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Toxicology of Chemical Stress to Monarch Butterflies (*Danaus plexippus* L.)

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TOXICOLOGY OF CHEMICAL STRESS TO MONARCH BUTTERFLIES (*Danaus plexippus* L.)

by

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TOXICOLOGY OF CHEMICAL STRESS TO MONARCH BUTTERFLIES (*Danaus plexippus* L.)

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Monarch butterfly (*Danaus plexippus* L.*)* population declines have caught the attention of the country and prompted nationwide conservation initiatives. The United States Fish and Wildlife Service has identified insecticide exposure and loss of milkweed (*Asclepias spp.*) reproductive habitat as primary threats to the monarch. In the Midwestern US, milkweed largely occurs around cropland borders where there may be a spatial and temporal overlap of monarch larvae, insecticide usage, and fertilizer applications. In this study, the acute toxicity and sub-lethal effects on growth and diet consumption of two commonly used pyrethroid insecticides, bifenthrin and beta-cyfluthrin, were characterized in fifth instar monarch larvae. While beta-cyfluthrin was more toxic than bifenthrin, foliar applications of formulated products, Baythroid (beta-cyfluthrin) and Brigade 2-EC (bifenthrin), would result in sub-lethal and lethal effects at similar distances from a treated field edge according to the United States Environmental Protection Agency AgDrift model. As monarch larvae consume milkweed leaves they also ingest insecticidal cardenolides, which are antagonized by potassium. We examined the effects of ouabain, a hydrophilic cardenolide, and potassium chloride (KCl), a commonly used potassium fertilizer, in monarch caterpillars following chronic oral exposure. Once effect thresholds of ouabain and KCl were determined, bifenthrin toxicity

was compared between different combinations of ouabain and KCl diets. Elevated concentrations of ouabain increased caterpillar growth and decreased development time whereas elevated concentrations of KCl decreased caterpillar growth and diet consumption. There was no difference in bifenthrin toxicity on different diet combinations. Milkweed species contain a variety of cardenolides that vary in concentration and polarity. Toxicity of bifenthrin and effects on detoxification enzymes were characterized in monarchs feeding on tropical milkweed (*A. curassavica*), a high cardenolide species, and swamp milkweed (*A. incarnata*), a low cardenolide species. Detoxification gene expression and enzyme activity significantly differed between milkweed species and between solvent control and bifenthrin treated caterpillars on each species. Understanding physiological differences in monarchs developing on different milkweed species is important for maximizing the benefits of habitat restorations among agricultural landscapes.

PREFACE

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CHAPTER 4 is currently under preparation for publication in *Pesticide Biochemistry and Physiology* (A. J. Krueger, L. C. Rault, E. A. Robinson, T. J. Weissling, A. M. Vélez, and T. D. Anderson *"Milkweed species affect detoxification enzyme activity and expression in monarch caterpillars"* (in preparation for submission to refereed journal).)

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CHAPTER 1 : LITERATURE REVIEW

Monarch biology

Distribution and migration

The monarch butterfly (*Danaus plexippus* L.*)* is a globally distributed species. It has become an icon due to its extensive migration across North America. There are three subpopulations in North America including the migratory population west of the Rocky Mountains, the migratory population east of the Rocky Mountains, and the residential (i.e., non-migratory) population in Florida. The migratory monarch butterflies residing east of the Rocky Mountains overwinter in vast numbers in Mexico. In the spring, these monarchs travel north into Texas and Oklahoma where the migrating females begin depositing eggs[1]. Each subsequent generation advances farther north, mating, laying eggs, and spreading throughout the Midwestern and Eastern United States. By August, the fourth generation from the initial spring migrants begin their southern migration and return to the oyamel firs in Mexico for overwintering[2].

Growth and development

Monarch butterflies are holometabolous insects that develop through five larval instars[3]. Mated females deposit eggs on milkweed that hatch within 3-4 d. Development time from egg to adult eclosion is highly correlated with temperature and growing degree day (GDD) models can account for this interaction with temperature, representing the accumulated "daily total of degrees (C^{o}) that occur between minimum and maximum temperature thresholds" [4]. Monarchs require 186 degree-days to develop from eggs to pupae, and 307 degree-days to develop from eggs to adult eclosion[5]. At a constant 19.5

ºC, first-instar caterpillars took 561 hours to reach pupation and 1084 hours to eclose whereas caterpillars maintained at 31.0 ºC pupated within 218 hours and eclosed in 361 hours[6]. Development time can vary further based on solar radiation[7,8]. Caterpillars can behaviorally thermoregulate and increase body temperature to 3-8 ºC above ambient temperature to reduce development time by 10-50%[6]. This behavior is largely facilitated by the caterpillars' aposematic coloration that allows individuals to remain exposed to predators throughout the day[6]. The general survival of caterpillars is quite low, with only around 5% of caterpillars developing to the last larval instar[9]. Predation, parasitism, and infection with *Ophyryocystis elektroschirra* (OE) contribute to the observed high mortality rate of monarch caterpillars[10,11]. There is a significant correlation between the development time and survival of caterpillars. For example, caterpillars that develop more quickly have higher pupation success and survival compared to individuals that take longer to develop[11]. In addition to development time, caterpillar body mass is an important predictor for adult longevity[12].

Cardenolide insensitivity

Caterpillars are obligate feeders on milkweed (*Asclepias* spp.), plants protected by toxic compounds known as cardenolides. Caterpillars require the equivalent of an entire milkweed stem to sustain their development from first through fifth instar stages [13]. Monarchs have co-evolved with milkweeds developing both target-site insensitivity to cardenolides and the ability to sequester these compounds[14]. Cardenolide-insensitive butterfly species (e.g., *Danaus gilippus*) possess a single mutation of glutamine (Q) to valine (V) at the 111 position in the ouabain binding site of the Na^+/K^+-ATPase[15]. Monarchs have the additional mutations of alanine (A) to serine (S) and asparagine (N) to

histidine (H) at the 119 and 122 positions, respectively[15,16]. These mutations confer cardenolide resistance and sequestration in genetically modified *Drosophila melanogaster*[16]. Cardenolide sequestration was only observed when all three mutations were present, which suggests modifications at the ouabain-binding site of the Na^+/K^+-ATPase are related to sequestration[17]. The mechanism of sequestration in monarchs and the environmental factors affecting sequestration, however, are still largely unknown[18,19].

Milkweed biology

Cardenolide structure and function

Cardenolides are secondary plant metabolites within a sub-class of cardiac glycosides derived from triterpenoids with broad-spectrum insecticidal activity [18]. The basic chemical structure (Figure 1.1a) contains a 23-carbon unit with 1) a steroid backbone structure of four fused C rings, 2) a five-membered lactone ring group in the β-position at C17, and 3) a carbohydrate or sugar moiety attached to C-3 of the first carbon ring [18]. These compounds act on the nervous system, specifically targeting the Na^+/K^+-ATPase and reversibly binding to the α-subunit to lock it in its phosphorylated E2-P conformation and disrupt ion translocation and nerve function [20,21]. The steroid nucleus 5β,14β-androstane-3β14-diol plays a critical role in binding at the target site and receptor recognition. There are over 500 identified cardenolide derivatives with a wide range in structural modifications [18,22].

Species differences

Cardenolides have been documented in 60 genera of 12 families of plants, although the re-classification of plants in recent years may have changed these estimates [18,23]. Within the former Asclepiadaceae family, now part of the Apocynaceae family,

there are 20 cardenolide-bearing genera[23]. Cardenolides produced in Apocynaceae differ structurally from *Digitalis spp.* (Plantaginaceae), containing the *trans*-conformation of A and B steroid rings (Figure 1.1b) instead of the *cis*-conformation (Figure 1.1c), resulting in decreased toxicity[18,23]. Plants in Apocynaceae also tend to have either highly polar or highly non-polar cardenolides, and *Asclepias* species that produce higher cardenolide concentrations tend to invest more in highly polar cardenolides [23,24].

Nearly all of the 108 *Asclepias* species of milkweed produce cardenolides albeit at different concentrations [25,26]. It is a challenge to compare cardenolide concentrations across studies due to different analytical techniques and reported units of concentration (e.g., µg equivalent of digitoxin/0.1 g dry weight, mg total cardenolide/g dry weight, mg total cardenolide/g fresh weight, µmol cardenolide/g dry weight). Despite these differences, North American *Asclepias sp.*, including the twiggy shrub-like *A. masonii, A. albicans, A. subulata, A. subaphylla, and A.linaria* of the southwest and the leafy shrub species *A. curassavica, A. asperula, A. humistrata,* and *A. viridis* in the east, are reported to have the highest cardenolide concentrations [23,25]. Pocius et al. (2017) used cardenolide values reported in Rasmann and Agrawal (2011) and ranked 8 *Asclepias spp.* in the Midwest by decreasing order of cardenolide production as follows: *A. hirtella, A. sullivantii, A. syriaca, A. speciosa, A. exaltata, A. incarnata, A. tuberosa, A. verticillate* with reported cardenolide values at 3.289, 2.149, 1.573, 1.112, 0.735, 0.511, 0.070, 0.031 µg/mg dry weight, respectively [27,28].

Cardenolide distribution within plants

Cardenolide content is highly variable across an individual plant and can change throughout the growing season and/or with increased herbivory. In a California milkweed

species, *A. eriocarpa*, the plant latex contained significantly greater quantities (i.e., 16 mg/g fresh weight in latex) than other parts of the plant such as the stems (i.e., 1.46 mg/g fresh weight)[29]. The distribution of polar and nonpolar cardenolides also varies across the plant, with more nonpolar cardenolides found in the plant latex[29]. Manson et al. (2012) compared cardenolide concentrations in leaves, flowers and nectar across 12 *Asclepias spp.* and determined cardenolide concentrations in nectar were significantly lower than in leaves and flowers, except for *A. pumilla*, which had relatively higher cardenolide concentrations in nectar[30]. In this same study, tropical milkweed, *A. curassavica*, had significantly higher cardenolide concentrations in flowers compared to leaves and no detectable cardenolides in nectar. The roots of *Asclepias spp.* are reported to have significantly lower amount of constitutive and inducible cardenolides compared to aboveground biomass[31].

Abiotic factors affect cardenolide production

In addition to varying across species and plant parts, cardenolide concentrations are modulated by soil nutrient concentrations and fertilizer applications. In *A. curassavica* [32] and *A. syriaca* [33], fertilizer applications containing higher phosphorous levels decreased total cardenolide levels however, nitrogen fertilizer could negate this effect on cardenolide concentration . In *Digitalis obscura*, Roca-Perez et al. (2005) observed a significant positive relationship between soil and leaf magnesium concentrations and higher leaf magnesium concentrations correlated with higher total cardenolide concentrations[34]. Furthermore, Roca-Perez and colleagues (2002) also found that cardenolide concentrations were significantly reduced with higher levels of copper in soil and phosphorous in leaf tissue [35]. This negative relationship with phosphorous

suggested a trade-off between primary and secondary metabolism in the plant, with higher phosphorous levels favoring primary metabolism and plant growth over the production of cardenolides [36].

The effects of elevated carbon dioxide (CO_2) levels on cardenolide production can vary between milkweed species. Decker et al. (2018) compared the effects of elevated CO_2 levels on cardenolide production in four *Asclepias spp.* and report cardenolide production was only affected by elevated CO_2 levels in one of the four species tested (*A. curassavica, A. speciosa, A. syriaca, A. incarnata*)[37]. The high cardenolide-producing milkweed species *A. curassavica* was shown to have a significantly lower concentration of total cardenolides (~ 2 mg/g fresh weight) when grown in 760 ppm CO_2 conditions compared to the concentration of total cardenolides (~ 3 mg/g fresh weight) in plants grown in ambient CO_2 levels of 400 ppm and this reduction of total cardenolides was specifically related to a decrease in the concentration of lipophilic cardenolides[37]. In *A. syriaca*, Vannette and Hunter (2011) found a significant 2-fold reduction in cardenolide concentration in 2 of 5 genotypes grown under elevated CO_2 conditions (775 ppm CO_2) and this decline was related to a decrease in polar cardenolide concentrations[38].

Water stress has also been shown to affect cardenolide concentrations. For *A. syriaca*, Couture and colleagues (2015) reported water stress to increase cardenolide production when combined with temperature stress[39]. This observed response was variable across milkweed populations and correlated with changes in plant biomass. Similarly, Agrawal and colleagues (2014) documented a 2-fold increase of cardenolide production in *A. syriaca* under drought conditions [40]. Ultimately, drought conditions

reduced plant biomass which in turn, increased cardenolide concentrations on a dry weight basis (i.e., ng/g dry weight).

When evaluating effects of cardenolides or milkweed species on monarch physiology, it is important to understand abiotic factors affecting cardenolide production and how these effects might differ between milkweed species. Elevated CO_2 levels and water stress are known to affect cardenolide concentrations[37-40]. As both of these abiotic factors are changing due to land use and climate change, it is critical that we understand the impact these factors will have on milkweed and the insect species that rely on milkweed as a host plant.

