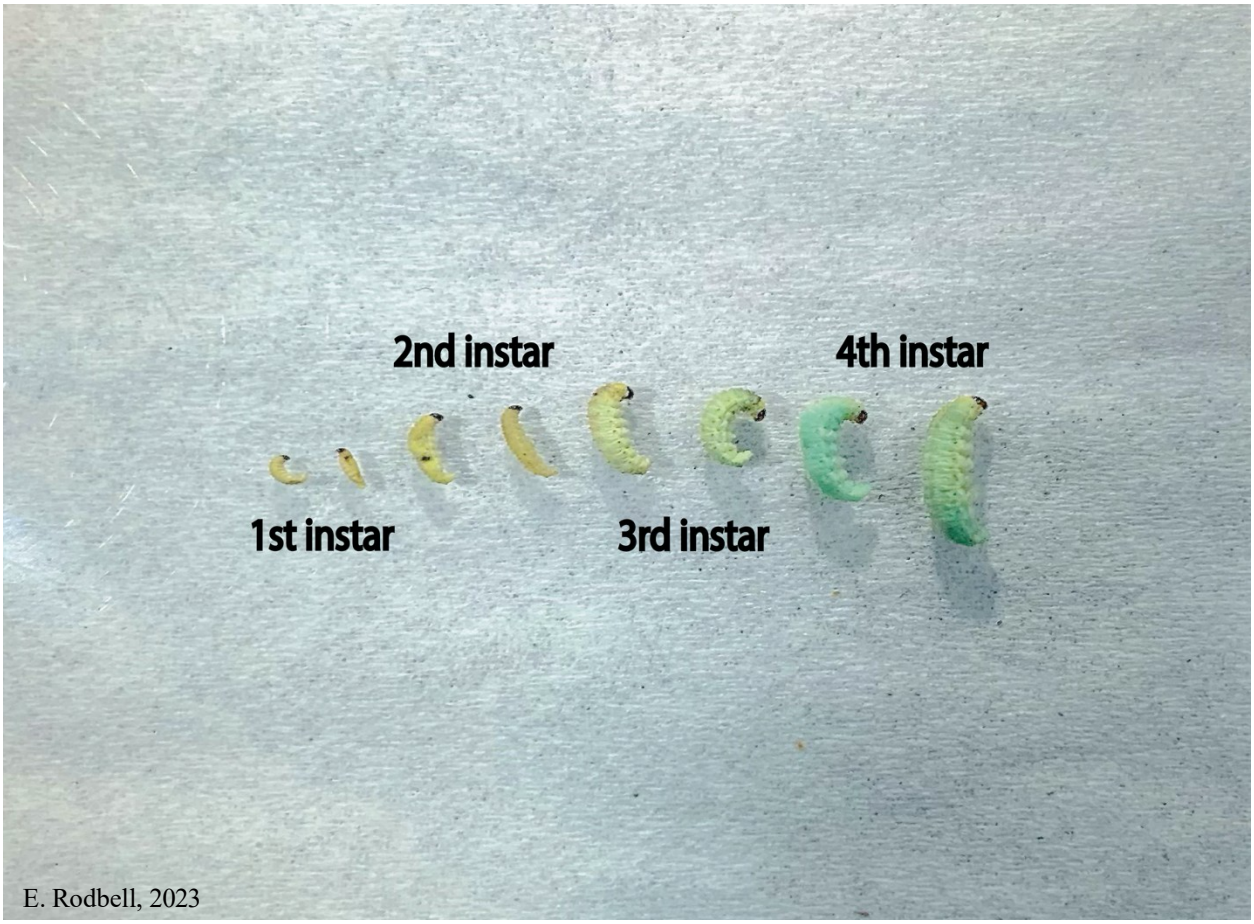


**Figure 3.** Alfalfa weevil larvae featuring their four larval instars. The first and second instars are generally found within the plant terminal and may need to be extracted from the plant to be seen. Third and fourth instar larvae are much larger and openly feed on open alfalfa leaves and can easily be seen while scouting. First instar alfalfa weevil larvae can be identified in the lab from the others as their head capsule width ranges between 0.18 to 0.22mm, second instar larval head capsule width ranges between 0.23 to 0.43mm, third instar larval head capsule width ranges between 0.35 to 0.43mm, and fourth instar head capsule width ranges from 0.46 to 0.62mm (Hamlin et al. 1949).