Biotic factors affecting cardenolide production

There are differential responses from insect herbivory depending on the insect and the milkweed species. Malcolm and Zalucki (1996) demonstrated the mechanical wounding of *A. syriaca* induced cardenolide defenses within 1 h[41]. Under more natural conditions, a variety of insect herbivores with different feeding behaviors feed on milkweed. Insect herbivory induces differential responses in cardenolide production among milkweed species depending on the species of insect causing feeding damage. The oleander aphid, *Aphis nerii,* and monarch caterpillars have evolved as milkweed specialists feeding on and sequestering high concentrations of cardenolides[42]. However, the differences in sucking versus chewing feeding behaviors between these two specialists yield different effects on plant cardenolide induction. Oleander aphid-mediated cardenolide induction also differs across milkweed species. Zehnder and Hunter (2007) found *Aphis nerii* feeding on *Asclepias viridis* resulted in a significant decrease in total cardenolide production although there were no significant changes in cardenolide

production for three other milkweed species tested in the study [43]. Similarly, Meier and Hunter (2019) observed *Aphis nerii* feeding on *Asclepias curassiva* to decrease cardenolide production, but not *Asclepias incarnata*[44]. Further, Mooney et al. (2008) reported *Aphis nerii* feeding did not change cardenolide production in *Asclepias syriaca* [45]. Based on these studies, the suppression of cardenolide defenses by *Aphis nerii* appears to be highly dependent on milkweed species and may be more clearly quantified in species with higher constitutive cardenolide production.

Monarch caterpillar feeding, in contrast to that of *Aphis nerii*, had only been observed to increase cardenolide production in milkweed. Mechanistically, the chewing of milkweed by monarch caterpillars has been shown to induce the jasmonic acid pathway[46]. An induction of the jasmonic acid pathway is reported to significantly increase cardenolide production in milkweed. Mooney et al. (2019) found exogenous applications of jasmonic acid that mimic a chewing-induced response increased total cardenolide content by 33% in *A. syriaca* after 13 d [45]. Bingham and Agrawal (2010) found cardenolide levels were 26% higher in *A. syriaca* plants after monarch feeding relative to control plants[47] and Vannette and Hunter (2011) measured a 31% increase in total cardenolide concentrations in *A. syriaca* under after monarch caterpillar herbivory, with a 50% increase in a highly polar cardenolide[38].

For other milkweed herbivores, Fordyce and Macolm (2000) showed an overall decrease of cardenolide concentration in *A. syriaca* following oviposition of the stem weevil, *Rhyssomatus lineaticollis* [48]. However, while total cardenolide concentration decreased, there was an increase of nonpolar cardenolide concentrations following

oviposition chemical cues. Mooney et al. (2008) found feeding by the beetle *Tetraopes tetraophthalmus* did not elicit a change in cardenolide content relative to plants without herbivory[45]. However, Rasmann et al. (2011) report *T. tetraopthalmus* larvae feeding on roots of high cardenolide-producing *A. syriaca* genotypes induced cardenolide production by 40% in root tissue 4 d after feeding[49].

Cardenolide exposure for monarch caterpillars will vary depending on abiotic factors as well as the presence and feeding of other milkweed arthropods. Milkweed communities consist of a diversity of arthropod species across several different insect orders. While monarch feeding will induce cardenolide defenses, feeding or oviposition by other arthropods may further alter the concentration and composition of cardenolides monarchs are exposed to. Cardenolide exposure is highly dynamic and interactions in milkweed communities may further complicate studies exploring effect of cardenolides on monarch physiology.

Decline of milkweed and current distributions

Over the past 70 years, the abundance of milkweed has been vastly reduced from the landscape, with the majority of remaining milkweed occurring near agricultural landscapes[50]. Across the monarch breeding habitats in the Eastern and Midwestern U.S., the increased use of the herbicide glyphosate couples with the expansion of farmland over the past two decades have greatly diminished the presence of milkweed[50–52]. Museum records demonstrate a significant decline in milkweed abundance since 1950, although the decline is variable among milkweed species[53]. For example, the abundance of *A. syriaca* has largely remained steady for the last 50 years, while over the same time

period the abundances of *A. incarnata, A. tuberosa,* and *A. verticillata* have greatly declined.

Monarch population decline and implicated stressors

USFWS assessment of decline

The reduction of overwintering monarchs in Mexico[54] and California[55] parallels the decline in milkweed abundance over the past 20 years. In 2014, the Center for Biological Diversity, Center for Food Safety, Xerces Society, and Dr. Lincoln Brower petitioned the U.S. Fish and Wildlife Service (USFWS) to consider the monarch butterfly as a threatened species to be listed under the Endangered Species Act of 1972[56]. The USFWS concluded the listing was warranted and initiated a range-wide status review to assess the global population of the monarch butterfly and to identify key influences contributing to the species' current condition[57]. The USFWS identified "loss and degradation of habitat (i.e., conversion of grasslands to agriculture, widespread use of herbicides, logging/thinning at overwintering sites in Mexico, senescence and incompatible management of overwintering sites in California, urban development, and drought), continued exposure to insecticides and effects of climate change" as the primary drivers affecting the health of North American monarch populations. Below, I will briefly review the key influences affecting the eastern migratory population.

Stressor: loss of overwintering habitat

The loss of habitat for the eastern monarch population includes both a loss of overwintering habitat (i.e., oyamel firs) in Mexico as well as breeding habitat (i.e., milkweed). Illegal logging has changed the microclimate in the Biosphere Reserve and, in combination with increased winter storms due to climate change, overwintering

monarchs are more exposed to the elements[58]. For example, a 2002 winter storm in the Biosphere killed more than 80% of the overwintering adults when storms produced prolonged cold and wet conditions that effectively froze adult butterflies[59]. Beyond winter storms, climate change will also produce lethal temperatures for the oyamel firs, reducing the forest by 69.2% by 2030, 87.6% by 2060 and 96.5% by 2090[60].

Stressor: loss of breeding habitat

The loss of breeding habitat throughout the U.S. has further challenged overwintering monarchs. Adult female monarchs lay one egg per milkweed stem and, thus, the monarch population is reliant on the number of milkweed stems present in a landscape[9]. The loss of milkweed stems is largely attributed to conversion of grassland to agriculture and the increased use of glyphosate, which in turn limits available oviposition substrates for monarchs [50]. Boyle et al. (2018) has shown that before the use of glyphosate, the abundance of several different milkweed species was already declining, however this decline was immediately followed by an increase in *A. syriaca* abundance[53]. The authors hypothesize the earlier decline in milkweed species is in part due to the consolidation of small farms and the loss of uncultivated land between farms, where many of these milkweed species flourished.

Stressor: insecticide usage

The monarch breeding season overlaps spatially and temporally with row crop production across the Eastern and Midwestern United States. Adult monarchs first arrive in the North Central U.S. from Mexico toward the end of May[1,61]. During this time, early season insecticides are applied to reduce true armyworm infestations exceeding economic thresholds[62]. These insecticide applications continue later in the season to manage

increasing insect pest populations, such as the soybean aphid[63,64], western bean cutworm[65], and western corn rootworm[66]

Given the nationwide distribution of monarchs during their breeding season, using 2018 NASS insecticide usage statistics[67,68], monarchs were potentially exposed to insecticides across 25 million acres of crop land and surrounding field margins, if individuals were present at the time of application. Direct exposure to adult monarchs is not likely a major route of exposure. However, spray drift following foliar applications may be deposited in milkweed in adjacent field margins, posing a chronic threat to monarchs on the downwind field edge[63]. Several studies in Europe have shown decreased butterfly abundance in margins of treated fields [69–71], suggesting milkweed bordering treated fields may expose monarch caterpillars to lethal levels of insecticide exposure.

Pyrethroids and agriculture

Mode of action

Pyrethroid insecticides are neurotoxic compounds that target the voltage-gated Na^+ channel to disrupt neurological function[72]. Pyrethroids fall in to two main classes, type I and type II, differing in both structure and symptomology: type I lack a cyano moiety, type II possess a cyano moiety at the α-positions[73]. Type I pyrethroids reversibly bind to the voltage-gated Na^+-channel which leads to type I poisoning syndrome (T syndrome), characterized by hyperexcitation, whole body tremors, and progressive paralysis in rats[74]. Examples of type I pyrethroids include allethrin, bifenthrin, resmethrin, and permethrin[75]. Type II pyrethroids cause prolonged interference with the gating kinetics of the voltage-gated Na^+-channel leading to increased toxicity as well as type II poisoning syndrome (CS-syndrome) characterized by hypersensitivity,

choreoathetosis and profuse salivation in rats[74–76]. Examples of type II pyrethroids include deltamethrin, cypermethrin, cyhalothrin, λ-cyhalothrin, cyfluthrin, and β-cyfluthrin[75].

Pyrethroids in agriculture pest management

Pyrethroids are broad-spectrum insecticides used to control insect pests in agriculture crops, such as corn and soybean[66,77,78], and in the control of disease vectors and structural pests in urban environments[79]. In 2018, 16% of corn acreage in Nebraska and 18% of corn acreage in Iowa were treated with foliar insecticides and 12% of acreage in both Nebraska and Iowa were treated specifically with bifenthrin in 2018[67,68]. In terms of acreage, 9.6 million acres were planted with corn in Nebraska, 1.5 million acres of corn were treated with foliar insecticides and 1.2 million acres of treated corn were treated with bifenthrin. In Iowa, 13.2 million acres were planted with corn, 2.4 million acres of corn were treated with foliar insecticides, and 1.6 million acres were specifically treated with bifenthrin.

For corn, pyrethroids have been commonly used to control western corn rootworm, western bean cutworm, and two-spotted spider mite[66]. For soybeans, pyrethroids have been used to control bean leaf beetle and soybean aphid[80,81]. Below, I will use the introduction of soybean aphid to the U.S. in 2000 as an example to examine the relatively recent increased insecticide usage occurring during the monarch breeding season.

Soybean aphid management

In 2000, soybean aphids were first reported in the North Central U.S. [82] and just nine years later, 30 states reported soybean aphid outbreaks [64]. Soybean aphids

overwinter as eggs on buckthorn, *Rhamnus cathartica* L., that hatch in the spring [83]. Populations build up for several wingless generations in buckthorn until soybean emerges and winged adults move into soybean. Once in soybean, aphids continue to reproduce for upwards of 15 generations producing all females [84]. During this time, female aphids can disperse to other soybean fields with assistance from the wind [64]. Under ideal laboratory conditions, without any natural control factors, soybean aphids are able to double their numbers in 1.5 d [85].

Soybean aphid feeding stunts plant growth and reduces yield by disrupting photosynthetic processes necessary for seed set [86,87]. Additional injury can occur from secondary infections caused by aphid feeding. Honeydew secretions create a medium for black sooty mold to develop and feeding wounds on the plant create entryways for pathogens [64]. The economic impact of soybean aphid can vary but if no treatment is applied, soybean aphid would cost producers $1.2 billion USD and consumers $546 million USD over 5 years [88]. And result in annual yield losses as high as 40%[64]. Prior to the arrival of soybean aphid, few growers applied insecticides and defoliating insect populations were naturally kept in check [83]. Since the arrival of soybean aphid, insecticide usage has increased 130-fold [80]. It is generally recommended to apply foliar insecticides (i.e., organophosphates and pyrethroids) 3-7 d after exceedance of the economic threshold or sooner due to rapidly increasing aphid populations [80]. The exceedance of economic thresholds and subsequent insecticide applications can often occur in late summer.

Soybean aphids are just one example of an insect pest that has increased usage of foliar insecticides. New emerging pests, such as soybean gall midge, may also increase insecticide usage in the Midwest when monarchs are present on the landscape[89]. However, insecticide applications particularly for controlling soybean aphids, may affect the fourth generation of monarchs that will migrate to the overwintering grounds in Mexico[62].

Potassium exposure and relevance

Milkweed habitat bordering crop fields can intercept agricultural inputs via spray drift, run-off or systemic uptake. When evaluating chemical exposure in habitat bordering agricultural fields, additional chemical inputs such as chemical fertilizers are often overlooked despite the massive quantities of nitrogen, phosphorous, and potassium fertilizers applied each year[90].

Potassium as a nutrient and fertilizer in plant health

Potassium is an essential nutrient in plant growth and stress physiology[91], however it is deficient in soils across several midwestern states[92]. Potassium fertilization can help increase drought tolerance and immune defense in agricultural crops and may be an important tool in combatting effects of climate change[93]. Currently, millions of tons of potassium fertilizer are applied in the Midwest and nationally, with 63% of corn acreage, 43% of soybean acreage, and 45% of cotton acreage receiving applications of potash fertilizer[90]. In states with potassium deficient soils such as Missouri, Alabama and North Carolina[92] that also support the eastern monarch butterfly migration, the percentage of cotton acreage receiving applications of potash fertilizer can be 80-96%[90]. With heavy rains in the spring, the timing of pre-plant fertilization is becoming more difficult and in

corn, studies have shown fertilizer applications at the V6 growth stage can be used when pre-planting fertilization is not possible[94]. Applications at this time would coincide with the timing of the monarch breeding season[62] and could increase potassium levels in milkweed. While foliar applications of potash are not widely used, there is evidence in soybean cropping systems for increased yield and protective benefits from fungal infections following foliar applications[95,96]. Spray drift from these applications may be deposited onto milkweed leaves or on developing monarchs near agriculture.

Role in insect physiology

Potassium ion regulation plays a key role in many physiological processes in insects. As in mammals, potassium ion balance across neurons dictates nerve function and signal transduction[97]. In addition to nerve function, potassium levels are highly regulated in hemolymph and play a critical role in molting[98,99]. Studies examining potassium regulation in Lepidoptera have quantified K^+ levels in larval tissues several days after feeding on KCl-spiked diets and demonstrated elevated levels of potassium in the hemolymph as well as tight regulation of ion balance[100–102]. However, increased levels of K^+ may impose an increased energetic cost of maintaining proper ion balance.

Pharmacology

Studies exploring pharmaceutical applications of cardenolides have identified potassium as an antidote for cardenolide poisoning in mammals, showing a reversal of effects and recovery of Na^+/K^+-ATPase function with increasing K^+ serum levels[103,104]. This reversal has been attributed to competitive binding and potassium antagonism at the target site[105]. Studies on monarchs and other Lepidoptera have also demonstrated this antagonism exists in insects, even with the structural modifications in the monarch

Na^+/K^+-ATPase[106]. Despite this evidence, the implications of potassium exposure for monarch physiology and sequestration have remained unexplored. In an agricultural setting, influxes of potassium through applications of potassium fertilizers have the potential to affect the sequestration and protective benefits of cardenolides for developing monarchs. While potassium is highly regulated in plants and insects, potassium fertilizers may expose monarchs to levels of K^+ above regulated biological limits that could alter monarch physiology or add additional energy requirements to maintain proper ion balance.

Detoxification

Cross resistance

The ability of the monarch butterfly to cope with milkweed cardenolides has been well characterized. However, implications of this evolved resistance on detoxification have largely remained unexplored. Insects with chemically defended host plants often have developed metabolic resistance to cope with phytotoxins[107]. Overproduction of esterase enzymes or cytochrome P450 monooxygenases have been documented in a number of species[108]. This phenomenon has prompted exploration of cross-resistance between plant allelochemicals and synthetic insecticides. Swallowtail butterflies, *Papilio glaucus canadensis*, evolved resistance to phenolic glycosides in the leaves of their host plants of the Salicaceae family through elevated esterase activity but when challenged with two different pyrethroids, this elevated activity had no effect on pyrethroid toxicity[109]. In insect pest species, there are several documented examples of cross-resistance. Corn earworm (*Helicoverpa zea*) larvae feeding on a diet containing the allelochemical xanthotoxin showed a higher tolerance to the pyrethroid α-cypermethrin,

and increased tolerance was observed in the offspring of xanthotoxin-exposed individuals[110]. In beet armyworm (*Spodoptera exigua*), larvae exposed to the flavonoid quercetin were more resistant to the pyrethroid λ-cyhalothrin with a more than 2-fold increase in the LD_{50}[111].

In monarchs, little is known about detoxification enzyme activity and the potential for cross-resistance or impact of cardenolide resistance on detoxification enzymes. Recent work has shown changes in expression of some detoxification genes in monarch caterpillars after feeding on different milkweed species[112]. Further, Krishnan et al. (2020) documented a significantly higher LD_{50} for imidacloprid when caterpillars fed on tropical milkweed, a high cardenolide milkweed species, compared to common milkweed[63]. These recent discoveries provide further reason to explore the potential for cross-resistance in monarchs.

Overview of research

Understanding the risk of habitat bordering agricultural landscapes to monarch caterpillars is critical for informing conservation efforts in the US Midwest. Monarchs are exposed to cardenolides as they feed on milkweed however cardenolide exposure will vary significantly depending on what milkweed species they feed on, where on the plant they feed, what other arthropods are feeding on the plant, and what environmental conditions the plant is growing under. Milkweed bordering agricultural fields may expose monarchs to agricultural inputs such as pyrethroid insecticides and potassium fertilizers. Pyrethroids like bifenthrin are used across millions of acres of corn and soybean production across the country and foliar applications may adversely affect monarch caterpillars developing in treated field margins. Additionally, potassium fertilizer

applications may also adversely affect monarchs through increased energic costs of osmoregulation or by altering cardenolide sequestration through antagonism of cardenolides at the Na^+/K^+-ATPase. Ultimately, understanding the role of milkweed species selection in habitat restoration near agriculture and how milkweed species could alter pyrethroid sensitivity will help inform conservation practices.

The second chapter of this dissertation investigates the acute contact toxicity of two pyrethroid insecticides to monarch growth and development. Using the USEPA AgDrift model, effect thresholds for both pyrethroids could be put into context on the landscape by predicting spray drift exposure in habitat bordering a treated field.

The third chapter of this dissertation explores how additional chemical interactions of potassium fertilizers and milkweed cardenolides in habitat bordering agriculture might affect bifenthrin toxicity. Monarch caterpillars were exposed to the cardenolide, ouabain, or the potassium fertilizer, potassium chloride (KCl), and effects on growth and development were assessed for each individual compound. Caterpillars were then exposed to combinations of ouabain and KCl to assess effects of dietary exposure on bifenthrin toxicity.

The fourth chapter of this dissertation explores the potential for cross resistance in monarch caterpillars by comparing bifenthrin toxicity and effects on detoxification enzymes when caterpillars feed on different milkweed species. Caterpillars developed on either a high cardenolide milkweed species, tropical milkweed (*A. curassavica*), or a low cardenolide milkweed species, swamp milkweed (*A. incarnata*) prior to bifenthrin exposure. Detoxification gene expression, enzyme activity, and survival were quantified.

The fifth chapter of this dissertation summarizes conclusions of each research chapter and provides an overview of future research directions.

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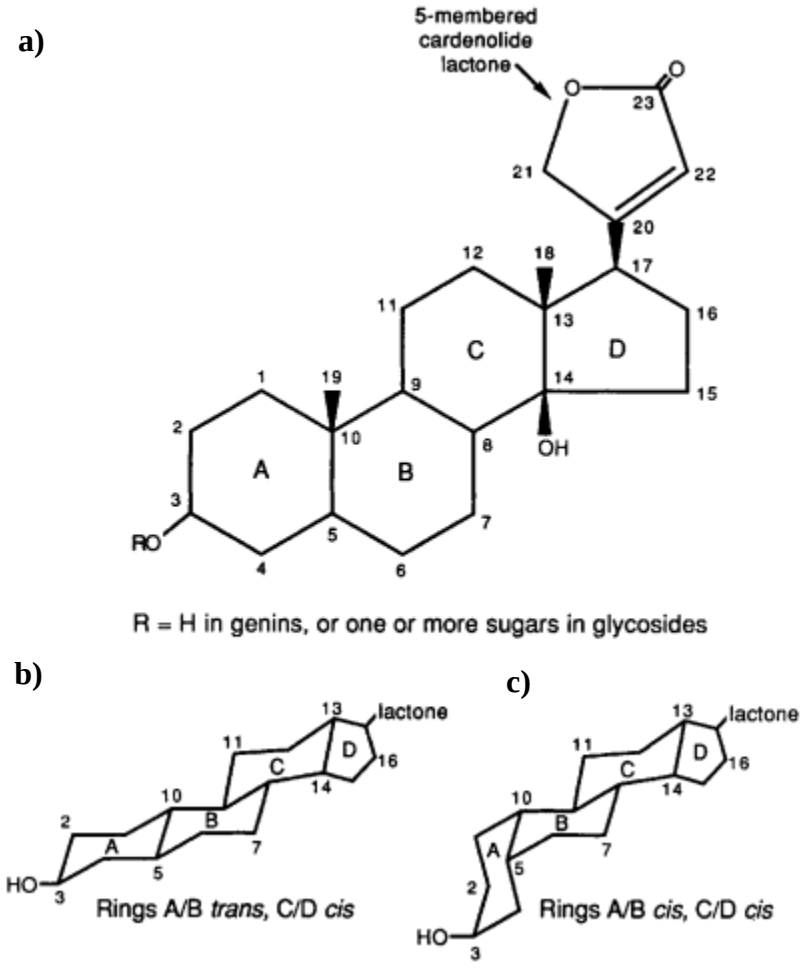

Figure 1.1 Generalized cardenolide structure (a) and specific conformation differences between *Digitalis* (b) and *Asclepias* (c) cardenolides. Figure adapted from Malcolm 1991, used with permission.

CHAPTER 2 : PYRETHROID EXPOSURE REDUCES GROWTH AND DEVELOPMENT OF MONARCH BUTTERFLY (Lepidoptera:Nymphalidae) CATERPILLARS

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1. INTRODUCTION

The monarch butterfly *Danaus plexippus* L. (Lepidoptera: Nymphalidae) is a globally distributed species, primarily in the Americas and Oceania. In North America, it has become an icon for extensive migration across the continent. Monarchs east of the Rocky Mountains overwinter in vast numbers in Mexico and travel north in the spring, covering most of the Midwest and east coast, advancing farther north with every generation (Oberhauser and Solensky 2004). By the fourth generation, the adults make the thousand-kilometer flight to return to overwintering grounds in Mexico (Alonso-Mejia et al. 1997). This unique life history has made the North American population more susceptible to multiple stressors, both in their overwintering grounds and breeding habitat. The monarch is a charismatic flagship for invertebrate conservation more broadly (Oberhauser and Guiney 2009) and the conservation of the monarch butterfly has been valued upwards of $4 billion according to a survey of U.S. households (Diffendorfer et al. 2014). An understanding of the threats to and conservation opportunities for the monarch butterfly is critical for securing further public engagement for invertebrate conservation.

In the U.S., the increased use of glyphosate and expansion of farmland over the past 40 years has greatly diminished the presence of milkweed in the breeding grounds

and removed it almost entirely within fields (Pleasants and Oberhauser 2013, Pleasants 2017, Thogmartin et al. 2017). Pleasants and Oberhauser (2013) documented a *ca*. 4-fold difference between egg densities on milkweed in agricultural fields compared to milkweed on roadsides or in pastureland. To make up for this loss of preferred oviposition habitat, researchers have set a 1.8 billion milkweed stem goal to restore and stabilize the overwintering monarch population (Thogmartin et al. 2017). While the number of milkweed stems on the landscape has been the focus of conservation efforts, the location of these stems and their proximity to commercial agriculture has raised concerns over the risk of agrochemicals to monarchs. In Europe, several studies have shown decreased butterfly abundance in margins of fields treated with foliar applied insecticides (Çilgi and Jepson 1995, Longley et al. 1997, Rundlöf et al. 2008). In the 1990's, *Bacillus thuringiensis* (*Bt*) crops and pollen expressing *Bt* Cry1 proteins targeting lepidopteran pests were heavily investigated for the risk to developing monarchs, however, the risk of most varieties on the market was considered negligible (Sears et al. 2001). Although the risk of *Bt* crops was heavily studied, toxicity data detailing the risk of other insecticide products to monarchs is limited. Braak et al. 2018 report insecticide data for a number of lepidopteran species and found only three available toxicity studies for monarchs using permethrin (Oberhauser et al. 2006), imidacloprid (Krischik et al. 2015), and clothianidin (Pecenka and Lundgren 2015). While more exhaustive toxicity (Krishnan et al. 2020) and exposure data (Olaya-Arenas and Kaplan 2019) are becoming available for different life stages and classes of insecticides, data are lacking on the sub-lethal effects of insecticide exposures to monarchs.

Pyrethroid insecticides are commonly used to control insect pests of corn and soybean across the U.S. (Ragsdale et al. 2011). These broad-spectrum insecticides are acutely neurotoxic, targeting the voltage-gated Na^+ channel and disrupting neurological function (Clements and May 1977). Pyrethroids are classified as type I or type II based on their chemical structure, effects on the central nervous system and subsequent symptomology (Gammon et al. 1981). Pyrethroid studies in butterfly species have focused on compounds largely used for mosquito management, including permethrin and deltamethrin (Braak et al. 2018). However, in agriculture, pyrethroids like bifenthrin and β-cyfluthrin are used in much larger quantities but toxicity data has only recently become available for β-cyfluthrin (Krishnan et al. 2020). Because of their acute toxicity, pyrethroids have been used to control a variety of insect outbreaks. For example, soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae) outbreaks and subsequent foliar applications of pyrethroids often occur in mid-July and again in mid-September when monarch caterpillars are present on the landscape (Nail et al. 2015, Bradbury et al. 2017). In Iowa, true armyworm populations *Mythimna unipuncta* Haworth (Lepidoptera: Noctuidae) can exceed economic thresholds in mid-May and late-June, prompting foliar insecticide applications at a time when monarchs are first beginning to colonize the Midwest US (Dunbar et al. 2016). AgDRIFT® is a model for estimating near-field spray drift from aerial applications and has been used as a modeling tool for risk assessment when residue data is unavailable (Teske et al. 2002). This model can be used as a screening tool at the Tier 1 level to provide a conservative assessment of off-field pesticide risk and has been used for non-target plant assessments (Brain et al. 2019).

Krishnan et al. 2020 reported the application of AgDRIFT® for the evaluation of pesticide risk to non-target insect communities, including monarch caterpillars.

The fifth larval instar is the longest larval development stage of monarch caterpillars that allows for changes in consumption and growth to be observed without confounding effects of molting (Zalucki 1982). The natural mortality rates of early instar monarch caterpillars, in the field, are significantly higher than that of fifth instar caterpillars surviving to adulthood (Nail et al. 2015). Therefore, this study was conducted to estimate the lethal and sub-lethal endpoints for fifth instar monarch caterpillars exposed to the type I and type II pyrethroids bifenthrin and β-cyfluthrin, respectively, and the potential effect of these insecticide on the biological fitness of monarch caterpillars. The AgDRIFT® model was used to predict spray deposition and to provide a landscape perspective for toxicity endpoints.

2. MATERIALS AND METHODS

2.1 Insects

Fifth instar caterpillars of the monarch butterfly were used for all laboratory experiments. A laboratory colony was established in the Department of Entomology at the University of Nebraska-Lincoln using eggs received from Iowa State University (Ames, IA). The monarch adults were maintained at 24 °C on a 12 h:12 h light:dark cycle, with an artificial nectar diet. For experiments, eggs were collected daily and stored at 16 °C for up to 14 d. The eggs were moved to room temperature and hatched within 2-3 d. Neonates were then placed on artificial diet within 24 h of hatching.