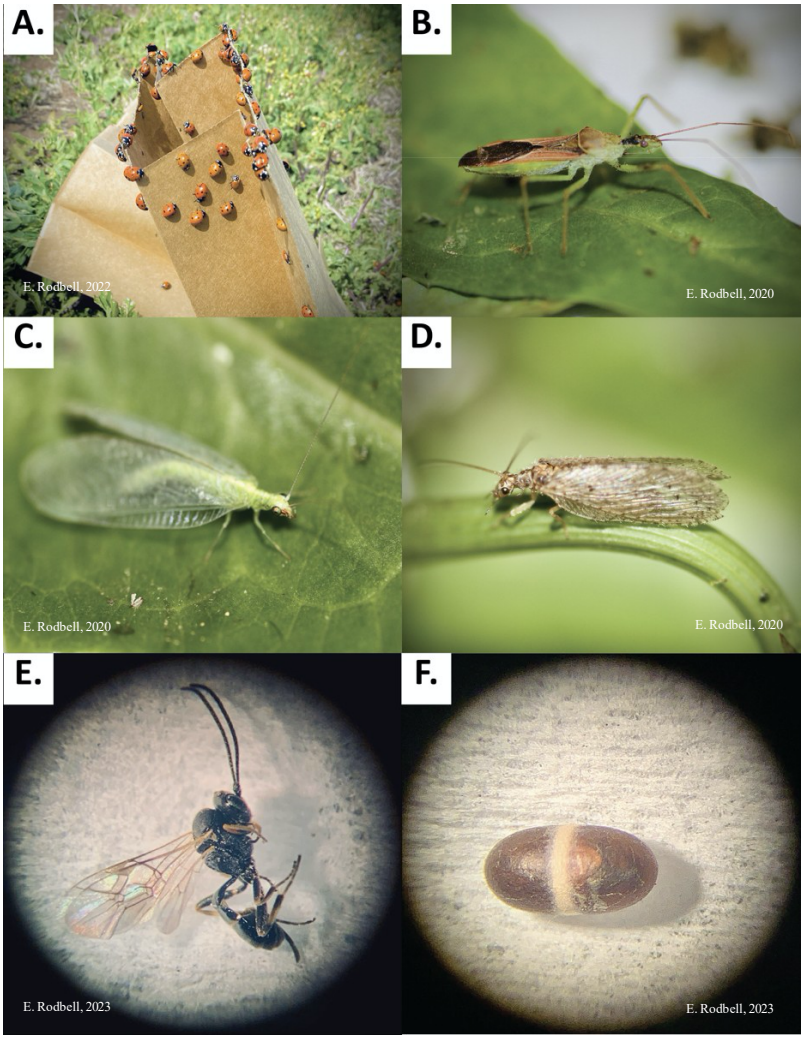


**Figure 4.** Common natural enemies of alfalfa weevils. In general, natural enemy predation on alfalfa weevil is most effective during the 1st and 2nd larval instars. Figure A. Features a collection of adult lady beetles (Coleoptera: Coccinellidae). Figure B. Features an adult assassin bug (Hemiptera: Reduviidae). Figure C. Features an adult green lacewing (Neuroptera: Chrysopidae). Figure D. Features an adult brown lacewing (Neuroptera: Hemerobiidae). The larval stages for all of these insects are also highly predacious. Figure E. Features an Ichneumonidae parasitoid wasp with Figure F representing a parasitized alfalfa weevil larvae often referred to as a mummy.