2.2 Artificial diet

The monarch caterpillar diet was prepared using Southland multi-species Lepidoptera diet (Southland Products Inc., Lake Village, AR, USA) with the addition of 15% (w/w) lyophilized tropical milkweed, *Asclepias curassavica* leaf powder. The leaves were collected from plants grown in a greenhouse throughout the year, washed in a 10% (v/v) bleach solution, rinsed thoroughly with water and soaked in a 10% (v/v) Sonne's No. 7 clay (Sonne's Organic Foods Inc., Liberty, MO USA) solution. After washing, the leaves were air-dried and stored at -80 °C before lyophilized and ground into a fine powder.

2.3 Chemicals

Bifenthrin (CAS# 82657-04-3, 99.5%) and β-cyfluthrin (CAS# 1820573-27-0, 98.0%) were purchased from Chem Service Inc. (West Chester, PA USA) and stored at room temperature. All stock solutions and dilutions were prepared in acetone (Sigma-Aldrich, St. Louis, MO USA).

2.4 Toxicity assays

One-day-old fifth instar monarch caterpillars were used to ensure individuals had fully finished their molt from the fourth instar and that insecticide residues on the cuticle remained for a 72-h observation period. A total of 50-60 individual 1-d-old fifth instar monarch caterpillars were weighed into pre-weighed diet cups. The caterpillars were stratified by weight and randomly assigned to treatment groups, 10 individuals per treatment group, to ensure an equal size distribution across all treatments. A 1 µl aliquot of acetone (solvent control) or each insecticide at 0.025, 0.05, 0.1, 0.2, 0.4 µg/µl bifenthrin or 0.0125, 0.025, 0.05, 0.1 µg/µL β-cyfluthrin prepared in acetone was applied

to the dorsal prothorax, between the anterior tentacles of each caterpillar. The mortality and behavior (i.e., normal, lethargic, immobile, loss of hemolymph) of the caterpillars was observed daily over a 72-h exposure period. Bifenthrin and β-cyfluthrin experiments were repeated in triplicate using caterpillars from two different generations for a total of 30 caterpillars per treatment.

2.5 Diet consumption and growth assays

The diet and frass of each caterpillar were weighed at 24, 48, and 72 h. To correct for evaporative loss of diet, additional diet cups were prepared and weighed at the same time points. The individual monarch caterpillars were weighed daily, with no adverse effects observed after handling caterpillars this frequently. The experiments were repeated in triplicate for a total of 30 caterpillars per treatment. The daily weight was not recorded for the 0.05 µg/µl bifenthrin treatment, but the initial and final weight was recorded for each caterpillar. There were no behavioral changes observed at this treatment level and daily weights at 24 h and 48 h were estimated using a generalized linear mixed model.

2.6 AgDRIFT® aerial and ground spray drift assessment

The AgDRIFT® Tier 1 aerial and ground spray drift assessment (AgDRIFT® ver. 2.1.1, US Environmental Protection Agency, 2003) was used as a conservative drift model to predict the spray deposition (mg/cm^2) for agricultural applications of bifenthrin and β-cyfluthrin formulations (Teske et al. 2002). The point deposition (µg/cm^2) of each insecticide estimated with AgDRIFT® was multiplied by the total surface area of a caterpillar (*ca*. 7.1 cm^2), as reported by Krishnan et al. (2020), to estimate the direct contact exposure of the insecticides to fifth instar monarch caterpillars. The label rates

from the common use pyrethroid formulations Brigade® 2-EC (0.1 lb/ac bifenthrin) and Baythroid® XL (0.022 lb/ac β-cyfluthrin) were used for the AgDRIFT® assessment. The spray deposition was modeled for low boom ground, high boom ground, and aerial applications at 0, 1, 3, 6, 12, 24 m from the edge of a field. In accordance of the manufacturer's label instructions for each insecticide formulation, the Tier 1 ground application assessment was calculated using an ASAE fine to medium-coarse droplet size and an ASAE medium to coarse droplet size was used for the Tier 1 aerial application assessment. The distances were selected to predict insecticide deposition on milkweed in ditches and field margins where milkweed is commonly found in the U.S. Midwest (Pleasants and Oberhauser 2013, Pleasants 2017).

2.7 Data analysis

The dose-response calculations and associated statistical analyses were conducted using SAS 9.4 PROC PROBIT (SAS, Cary, NC). The caterpillar weight and diet consumption were analyzed using SAS 9.4 PROC GLIMMIX (SAS, Cary, NC). A Gaussian distribution was assumed for both outcomes. A repeated measures analysis was conducted for weight and diet consumption on individual monarch caterpillars over time. The treatments were analyzed as a continuous effect. The initial model included fixed linear, quadratic, and cubic treatment dose effects, time effect, interaction between linear, quadratic, and cubic treatment dose with time, and initial monarch caterpillar weight as a covariate. Experiment was used as a significant blocking factor in all analyses. A first order Antedependence pattern was chosen to model the covariance structure. The non-significant terms ($p > .05$) were dropped from the initial model for the final analysis. Total diet consumption was analyzed with an initial model that included fixed linear,

quadratic, and cubic treatment dose effects, and initial caterpillar weight as a covariate. The assessment estimates for each treatment level were compared to the control group at each time point using Scheffe's multiple comparison procedure (Scheffé 1953).

3. RESULTS

3.1 Toxicity assays

The results of the toxicity assays for bifenthrin and β-cyfluthrin are presented in Table 1. The toxicity of bifenthrin (LD_{50} = 0.44 µg/µl (0.32-0.65), slope = 1.86 (1.34-2.37)) was significantly less for the monarch caterpillars compared to β-cyfluthrin (LD_{50} = 0.14 µg/µl (0.12-0.19), slope = 3.59 (2.39-4.80)) 72 h after application of the insecticides based on non-overlapping 95% confidence intervals. There were symptoms of intoxication, including hemolymph bleeding and spasming, observed for the monarch caterpillars treated with $\geq$ 0.2 µg/µL bifenthrin and $\geq$ 0.025 µg/µL β-cyfluthrin at 0-1 h post-treatment. Monarch caterpillars treated with 0.2 µg/µl and 0.4 µg/µl bifenthrin exhibit 27% and 36% mortality, respectively. There was no mortality observed for monarch caterpillars treated with β-cyfluthrin at 0.025 µg/µl although there was 7% mortality observed for caterpillars treated with 0.05 µg/µl β-cyfluthrin, despite hemolymph bleeding and an upright posturing observed for these individuals.

3.2 Diet consumption and growth assays

The results of the daily diet consumed by monarch caterpillars after treatment with bifenthrin and β-cyfluthrin are presented in Figure 2.1. The final model for the effect of bifenthrin and β-cyfluthrin on daily diet consumption, included the covariate of starting weight for individual monarch caterpillars ($p < 0.001$) in addition to significant linear ($p < 0.001$) and quadratic ($p < 0.005$) treatment by time interaction terms.

Experiment was a significant ($p < 0.0001$) blocking factor for bifenthrin diet consumption, but not for β-cyfluthrin ($p = 0.22$). A significant 9%, 33%, 58%, and 87% reduction in diet consumption was observed for monarch caterpillars treated with 0.025, 0.1, 0.2 and 0.4 µg/µl β-cyfluthrin ($p < 0.005$), respectively, at 24 h post-treatment compared to the solvent-treated individuals. Monarch caterpillars treated with 0.4 µg/µl bifenthrin also consumed significantly less diet after 48 h (91%, $p < 0.0001$) and 72 h (75%, $p = 0.0016$) compared to the solvent-treated individuals. The daily diet consumption was not significantly different than that of the untreated monarch caterpillars for any other concentration or time-point. A significant 33%, 59%, 94%, and 92% reduction in diet consumption was observed for monarch caterpillars treated with 0.0125, 0.025, 0.05, and 0.1 µg/µl β-cyfluthrin ($p < 0.0001$), respectively, at 24 h post-treatment compared to the solvent-treated individuals. A significant reduction in diet consumption for monarch caterpillars in all treatment groups was observed at 48 h post-treatment ($p < 0.0001$) compared to the solvent-treated individuals. However, at 72-h post-treatment, a significant decrease in diet consumption was observed for monarch caterpillars treated with 0.1 µg/µl β-cyfluthrin (59%, $p = 0.0034$) compared to the solvent-treated individuals.

A model including a linear treatment effect ($p < 0.0001$) and the individual starting weight covariate ($p < 0.0001$) was used to predict total diet consumption for bifenthrin. A model including both a linear ($p < 0.0001$) and quadratic ($p = 0.0004$) treatment effect and the individual starting weight covariate ($p = 0.0021$) was fit for β-cyfluthrin. Again, experiment was a significant ($p < 0.005$) blocking factor for bifenthrin

total diet consumption, but not for β-cyfluthrin total diet consumption ($p = 0.88$) and was removed from the β-cyfluthrin diet models. The reduction in total diet consumed by monarch caterpillars was 5%, 20%, 39% and 79% for caterpillars treated with bifenthrin at 0.025, 0.1, 0.2 and 0.4 µg/µl, respectively, compared to the solvent-treated caterpillars (Fig. 2.1C). The total diet consumption was significantly reduced ($p < 0.0001$) by 18%, 34%, 60%, and 86% for monarch caterpillars treated with β-cyfluthrin at 0.0125, 0.025, 0.05, 0.1 µg/µl, respectively, compared to the solvent-treated caterpillars (Fig. 2.1D). The total diet consumed between monarch caterpillars was variable for each experiment, but part of the variability was accounted for using the initial weight of each caterpillar.

The results of the monarch caterpillar body weights after treatment with bifenthrin and β-cyfluthrin are shown in Figure 2.2. The final model for the effect of bifenthrin and β-cyfluthrin on monarch caterpillar weight included the covariate of individual starting weight ($p < 0.0001$), blocking factor of experiment ($p < 0.005$) and significant linear ($p < 0.005$) and quadratic ($p < 0.005$) treatment by time interaction. There was a significant reduction in body weight for monarch caterpillars treated with 0.2 µg/µl (13%, $p = 0.0085$) and 0.4 µg/µl bifenthrin (22%, $p < 0.0001$) for 24 h, but only a significant reduction for caterpillars treated with 0.4 µg/µl bifenthrin for 48 h (24%, $p < 0.0001$) and 72 h (24%, $p = 0.0003$) compared to the solvent-treated individuals (Fig. 2.2A). A significant decrease ($p < 0.0001$) in body weight was observed for monarch caterpillars treated with 0.0125, 0.025, 0.05, and 0.1 µg/µl β-cyfluthrin, respectively, after 24 h and 48 h as compared to the solvent control individuals (Fig. 2.2B). At 72 h post-treatment, there was a significant 15% ($p = 0.047$) and 45% ($p < 0.0001$) reduction in body weight

for monarch caterpillars that were treated with, and survived, 0.05 and 0.1 µg/µl β-cyfluthrin, respectively, compared to the solvent-treated caterpillars.

3.3 AgDRIFT® aerial and ground spray drift assessment

The results of the AgDRIFT® Tier 1 aerial and ground spray drift assessment are presented in Figure 3. The aerial assessment predicted bifenthrin deposition could exceed 0.44 µg/caterpillar, the estimated LD_{50}, for fifth instar monarch caterpillars on milkweed up to 28 m from the treated edge of a field (Fig. 2.3A). Additionally, the aerial assessment predicted β-cyfluthrin deposition could exceed 014 µg/caterpillar, the estimated LD_{50}, for fifth instar monarch caterpillars on milkweed up to 23 m from the treated edge of a field (Fig. 2.3B). These exposure distances are reduced in the ground assessment with the high boom deposition of bifenthrin and β-cyfluthrin predicted to be lethal at 3 m and 2 m, respectively, from the treated edge of a field. For the low boom deposition of bifenthrin and β-cyfluthrin, these distances are reduced to 2 m from the treated edge of a field.

The most sensitive endpoint for bifenthrin was monarch caterpillar survival and, thus, the NOED (0.10 µg/caterpillar) and LOED (0.20 µg/caterpillar) were estimated based on survival 72 h after insecticide treatment. However, the most sensitive endpoint for β-cyfluthrin was monarch caterpillar weight and, thus, the NOED (0.025 µg/caterpillar) and LOED (0.05 µg/caterpillar) were estimated based on weight following 72 h of insecticide treatment. The aerial assessment predicts the deposition of bifenthrin on milkweeds at distances up to 60 m from the treated edge of a field to be lethal to monarch caterpillars, but the insecticide would not be lethal at distances > 105 m from the treated edge of a field. For β-cyfluthrin, the aerial assessment predicts deposition on

milkweeds at distances up to 55 m from the treated edge of a field to affect monarch caterpillar growth, but the insecticide would not affect growth at distances > 94 m from the treated edge of a field. The low and high boom ground assessment predicts the deposition of bifenthrin to milkweeds at distances up to 4 m and 6 m, respectively, from the treated edge of a field to be lethal to monarch caterpillars. Bifenthrin would not be lethal at distances beyond 8 m for low boom and 15 m of for high boom applications. The low and high boom ground assessment predicts the deposition of β-cyfluthrin to milkweeds at distances up to 3 m and 6 m, respectively, from the treated edge of a field to reduce monarch caterpillar growth. β-cyfluthrin deposition would not affect growth if deposition was > 7 m and 13 m from the edge of a treated field for high boom and low boom applications, respectively. However, if the only dorsal side of the monarch caterpillar is exposed to the insecticides, there would be a substantial decrease in these predicted distances.

4. DISCUSSION

This study not only provides the first report of bifenthrin toxicity to monarch caterpillars, but it also confirmed that pyrethroid insecticides affect the growth and development of monarch caterpillars as reported by Oberhauser et al. (2006) and Krishnan et al. (2020). Bifenthrin was found to be less toxic than β-cyfluthrin to fifth instar caterpillars as documented in other insect species (Clements and May 1977, Gammon et al. 1981). There were observations of monarch caterpillar mortality 12 h after bifenthrin treatment, whereas monarch caterpillar mortality was observed within 6 h of β-cyfluthrin treatment. Type II pyrethroids, such as β-cyfluthrin, can cause prolonged interference with the gating kinetics of the voltage-gated Na^+-channel leading to a greater

influx of Na^+ and more prolonged convulsions. Furthermore, alternative neuronal target sites have been identified with type II pyrethroids which leads to the CS-syndrome observed with β-cyfluthrin and may explain the increased toxicity observed with the caterpillars (Soderlund et al. 2002, Davies et al. 2007).

Bifenthrin and β-cyfluthrin were observed to significantly affect monarch caterpillar growth and development throughout the 72-h exposure period. A reduction in body size and diet consumption can affect pupation success (Rhainds et al. 1999), adult lifespan (McKay et al. 2016) and immune function (Adamo et al. 2016). Since reduced body size and diet consumption were observed at the final larval instar stage, it is likely the surviving individuals could have challenges with pupation success and, in turn, lead to higher mortality. While our study did not focus on pyrethroid effects to caterpillars infected with the protozoan *Ophryocystis elektroscirrha* (OE), a challenged immune system in response to infection may affect the susceptibility of caterpillars to insecticide exposures. It should be noted that our adult monarchs are routinely checked for the OE, which has never been observed in the colony, and that field-collected adults are not introduced to the colony. However, further studies would be important for determining if the reduced weight resulting from pyrethroid exposure affects pupation, adult emergence, and fitness as well as OE infection affecting pyrethroid susceptibility.

The performance of a monarch butterfly colony can fluctuate throughout the year, and growth rates can be influenced by changes in humidity and ambient temperatures (Kingsolver 2007). Growth rates in solvent-treated monarch caterpillars differed between the bifenthrin and β-cyfluthrin experiments. For the bifenthrin experiments, the solvent-

treated monarch caterpillars were 1.3-fold higher than their original starting weight at the end of the experiment. However, the solvent-treated monarch caterpillars exposed to β-cyfluthrin were 2.1-fold higher than their original starting weight at the end of the experiment. The bifenthrin experiments were conducted prior to the β-cyfluthrin experiments and, thus, the natural variability in the caterpillar growth rate may explain the differences observed with each experiment. Despite this variability, there were statistically significant differences observed between the solvent and bifenthrin treatments for the three cohorts of monarch caterpillars used in this study.

In this study, the 72 h LD_{50} for β-cyfluthrin (0.15 µg/caterpillar or 0.21 µg/g) was found to be significantly higher than the 96 h LD_{50} (0.048 µg/g caterpillar) reported by Krishnan et al. (2020). However, there cannot be a direct comparison between the two studies due to differences between the experimental approach. Our study was designed to exclude post-pupation observations due to the high pupation mortality observed within the monarch colony. There is *ca*. 20% pupation mortality observed with the monarch colony, which is often attributed to caterpillars in the J-state falling mid-pupation from the top of the test chamber (Greiner et al. 2019). Thus, in our study, the mortality of monarch caterpillars that would have failed to initiate pupation (i.e., laggers) or would die during pupation is not captured in our 72-h mortality observations and, instead, these individuals are recorded as alive. In contrast, Krishnan et al. (2020) recorded mortality for fifth instar monarch caterpillars after pupation, which includes this additional source of mortality. Similar to the study of Krishnan et al. (2020), the monarch caterpillars treated with the highest three concentrations of β-cyfluthrin were observed to bleed (i.e.,

loosing hemolymph) less than 1 h after treatment, which contributes to the weight loss recorded at 24 h. Monarch caterpillars exposed to the LOED of β-cyfluthrin did recover from this loss of hemolymph and were observed to gain weight. Hemolymph is critical for molting, immunity, thermal regulation, maintaining turgor pressure, and a number of other physiological processes (Klowden 2008, Kanost 2009). A loss of hemolymph, and possibly turgor pressure, could significantly impact the molting and pupation success of the caterpillars. While it is unclear how hemolymph loss might affect pupation, McKay et al. (2016) reported monarch caterpillar hemolymph loss to reduce pupal mass and increase infection of OE. Additionally, a delay in development could increase the risk of predation or parasitism of monarch caterpillars in the field (Geest 2017).

The AgDRIFT® Tier 1 aerial spray drift assessment predicts the aerial application of bifenthrin and β-cyfluthrin to be a potential risk for monarch caterpillar development on the leaf surface of milkweeds that border pyrethroid-treated crops. This prediction is based on a worst-case scenario for the whole-body surface area of the monarch caterpillar to be exposed to bifenthrin or β-cyfluthrin either by direct deposition or with the caterpillar walking across the pyrethroid-treated surface of a milkweed leaf. If a less conservative exposure scenario is considered for the deposition of the insecticides on the dorsal half of the monarch caterpillar following a low ground boom application, the risk of lethal exposure is predicted to be within 2 m for a bifenthrin- or β-cyfluthrin treated crop. If the risk of exposure is based on the β-cyfluthrin LOED of 0.05 µg/µl, then the deposition affecting monarch caterpillar growth after a ground application is predicted to be 3 m for low boom and 6 m for high boom from the edge of the insecticide-treated

field. The AgDRIFT® Tier 1 aerial and ground deposition assessments are conservative assessments and other studies have found deposition estimates from this model to be 20-40 times higher than what is detected in spray drift residue trials (Brain et al. 2019). While the buffer distances calculated in this study would not be applicable for every field scenario, these distances provide a worst-case estimate for the risk of pyrethroid exposure and provide an opportunity to test laboratory toxicity data in an agricultural landscape. Krishnan et al. (2020) documented larger buffer distances and greater risk down-wind to fifth instar monarch caterpillars near a β-cyfluthrin treated field. However, the different estimates are due to the lower toxicity values determined in the earlier study (Krishnan et al. 2020). Aside from these two models, there is minimal pyrethroid residue data and minimal toxicity data for monarch butterflies, which provides a challenge for determining the actual risk of exposure to caterpillars. Additionally, application timing, frequency and resistance management programs further complicate exposure predictions for monarch caterpillars and determining temporal and spatial overlap near agriculture. A recent study reports the residue levels of deltamethrin on milkweeds that border agricultural crops (Olaya-Arenas and Kaplan 2019), but there are no data collected for other pyrethroids, including bifenthrin and β-cyfluthrin. Additional studies are needed to examine the persistence and stability of these pyrethroids to determine the duration of exposure to monarch caterpillars following the application of these insecticides. Previously, (Oberhauser et al. 2006) found the pyrethroid permethrin, used for mosquito control, to persist on milkweed leaves for 21 d following application. Terrestrial field dissipation studies have reported the half-life of bifenthrin and β-cyfluthrin to be 78-325 d and 4-24

d, respectively (US EPA 2016). The dissipation half-life for β-cyfluthrin is less than that for bifenthrin and, according to the Baythroid XL® label, there can be multiple applications of the insecticide to pest-infested soybean fields at 7-d intervals. Additionally, the deposition assessment with AgDRIFT® and the field deposition reported in the "EPA Environmental Fate and Ecological Effect Assessment" (US EPA 2016) raises concerns for the risk of bifenthrin and β-cyfluthrin to monarch caterpillars on milkweeds that border agricultural crops. Future work should focus on testing these drift assessments and the application of drift reduction technologies to reduce pyrethroid exposures to monarch caterpillars.

Here, we report the significant effects that the pyrethroids bifenthrin and β-cyfluthrin, at field-relevant concentrations, have on the growth and survival of fifth instar monarch caterpillars. These data are important for the ecological risk characterization of foliar-applied insecticides in agriculture-dominated landscapes. Our findings provide evidence that pyrethroids are a potential risk to monarch caterpillars in these landscapes. However, this risk can be mitigated if prevailing wind direction is considered when establishing milkweed near conventional agricultural fields and, when possible, pyrethroids should be applied using low boom ground applications. The conservation efforts to restore monarch butterfly populations require *ca.* 1.8 bill new milkweed stems on the landscape, a goal that can only be reached with the cooperation of agricultural land managers (Thogmartin et al. 2017).

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Table 2.1 Contact toxicity of bifenthrin and β-cyfluthrin to fifth-instar monarch caterpillars[a].

Insecticide	*N*	LD10 95% CI	LD25 95% CI	LD50 95% CI	LD75 95% CI	LD90 95% CI	Slope 95% CI	Chi-Square (b) *Pr* > Chi-Square
Bifentrhin	200	0.08 0.05 - 0.12	0.19 0.14 - 0.25	0.44 0.32 - 0.65	1 0.67 - 1.90	2.10 1.20 - 5.30	1.86 1.34-2.37	50.42 < 0.0001
β-Cyfluthrin	170	0.06 0.05 - 0.08	0.09 0.07 - 0.11	0.14 0.12 - 0.19	0.22 0.17 - 0.35	0.32 0.23 - 0.61	3.59 2.39-4.80	34.38 < 0.0001

[a] Pyrethroid toxicity data are presented as LD_{10}, LD_{25}, LD_{50}, LD_{75}, and LD_{90} and their 95% confidence intervals (95% CI) in micrograms per microliter (µg/µl).

[b] Pearson's chi-square and the probability of chi-square. The probability of > 0.05 indicates that the observed regression model is not significantly different from the expected model.

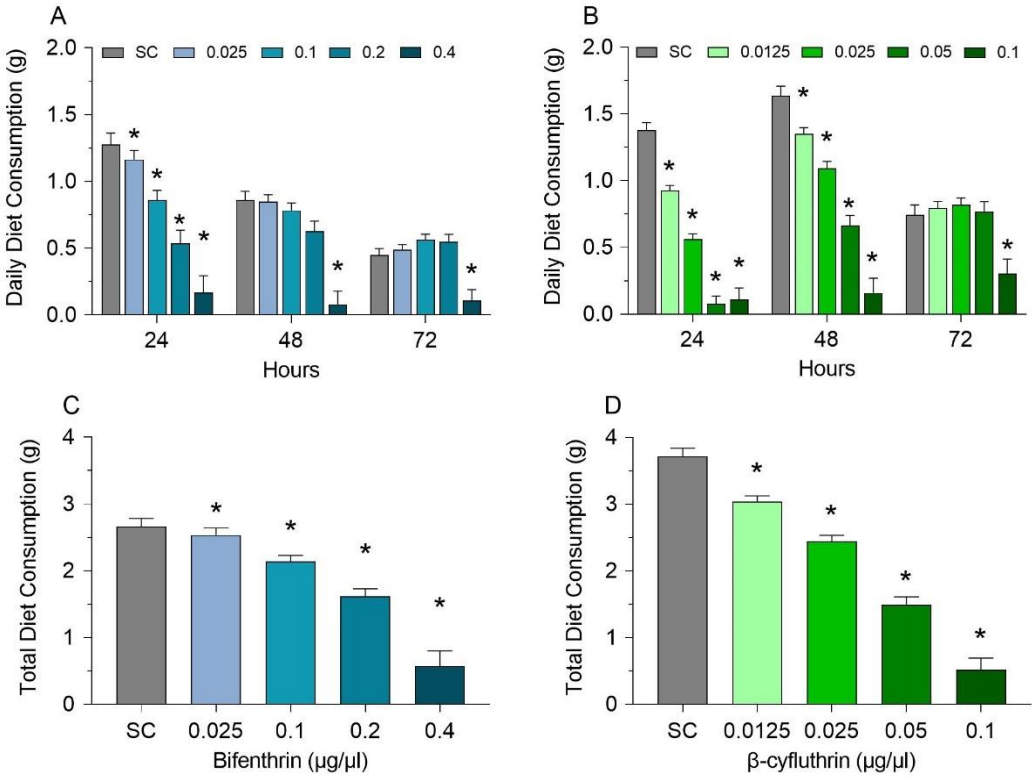

Figure 2.1 Daily and total diet consumption of fifth instar monarch caterpillars after topical exposure to bifenthrin (A, C) and β-cyfluthrin (B, D). Vertical bars represent the mean ± standard error (n = 20) and asterisks indicate significant differences between the solvent control (SC) and treatment means (SAS PROC GLIMMIX, $P < .05$).