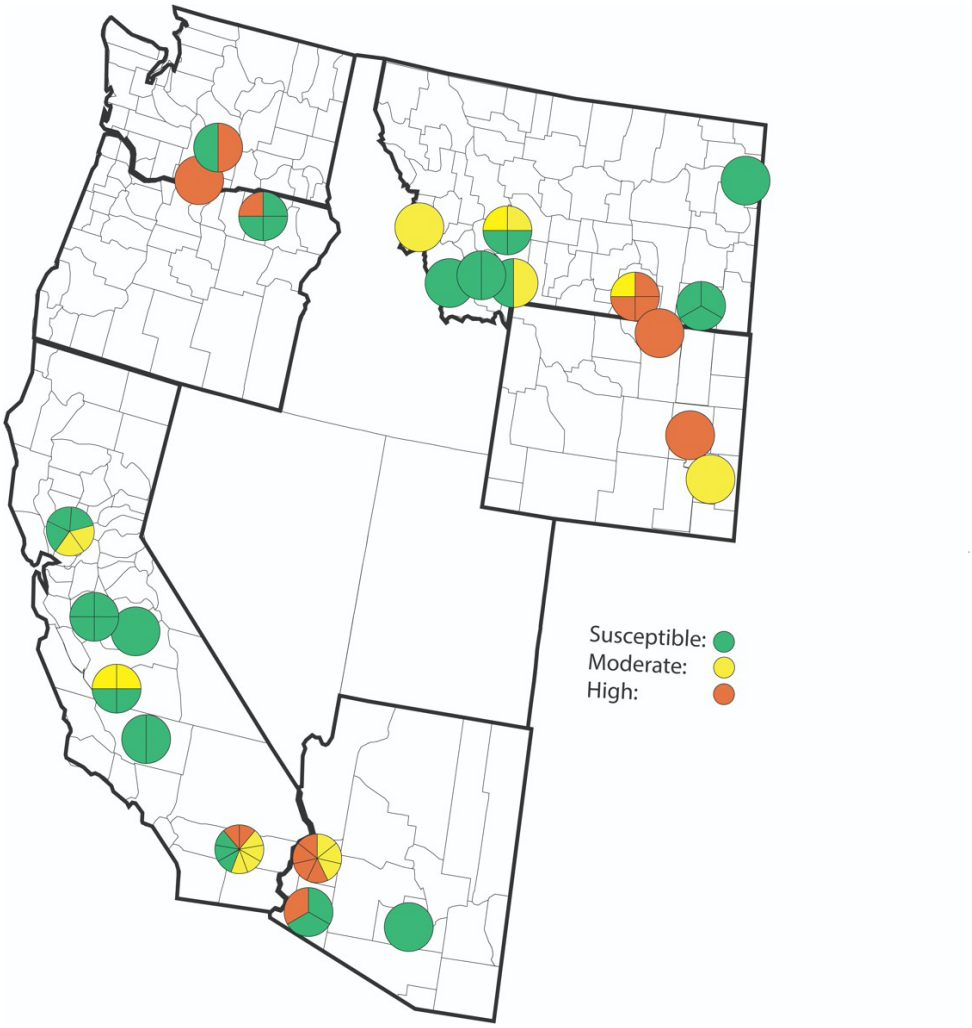


**Figure 5.** Alfalfa weevil resistance levels to type II pyrethroid, lambda-cyhalothrin (Rodbell et al. 2022). Locations susceptible to the toxin will be well controlled by that active ingredient, locations moderately resistant to the toxin, may still be impacted by the exposure, but the degree to which they are impacted will be reduced. Finally, highly resistant populations will not be negatively impacted when exposed to the insecticide. This map highlights how locationally specific lambda-cyhalothrin resistance is in the west, and how important susceptible locations are to conserve.

# CHAPTER 6

# CONCLUSION AND FUTURE WORK

In this dissertation, I identified that alfalfa weevil (*Hyper postica* Gyllenhal [Coleoptera: Curculionidae]) has developed resistance to a type II pyrethroid mode of action (MoA) group 3A insecticide, lambda-cyhalothrin in Arizona, California, Montana, Oregon, Washington, and Wyoming. Additionally, I have identified multiple resistance among type II active ingredients, a moderate degree of multiple resistance between lambda-cyhalothrin and permethrin, and finally, that bifenthrin (type I pyrethroid) and indoxacarb (mode of action (MoA) 22A) were highly efficacious in lambda-cyhalothrin resistant systems. Cumulatively, these data illustrate that MoA 3A insecticides registered for the control of alfalfa weevil in forage alfalfa systems will not be efficacious if the population is resistant to lambda-cyhalothrin. To address this challenge in forage alfalfa production this dissertation provides integrated resistance management and integrates pest management strategies to limit the further development of alfalfa weevil pyrethroid resistance in the western United States.