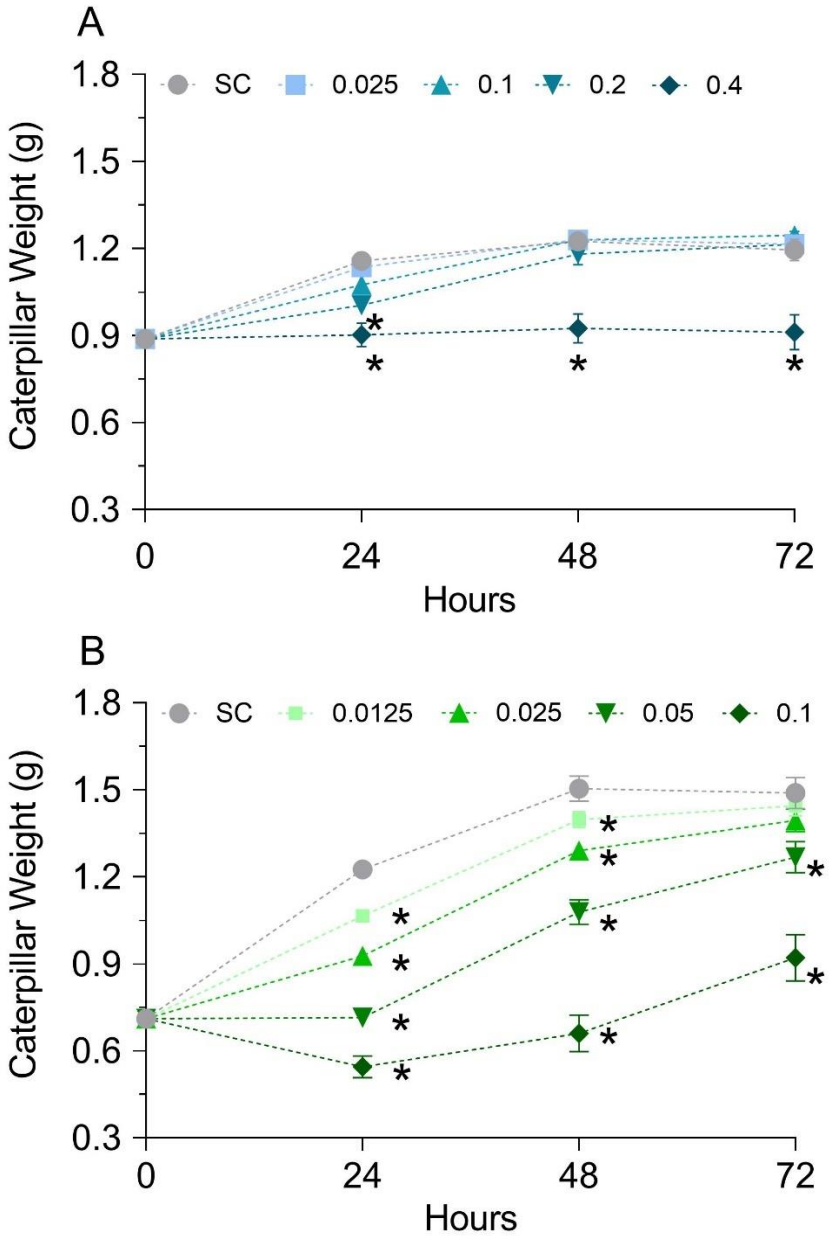

Figure 2.2 Weight of fifth instar monarch caterpillars after topical exposure to bifenthrin (A) and β-cyfluthrin (B). Symbols represent the mean ± standard error (n = 20) and when absent the error bars are within the size of the symbol. Asterisks indicate significant differences between the solvent control (SC) and treatment means (SAS PROC GLIMMIX, $P < .05$).

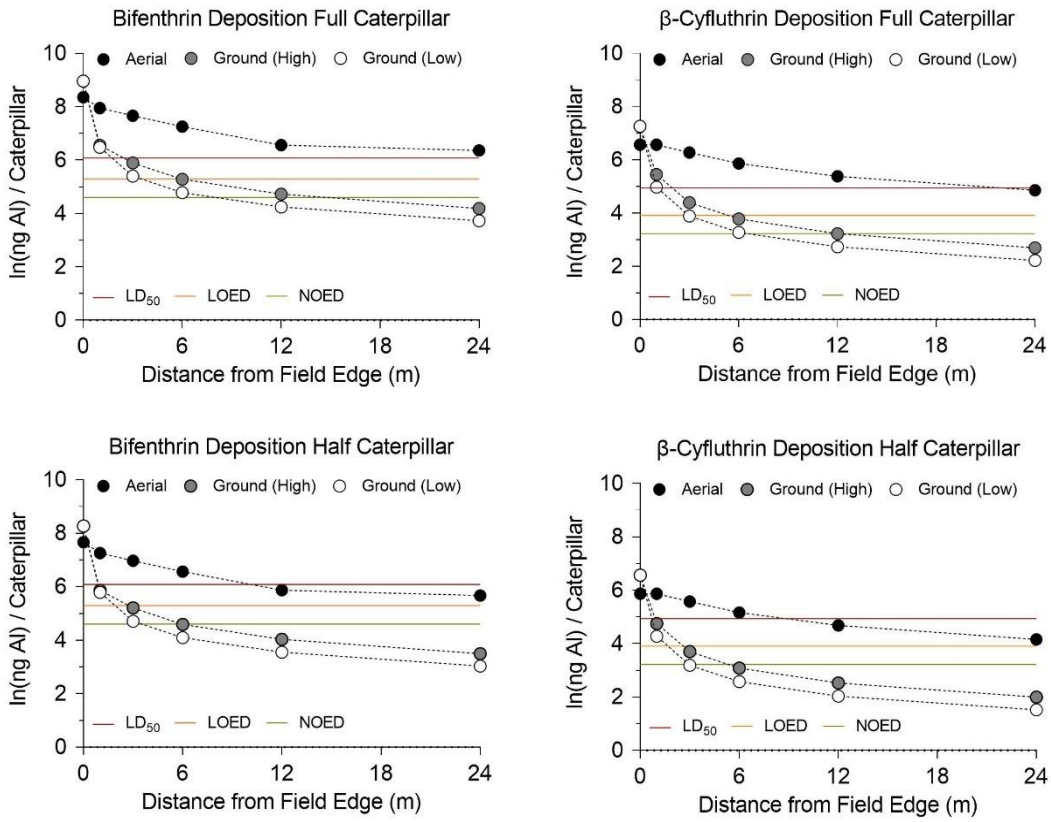

Figure 2.3. **Spray-drift exposure estimates of bifenthrin and β-cyfluthrin for fifth instar monarch caterpillars using the AgDRIFT® model.** Deposition (µg/cm^2) was multiplied by either the full caterpillar surface area (7.10 cm^2) or one-half caterpillar surface area (3.55 cm^2). Exposure values were log transformed to account for orders of magnitude differences in deposition estimates. Effect thresholds, LD_{50} (red line), LOED (orange line) and NOED (green line), are overlaid for each insecticide.

CHAPTER 3 : CARDENOLIDE, POTASSIUM, AND PYRETHROID INSECTICIDE COMBINATIONS REDUCE GROWTH AND SURVIVAL OF MONARCH BUTTERFLY CATERPILLARS [Lepidoptera: Nymphalidae]

This chapter is in review at Economic Entomology: Krueger, A. J., Robinson, E. A., Weissling, T. J., Vélez, A. M., & Anderson, T. D. (2021). Cardenolide, potassium, and pyrethroid insecticide combinations reduce growth and survival of monarch butterfly caterpillars [Lepidoptera: Nymphalidae]. Economic Entomology. Used with permission.

Introduction

The monarch butterfly (*Danaus plexippus)* is a well-known specialist of milkweed (*Asclepias* sp.) [Gentianales: Apocynaceae]. The reduction of milkweed stems throughout the US are implicated in the decline of the monarch butterfly population (Pleasants and Oberhauser 2013). Throughout the US Corn Belt landscape, the remaining milkweed species are primarily confined to field margins forcing monarchs to concentrate near row-crop production (Thogmartin et al. 2017). Because of this proximity to agricultural landscapes, the USFWS has identified agrochemicals to be one of five main stressors contributing to the decline of monarchs (USFWS 2017). The monarch is a well-known flagship for insect conservation and raising public awareness about the decline of insect populations (Oberhauser and Guiney 2009). A public survey found that US households valued the conservation of the monarch butterfly at $4-6 billion USD (Diffendorfer et al. 2014). The cost of monarch butterfly conservation and habitat restoration will require substantial funding from government programs to support these actions on public and private lands (Pindilli and Casey 2015). An understanding of the potential stressors and their interactions within habitat restoration sites is critical for maximizing the benefits of this economic investment.

Monarch caterpillars and other herbivorous insects feeding on milkweed are exposed to the cardenolide defenses of milkweeds. Cardenolides are secondary plant metabolites within a sub-class of cardiac glycosides derived from triterpenoids with broad-spectrum insecticidal activity (Agrawal et al. 2012). These compounds act on the nervous system targeting Na^+/K^+-ATPase, where they reversibly bind to the α-subunit locking it in a phosphorylated conformation and disrupting ion translocation and nerve function (Horisberger 2004, Dobler et al. 2011). This active site for cardenolide toxicity has been identified with ouabain, a foxglove (*Digitalis sp.*) [Lamiales: Plantaginaceae] cardenolide which selectively binds to Na^+/K^+-ATPase (Lingrel 2010). There are *ca*. 500 identified cardenolide derivatives with diverse structural conformations (Schönfeld et al. 1985, Agrawal et al. 2012). Of the 73 native milkweed species in North America, nearly all *Asclepias* species produce cardenolides albeit at different compositions and concentrations depending on the milkweed species (Brower et al. 1982, Seiber et al. 1983, Frick and Wink 1995, Agrawal et al. 2012). The monarch is insensitive to cardenolides, resulting from their co-evolution with milkweeds, and have the capacity to sequester these compounds (Holzinger and Wink 1996).

Pyrethroid insecticides are commonly used to control insect pests in corn and soybean across the Midwestern US (Ragsdale et al. 2011). These broad-spectrum insecticides are neurotoxic and target the voltage-gated Na^+ channel to disrupt neurological function (Clements and May 1977). Because of their acute toxicity, pyrethroids have been used to control a variety of insect pests (Kogan and Turnipseed 1987, Meinke et al. 2021). For example, the timing of soybean aphid infestations and

subsequent foliar applications of pyrethroids can occur when monarch caterpillars are present on the landscape (Bradbury et al. 2017). Pyrethroid field residue and exposure modeling data show monarchs developing in field margins 0-10 m from the field may be adversely affected by foliar applications during the breeding season (Olaya-Arenas and Kaplan 2019, Krishnan et al. 2020). Krueger et al. (2021) have shown the pyrethroid insecticides bifenthrin and β-cyfluthrin, at field realistic concentrations, to significantly affect the survival, growth, and development of fifth-instar monarchs.

Potassium is an essential nutrient for plant growth and stress physiology (Pettigrew 2008). However, potassium is deficient in soils across several Midwestern states (Woodruff et al. 2015). Potassium fertilization can help increase drought tolerance and immune defense in agricultural crops and may be an important tool for mitigating the effects of climate change on crops (Wang et al. 2013). Millions of tons of potassium fertilizer are applied across the US, with potash fertilizer applied to 63% corn, 43% soybean, and 45% cotton acreage annually (USDA ERS 2019). Pharmacological studies have shown potassium antagonism of cardenolides at the target site in several mammalian systems and, in turn, decrease cardenolide toxicity and inhibition of Na^+/K^+-ATPase by orders of magnitude (Glynn 1957, Baker and Willis 1970). These studies have shown increasing K^+ serum concentrations administered after cardenolide exposure to reverse cardenolide inhibition and recover the function of Na^+/K^+-ATPase (Glynn 1957, Baker and Willis 1970). These results demonstrate the competitive binding of potassium and antagonism of cardenolides at the Na^+/K^+-ATPase (Songu-Mize et al. 1989). Previous studies documented cardenolide-binding site modifications for Na^+/K^+-ATPase in

monarchs (Vaughan and Jungreis 1977). These modifications of Na^+/K^+-ATPase are reported to decrease the toxicity of the cardenolide ouabain, and as observed in mammals, the inhibition of Na^+/K^+-ATPase by ouabain can be reversed with increasing concentrations of K^+ (Vaughan and Jungreis 1977). There are 4.6 bill. lbs. and 3.2 bill. lbs. of potassium fertilizer applied annually to corn and soybean crops, respectively, in 18 states along the monarch flyway (USDA ERS 2019). However, there are no studies focusing on the effects of potassium on cardenolide toxicity to monarchs in agricultural landscapes. Previous studies show that other lepidopterans can cope with increased concentrations dietary K^+ (Jungreis et al. 1973, Harvey et al. 1975, Dow and Harvey 1988) albeit with an energetic cost for the maintenance and regulation of osmolarity. The influx of potassium fertilizer applications in agricultural landscapes may affect the sequestration and protective benefits of cardenolides in developing monarchs.

This study evaluated the effects of cardenolide ouabain and potassium chloride (KCl) combinations on the sensitivity of monarch caterpillars to the pyrethroid insecticide bifenthrin. Bifenthrin was chosen as a representative pyrethroid not only due to its relevance as a crop-protection insecticide in Nebraska and Midwest agricultural landscapes, but also a continuation of our previous studies on the sub-lethal toxicity of bifenthrin to caterpillars (Krueger et al. 2021). First, we examined the weight and diet consumption of caterpillars exposed to ouabain, KCl, and bifenthrin. Second, we examined the weight, diet consumption, and survival of caterpillars exposed combinations of KCl and bifenthrin. Third, we examined the weight, diet consumption,

and survival of caterpillars pre-exposed to ouabain followed by treatment to combinations of KCl and bifenthrin.

Materials and Methods

Chemicals

Bifenthrin (CAS# 82657-04-3, 99.5%) was purchased from Chem Service Inc. (West Chester, PA) and stored at room temperature. Ouabain (CAS# 11018-89-6) and potassium chloride (KCl) (CAS#7447-40-7, 99.0%) were purchased from Sigma-Aldrich (St. Louis, MO) and stored at room temperature. Stock solutions of bifenthrin were prepared in acetone. Ouabain and KCl solutions were dissolved in deionized water for incorporation into the diet. Deionized water was used as a solvent control for ouabain and KCl treatments and acetone was used as a solvent control for bifenthrin treatments.

Insects and Artificial Diet

Monarch caterpillars were sourced from a laboratory colony in the Department of Entomology at the University of Nebraska-Lincoln and maintained as described in Krueger et al. (2021). Briefly, eggs were collected daily and stored at 16 °C for up to 14 d. The eggs were moved to room temperature and hatched within 2-3 d. Neonates were then placed on artificial diet within 24 h of eclosion and maintained on the diet through the third- and fifth-instar stages for the experiments. The number of caterpillars per treatment and replications were different for each experiment due to asynchrony of development in the third- to fifth-instar stages. The number of caterpillars tested per treatment and replications are shown in Appendix 1.

The monarch caterpillar diet was prepared following methods outlined in Krueger et al. (2021) with the following modifications. Diet was prepared using Southland multi-species Lepidoptera diet (Southland Products Incorporated, Lake Village, AR) with the addition of 15% (w/w) lyophilized common milkweed (*Asclepias syrica*) leaf powder. . Milkweed leaves were collected from garden spaces or field sites receiving no insecticide application, washed in a 10% (v:v) bleach solution, and stored at -80 ºC. Leaves were freeze dried, ground to a powder, and stored at -20 ºC.

Monarch caterpillars on artificial diet have been shown to develop significantly slower than caterpillars feeding on milkweed leaves (Greiner et al. 2019). Newly hatched caterpillars take approximately 4-5 days to develop and molt to a third-instar caterpillar, 8-10 days to develop and molt to a fifth-instar stage caterpillar, and 15-17 days to pupation. All experiments used caterpillars that had molted approximately 24 h prior to the start of the experiment to avoid confounding effects from molting.

Single and Combination Chemical Treatments

Single bifenthrin treatments were reported in Krueger et al. (2021). Briefly, stock solutions were prepared in acetone at 0.025, 0.05, 0.1, 0.2, and 0.4 µg/µl and 1 µl was applied topically with a pipette to the dorsal prothorax of each fifth-instar caterpillar to determine effect thresholds. Stock solutions of ouabain were prepared in deionized water and mixed with the diet to achieve concentrations of 0.03, 0.1, 0.3, 1, and 3 mg ouabain/g diet. Concentrations were selected to mimic a range of total cardenolide concentrations documented across *Asclepias sp.* (Rasmann and Agrawal 2011). Artificial diet was prepared in a single batch and cooled to 15-17 °C before aliquots of diet were removed to

prepare individual diet treatments. Individual diet treatments were dispensed using a 60 ml syringe (BD Biosciences, San Jose, CA) in 2.5 ml aliquots into 1 oz. condiment cups (Dart, Mason, MI). Diet was either fully consumed or beginning to dry out. after 6 days. If the diets were observed to dry out, then the diet was prepared again on day 6. Treated diet that was prepared again on day 6 was prepared following the same methods as described for day 0; however, diet was dispensed in 4 ml aliquots into 2 oz. condiment cups (Dart, Mason, MI) to provision caterpillars through the conclusion of the study. KCl was dissolved in deionized water at 0.4, 2, 10, and 50 mg KCl/g diet and treated diets were prepared in separate batches. Since the effects of KCl on caterpillar growth and diet consumption were unknown, KCl concentrations were selected to span a wide range, with the highest concentration approaching limits of solubility in water. KCl was weighed for each treatment, dissolved into deionized water, and boiled before the addition of artificial diet mix and milkweed powder. Diets were dispensed in 4 ml aliquots into 2 oz. condiment cups (Dart, Mason, MI) using a 60 ml syringe (BD Biosciences, San Jose, CA). The number of caterpillars tested per treatment and replications are shown in Appendix 1.

Fifth-instar caterpillars were exposed to either 0 or 10 mg/g KCl and 0, 0.1, 0.2, or 0.4 µg/µl bifenthrin to yield 8 treatment groups. KCl concentration was selected to mimic a realistic, environmental exposure, and to be the threshold for significant effects on caterpillar growth and diet consumption. Bifenthrin concentrations were selected based on effects documented in Krueger et al. (2021), which are shown to affect the growth, development, and survivorship of fifth-instar caterpillars. KCl-treated diet was

prepared as previously described for the single compound treatments. Bifenthrin was dissolved in acetone at 0.1, 0.2, and 0.4 µg/µl and topically applied to the dorsal prothorax as described in Krueger et al. (2021). The number of caterpillars tested per treatment and replications are shown in Appendix 1.

On day 0, 100-120 third-instar caterpillars were weighed, and 50-60 individuals were placed on 2.5 ml of untreated diet. Another 50-60 individuals were placed on 2.5 ml diet treated with 1 mg ouabain/g diet. Ouabain diets were prepared following the same method previously described for single ouabain treatment. On day 6 of the experiment, KCl and bifenthrin treatments were started when caterpillars reached the fifth-instar stage. For each untreated and ouabain-treated group, caterpillars were randomly assigned to 1 of 4 different KCl-bifenthrin treatment groups: 1) no KCl + no bifenthrin, 2) no KCl + 0.1 µg/µl bifenthrin, 3) 10 mg/g KCl + no bifenthrin, or 4) 10 mg/g KCl + 0.1 µg/µl bifenthrin. There was a total of 8 different treatment combinations of ouabain, KCl, and bifenthrin. Ouabain concentration was selected to mimic a high cardenolide exposure found in native milkweed species. KCl concentration was selected based on results from the KCl + bifenthrin experiments, and bifenthrin concentration was selected as a more field realistic exposure that consistently produced effects on caterpillar growth and diet consumption with minimal effects on survival. KCl and bifenthrin were prepared on day 6 as previously described for the single compound treatments. The number of caterpillars tested per treatment and replications are shown in Appendix 1.

Diet Consumption and Growth Experiments

Third-instar caterpillars were used to mimic a sub-chronic ouabain exposure and fifth-instar caterpillars were used for KCl and bifenthrin treatments as reported in

Krueger et al. (2021). First-instar caterpillars were reared in a 128-well bioassay tray (Frontier Agricultural Sciences, Newark, DE) to the second-instar stage on 0.5 g untreated artificial diet. For ouabain treatments, third-instar caterpillars were stratified by weight on day 0 and 10 individuals were randomly assigned to each treatment group after molting to the third-instar stage to ensure equal size distribution across treatments. For KCl + bifenthrin treatments, third-instar caterpillars were placed on 2.5 g untreated diet in 32-well bioassay trays (Frontier Agricultural Sciences, Newark, DE) to continue developing to the fifth-instar stage. After molting, fifth-instar caterpillars were stratified by weight and individuals were randomly assigned to the treatment groups. For ouabain + KCl + bifenthrin, caterpillars were randomly assigned to ouabain treatment groups on day 0 and stratified by weight on day 6 for KCl and bifenthrin treatment groups. Caterpillars, diet, and frass were weighed daily for single compound treatments with ouabain and KCl, and 72-h KCl + bifenthrin treatments. Caterpillars, diet and frass were weighed every other day on days 0-6 for ouabain + KCl + bifenthrin experiments and weighed every day from day 6-10 after KCl and bifenthrin treatments were administered. Mortality was recorded daily for each experiment. To quantify diet consumption, three evaporative control containers were setup for each experiment to quantify weight loss from evaporation. Diet consumption is reported as the difference in diet weight minus the difference in evaporative controls over the same time frame. Ouabain, KCl + bifenthrin, and ouabain + KCl + bifenthrin experiments were repeated in triplicate and KCl experiments were repeated in quadruplicate using caterpillars from three different

generations. The number of caterpillars tested per treatment and replications are shown in Appendix 1.

Data Analysis

Caterpillar weight, diet consumption, and survival for each treatment were analyzed in R 4.0.1 (R Core Team 2020). For each treatment, a repeated measures analysis was conducted for weight and diet consumption on individual caterpillars over time, assuming a Gaussian distribution with an AR-1 covariance structure to account for correlation between days. The linear mixed model repeated measures analyses were conducted using the nlme package (Pinheiro et al. 2021) with the lme function. Proportion survival was analyzed for both the KCl + bifenthrin and ouabain + KCl + bifenthrin treatments using a generalized linear mixed model assuming a binomial distribution with a PROBIT link function using the glmer function in the lme4 package (Bates et al. 2015). There was no mortality observed in solvent control treatments (i.e., standard deviation = 0) so survival was only analyzed for treatment groups exposed to bifenthrin for KCl + bifenthrin and ouabain + KCl + bifenthrin experiments. Each treatment was replicated on three or four separate occasions, with experimental replicate treated as a fixed block across all analyses. Baseline caterpillar weight (i.e., weight on day 0) was used as a covariate for all analyses. For both ouabain and the ouabain + KCl + bifenthrin treatments, caterpillar growth was exponential over the 9-10-d treatment period and, thus, the caterpillar weight response variable and the baseline caterpillar weight covariate were both log-transformed to satisfy assumptions of normality. For the ouabain-only and KCl-only treatments, respective concentrations were log-transformed in the analysis for equal spacing of treatment levels. The AIC was then used as model selection

criteria to fit the best polynomial regression. Final reduced model fit for each analysis is provided in Appendices 2-5. The assessment estimates for each treatment level were compared to the control group at each time point using Dunnett's multiple comparison procedure and reported at the $\alpha = 0.05$ significance level. Caterpillar weight and diet consumption analyses for ouabain-only and KCl-only concentrations were log transformed and treated as quantitative treatment variables since at least 5 concentrations were used in these experiments. In addition to the treatment comparisons, a threshold concentration was determined for each analysis as the lowest predicted concentration where caterpillar weight or diet consumption significantly differed ($p < 0.05$) from the control. For KCl + bifenthrin and ouabain + KCl + bifenthrin treatments, KCl, ouabain, and bifenthrin treatment variables were treated as qualitative since both experiments had fewer than 5 concentrations. Therefore, treatment comparisons were tested between the treatments and the control. Conditional residual plots were used to assess model fit. All figures were generated using the estimates obtained using the estimated marginal means (emmeans) package (Lenth 2020) from the model outputs at the average base caterpillar weight for that treatment experiment and the ggplot2 package (Wickham 2016) for plotting.

Results

The results of caterpillar weights after receiving an ouabain diet are shown in Fig. 3.1A. There was no significant caterpillar mortality observed after each treatment for the duration of the experiment. There was a significant increase in body weight (36-57%) for caterpillars feeding on the 3 mg ouabain/g diet for 3-10 d compared to the individuals receiving the untreated diet ($p < 0.05$) (Fig. 3.1A). Similarly, the caterpillars feeding on

the 1 mg ouabain/g diet for 4-10 d had significantly higher body weight (25-30%) compared to the individuals receiving the untreated diet ($p < 0.05$) (Fig. 3.1A). The final generalized linear mixed model showed a significant linear relationship between log(ouabain) and caterpillar weight ($F = 13.29$; $df = 1, 174$; $p < 0.001$) as well as log(ouabain) x day and caterpillar weight ($F = 3.92$; $df = 9, 1580$; $p < 0.0001$). Day, experimental replicate, and starting caterpillar weight also had a significant effect on caterpillar weight ($p < 0.0001$). The quadratic log(ouabain) ($F = 0.47$; $df = 1, 174$; $p = 0.45$) and quadratic log(ouabain) x day ($F = 1.76$; $df = 9, 1580$; $p = 0.072$) terms were kept in the model based on AIC. Using the model to estimate caterpillar weight across ouabain concentrations, after 3-d of feeding on an ouabain diet, the model estimated concentrations above 1.25 mg ouabain/g diet will significantly ($p < 0.05$) increase caterpillar weight over the course of the 10-d exposure period. The exact concentration threshold for significance varies from 1.26 to 2.50 mg ouabain/g diet between 3-10 d and is shown as the dotted vertical line each day in Fig. 3.1A.

The results of daily diet consumed by caterpillars after receiving an ouabain-treated diet are presented in Fig. 3.1B. A significant 37% ($t = 2.88$; $df = 173$; $p = 0.020$) and 47% ($t = 3.08$; $df = 173$; $p = 0.011$) increase in diet consumption was observed for caterpillars exposed to a 1 and 3 mg ouabain/g diet, respectively, after 8 d compared to the individuals receiving an untreated diet (Fig. 3.1B). However, the caterpillars exposed to a 0.1 mg ouabain/g diet exhibited a significant 46% reduction ($t = -2.70$; $df = 173$; $p = 0.034$) in diet consumption after 6 d compared to the caterpillars receiving an untreated diet (Fig. 3.1B). After 7 d, the caterpillars exposed to 0.03, 0.10, and 0.30 mg ouabain/g

diet were observed to exhibit a significant 45% (t = -4.85; df = 173; $p < 0.0001$), 49% (t = -460; df = 173; $p < 0.0001$), and 41% (t = -2.89; df = 173; $p = 0.019$) reduction in diet consumption, respectively, compared to the individuals receiving an untreated diet (Fig. 3.1B). The final generalized linear mixed model used a significant quadratic log(ouabain) (F = 5.09; df = 1, 173; $p = 0.025$) term as well as linear (F = 3.96; df = 9, 1572; $p < 0.0001$), quadratic (F = 2.72; df = 9, 1572; $p = 0.0032$) and cubic log(ouabain) x day (F = 1.99; df = 9, 1572; $p = 0.037$) interaction terms. Day and starting caterpillar weight also had a significant effect on diet consumption in the model ($p < 0.01$). Given the significance of the cubic and quadratic interaction terms and limited significant comparisons, model predictions were not determined.