At the commencement of my work with alfalfa weevil there were reports of pyrethroid resistant alfalfa weevils in the Alberta, Canada, Scott Valley California, and isolated pockets in the northwestern United States. However, none were scientifically substantiated nor were the published works peer reviewed. To address this concern, my research was focused on identifying the severity of pyrethroid resistance in the region. However, no published methods had be developed for the alfalfa weevil regarding pyrethroid bioassays. As such, effective laboratory methodologies needed to be developed for this pest species.

In 2020, we developed laboratory methodologies and protocols and shared them with our collaborating research lab at University of California, Davis. At that point, we had identified two

locations in Big Horn County, MT that were resistant to lambda-cyhalothrin and zeta-cypermethrin. That same year, we conducted a field trial at one of the resistant locations to confirm our findings that lambda-cyhalothrin was no longer efficacious at this location. The results were published in 2021 (see chapter 2).

Research efforts expanded beyond Montana in 2020 and 2021. The focus of this work was to evaluate alfalfa weevil susceptibility to lambda-cyhalothrin in multiple states in the western region. Using the methodologies established in chapter 2, we identified resistant locations in every state that we evaluated. We additionally illustrated that zeta-cypermethrin, another type II pyrethroid, was also not efficacious in lambda-cyhalothrin resistant locations (see chapter 3). Illustrating the potential for cross or multiple resistance for these location samples.

With documented resistance to lambda-cyhalothrin in the western region, we needed to establish the extent of cross or multiple resistance among pyrethroid active ingredients within resistant locations. In three distinct alfalfa production zones, we utilized the same laboratory bioassay methods in chapter 2, included three field trials to corroborate our laboratory findings, and utilized molecular biology to identify alfalfa weevil strains in the field trial locations. These efforts illustrated that regardless of location within the western United States and strain, the pattern of multiple resistance among type II pyrethroid active ingredients was consistent in both our laboratory bioassays and in all three field trials. Meaning, that with resistance to lambda-cyhalothrin in the western United States, all MoA 3A insecticides registered for alfalfa weevil control in forage alfalfa systems will also fail (see chapter 4). Thus, challenging the economic profitability of forage alfalfa production.

To provide resources and tools to stakeholders in the region we developed an integrated resistance management and integrated pest management focused peer reviewed publication (see chapter 5). This document includes equations to aid producers to determine when chemical control measures are required and what alternative tools are available. The goal here, is to reduce producer reliance on chemical control tactics to improve pyrethroid efficacy in resistant systems and to help protect indoxacarb, MoA 22A, efficacy. Overall, the synthesis of the work included in this dissertation provides scientific evidence that confirms early reports of pyrethroid failure in California, Alberta Canada, and the Pacific Northwest. Specifically, we 1) identified lambda-cyhalothrin resistance in Montana; 2) we identified lambda-cyhalothrin resistance in Arizona, California, Montana, Oregon, Washington, and Wyoming; 3) we identified that all MoA3A pyrethroid insecticides registered for forage alfalfa systems are no longer effective in lambda-cyhalothrin resistant locations in the western region; and 4) we provide recommendations and tools to help address this emerging agricultural challenge.

## Future Work in Alfalfa Weevil Pyrethroid Resistance

The work shown here illustrates the severity of alfalfa weevil (*Hyper postica* Gyllenhal [Coleoptera: Curculionidae]) pyrethroid resistance in the western region. While beyond the scope of the dissertation, the data generated here can be used to inform forage alfalfa producers, crops advisors, Extension agents, and industry representatives of this emerging agricultural challenge. For future research efforts, this data can aid in selecting location samples to identify mechanisms of resistance, future research field sites to identify optimal timing for insecticide applications, and to determine the economic burden of pyrethroid resistance for producers. In all, this research

has provided valuable data to help direct future research efforts focused on the control of alfalfa weevils in the western United States.

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