The results of caterpillar weights after receiving a KCl diet are shown in Fig. 3.2A. A significant 39% (t = -8.16; df = 143; $p < 0.0001$), 51% (t = -13.2; df = 143; $p < 0.0001$), and 55% (t = -14.5; df = 143; $p < 0.0001$) decrease in body weight was observed for caterpillars exposed to a 50 mg KCl/g diet at 24, 48 and 72 h, respectively, relative to the untreated individuals. The final generalized linear mixed model used significant linear, quadratic, cubic, and quartic log(KCl) and log(KCl) x day interaction terms ($p < 0.05$). Day, experimental replicate, and starting caterpillar weight also had a significant effect on caterpillar weight ($p < 0.01$). Using the model to estimate caterpillar weight across KCl concentrations, the model estimated concentrations between 21 and 26 mg KCl/g diet will decrease caterpillar weight relative to controls over the 72-h exposure period.

The results of daily diet consumed by caterpillars after receiving a KCl-treated diet are presented in Fig. 3.2B. A significant 88% ($t = -8.00$; $df = 143$; $p < 0.0001$), 90% ($t = -9.38$; $df = 143$; $p < 0.0001$), and 91% ($t = -6.79$; $df = 143$; $p < 0.0001$) decrease in diet consumption was observed at 24, 48, and 72 h, respectively, for caterpillars exposed to 50 mg KCl/g diet compared to individuals receiving the untreated diet. The final generalized linear mixed model used significant linear, quadratic, cubic and quartic log(KCl) terms ($p < 0.05$). Day, experiment, and starting caterpillar weight also had a significant effect on caterpillar weight ($p < 0.01$). Using the model to estimate diet consumption across KCl concentrations, the model estimated concentrations between 22 and 30 mg KCl/g diet will decrease diet consumption relative to untreated individuals.

The results of caterpillar weight after receiving a KCl-treated diet and bifenthrin treatment are presented in Fig. 3.3A. There were no significant differences in weight between caterpillars that received a KCl-treated diet relative to caterpillars receiving an untreated diet at any time point during the 72-h experiment. The results of daily diet consumed by caterpillars receiving a KCl-treated diet and treated with bifenthrin are presented in Fig. 3.3B. A significant 41% ($t = -1.99$; $df = 146$; $p = 0.048$) and 52% ($t = -2.77$; $df = 146$; $p = 0.0063$) decrease in daily diet consumption was observed at 48 h and 72 h, respectively, for caterpillars on a KCl-treated diet and exposed to 0.2 µg/µl bifenthrin compared to caterpillars receiving an untreated diet and treated with 0.2 µg/µl bifenthrin. Caterpillars exposed to KCl only and not treated with bifenthrin exhibited a significant 43% ($t = -5.08$; $df = 146$; $p < 0.0001$) reduction in diet consumption after 48 h compared to caterpillars receiving an untreated diet. The results of survival for

caterpillars receiving a KCl-treated diet and treated with bifenthrin are presented in Fig. 3.4. There were no significant differences in survival between untreated caterpillars and caterpillars exposed to KCl at 0.1 ($z = 0.85$; $p = 0.39$), 0.2 ($z = 1.55$; $p = 0.12$), and 0.4 ($z = -1.71$; $p = 0.088$) µg/µl bifenthrin. On the untreated diet, the binomial model predicts 75%, 72%, and 25% of caterpillars will survive 0.1, 0.2, and 0.4 µg/µl bifenthrin, respectively, 72 h after topical treatment. On the KCl-treated diet, the model predicts 86%, 91% and 4% of caterpillars will survive 0.1, 0.2, and 0.4 µg/µl bifenthrin, respectively, 72 h after topical treatment.

The results of caterpillar weight after receiving a KCl- and ouabain-treated diet and treated with bifenthrin are presented in Fig. 3.5A. There were no significant ($p > 0.05$) differences in growth between any treatments on day 2, 4, or 6 prior to bifenthrin or KCl treatments. Ouabain had no significant effect ($p > 0.05$) on caterpillar growth or diet consumption for any combination of KCl and bifenthrin. On day 7, 24 h after KCl and bifenthrin treatment, a significant 32% ($t = -5.14$; $df = 208$; $p < 0.0001$), 28% ($t = -4.35$; $df = 208$; $p < 0.0001$), 26% ($t = -3.94$; $df = 208$; $p < 0.0001$) and 29% ($t = -4.54$; $df = 208$; $p < 0.0001$) decrease in body weight was observed for caterpillars exposed to an untreated diet, KCl-treated diet, ouabain-treated diet, and ouabain plus KCl-treated diet, respectively, relative to individuals that were topically treated with acetone. Similarly, on day 8, 48 h after treatment with bifenthrin, a significant 27% ($t = -4.07$; $df = 208$; $p < 0.0001$), 23% ($t = -3.38$; $df = 208$; $p = 0.0009$), 17.1% ($t = -2.42$; $df = 208$; $p = 0.0163$) and 26% ($t = -3.82$; $df = 208$; $p = 0.0002$) decrease in body weight was observed for caterpillars exposed to an untreated diet, KCl treated diet, ouabain treated diet, and

ouabain plus KCl-treated diet, respectively. However, on day 9 a significant 23% (t = -3.25; df = 208; p = 0.0014) and 13% (t = -2.25; df = 208; p = 0.0257) decrease in body weight was only observed for caterpillars exposed to a KCl treated diet and an ouabain plus KCl-treated diet, respectively. The final categorical model for the effect of ouabain, KCl, and bifenthrin on caterpillar weight used significant day x ouabain x KCl (F = 0.77; df = 5, 954; p = 0.0421), day x KCl (F = 2.83; df = 5, 954; p = 0.0151), and day x bifenthrin (F = 8.37, df = 5, 954, p < 0.0001) interaction terms. Day, experimental replicate, and caterpillar starting weight also had a significant effect on caterpillar weight (p < 0.0001). The ouabain x KCl (F = 2.15; df = 1, 208; p = 0.14), ouabain x KCl x bifenthrin (F = 0.201; df = 1, 208; p = 0.65) and day x ouabain x KCl x bifenthrin (F = 0.774; df = 5, 954; p = 0.0151) interaction terms were not significant in the model.

The results of daily diet consumed by caterpillars receiving a KCl- and ouabain-treated diet and treated with bifenthrin are presented in Fig. 3.5B. Caterpillars exposed to ouabain and KCl exhibited a significant 32% (t = -3.25; df = 208; p = 0.0014) and 28% (t = -3.25; df = 208; p = 0.0014) reduction in diet consumed on day 7 and 8, respectively, 24 and 48 h after treatment with bifenthrin relative to caterpillars receiving the same diet treated with acetone. Caterpillars exposed to ouabain, KCl and bifenthrin exhibited a significant 32% (t = -2.74; df = 208; p = 0.0067) and 28% (t = -2.26; df = 208; p = 0.0243) reduction in diet consumption on day 8 and 9, respectively, relative to individuals exposed to only ouabain and bifenthrin. A significant 17% (t = -2.14; df = 208; p = 0.0334) and 21% (t = -2.96; df = 208; p = 0.0035) reduction in diet consumption was observed for caterpillars exposed to KCl on day 8 and 9, respectively, relative to

caterpillars receiving an untreated diet. The final categorical model for the effect of ouabain, KCl, and bifenthrin on diet consumption used significant bifenthrin ($F = 43.7$; df =1, 208; $p < 0.0001$), day x bifenthrin ($F = 54.1$; df = 5, 952; $p < 0.0001$), and day x KCl ($F = 3.23$; df = 5, 952; $p = 0.0068$) interaction terms. Day, experimental replicate, and caterpillar starting weight also had a significant effect on diet consumption ($p < 0.0001$). No other ouabain, KCl, bifenthrin, or day interactions were significant ($p > 0.05$). The results of caterpillar survival after receiving a KCl- and ouabain-treated diet and treated with bifenthrin are presented in Fig. 3.6. Survival did not significantly differ ($p < 0.05$) following treatment with 0.1 µg/µl bifenthrin on any ouabain or KCl diet. The binomial model predicts 89%, 84%, 83% and 83% of caterpillars will survive treatment with 0.1 µg/µl bifenthrin on untreated diet, 1 mg/g ouabain diet, 10 mg/g KCl diet, and 1 mg/g ouabain + 10 mg/g KCl diet, respectively.

Discussion

This study provides the first report of potassium to affect the growth and development of monarch caterpillars. Here, we also show a concentration-dependent increase in the body weight of caterpillars exposed to the polar *Digitalis*-derived cardenolide ouabain. We observed a significant interaction of KCl + bifenthrin on caterpillar diet consumption and a significant interaction of ouabain + KCl + bifenthrin on caterpillar weight. While these interaction terms were significant in the mixed model analyses, there was no significant interaction observed for KCl and ouabain on the sensitivity of caterpillars to bifenthrin.

We have observed significant increases in caterpillar body mass starting on day 3 and continuing over the 10-day period when caterpillars were exposed to 1 and 3 mg ouabain/g diet. In addition to increased caterpillar weight, we observed an accelerated

development time of caterpillars that were exposed to 1 and 3 mg ouabain/g diet compared to lower concentrations. There were more caterpillars that developed to the fifth-instar stage on day 5 and 6 when feeding on the elevated concentrations of ouabain compared to those that fed on lower concentrations. The 1 mg ouabain/g diet used in the interaction experiments was chosen to mimic total cardenolide concentrations of approximately 1 mg/g reported in *A. curassavica* (Rasmann and Agrawal 2011, Tan et al. 2019). Higher cardenolide concentrations in milkweed species have been associated with reduced growth and survival, particularly with early-instar caterpillars (Zalucki et al. 1990, 2001, Pocius et al. 2017). However, polar cardenolides, such as ouabain, are less toxic to and readily sequestered by monarch caterpillars (Frick and Wink 1995) compared to nonpolar cardenolides (Jones et al. 2019). The prevalence of polar cardenolides compared to non-polar cardenolides is variable between milkweed species (Agrawal et al. 2012). It has been reported that milkweed species with higher cardenolide defenses also contain higher amounts of non-polar cardenolides (Rasmann and Agrawal 2011). It is challenging to extrapolate our findings with ouabain to native milkweed species. However, it has been reported that tropical milkweed (*Asclepias curassavica*) and white swamp milkweed (*Asclepias perennis*) have a higher proportion of polar cardenolides (i.e., low polarity score) (Jones et al. 2019). Our data suggests that caterpillars grow faster and have higher body weight when feeding on milkweeds with high levels of polar cardenolides.

We have observed significant effects on caterpillar growth and diet consumption after exposure to 50 mg KCl/g diet with the final model analysis predicting adverse

effects to caterpillars at concentrations exceeding 21 mg KCl /g diet. Interestingly, the final models for the effect of KCl on caterpillar weight and diet consumption show a significant quartic relationship with KCl. This quartic relationship estimates increased caterpillar growth and diet consumption after exposure to low concentrations of KCl and before the onset of adverse effects elicited from higher KCl concentrations. Lepidopterans require higher concentrations of salt in artificial diets (Beck et al. 1968, Banu 2004, Han et al. 2012), which is due to the higher K^+:Na^+ ratio maintained in their hemolymph (Harvey et al. 1975). The salt requirements in artificial diets suggest there are elevated concentrations of salts already present in the diet before the addition of potassium. An incremental increase of KCl might adjust the overall concentration to an optimal concentration range of KCl in the diet for the caterpillars. As a result, it is also unclear how much potassium caterpillars receive during the exposure period. Potassium fertilizer exposure is dynamic in the field and the bioavailability to caterpillars is unknown following the application of fertilizers. Future studies are warranted to estimate the bioavailability of KCl to caterpillars in the field.

Caterpillar weight and diet consumption were used as metrics of sub-lethal toxicity for each experiment as described by Krueger et al. (2021). These metrics provide similar results for effect thresholds when exposure is limited to a single instar stage of caterpillars (i.e., 72-h treatment for fifth-instar caterpillars). This is evident with the KCl data and the congruent model predictions of effect thresholds for caterpillar diet consumption and weight. However, caterpillar diet consumption can be highly variable across multiple instar stages and longer exposure periods. Caterpillars are observed to

stop feeding on the diet as they approach the next molting stage. During these pre-molt stages, any treatment effects reducing consumption will be confounded by a naturally lower consumption of diet. Additionally, caterpillars within a treatment group will be variable in the molting time, which can lead to differences in daily consumed diet (Fig. 3.1B). The results from the chronic ouabain exposure suggest caterpillar weight can capture delayed development effects and have reduced margins of error (Fig. 3.1A).

We observed a significant interaction of KCl x bifenthrin on diet consumption. However, there were no significant interactions observed for caterpillar weight or survival. Caterpillars exposed to 10 mg KCl/g diet and treated with 0.2 µg/µl bifenthrin consumed significantly less diet compared to those provided an untreated diet. Despite the differences in diet consumption, there were no significant differences in caterpillar weight for individuals provided an untreated and KCl-treated diet and caterpillars treated with 0.2 µg/µl bifenthrin. While not statistically significant, there was a trend of increased survival on the KCl-treated diet at 0.1 and 0.2 µg/µl bifenthrin and reduced survival on the KCl-treated diet at 0.4 µg/µl bifenthrin. Padhy et al. (2014) report a reduction in carbamate toxicity to cyanobacterium when co-exposed to potash fertilizers. For the caterpillars treated with combinations of ouabain + KCl + bifenthrin, there was a significant day x KCl x ouabain interaction on caterpillar individual weights, but there was no significant interaction on diet consumption or survival. Overall, the interactions observed do not show KCl or ouabain to affect the sensitivity of caterpillars to bifenthrin. Herbivorous insects feeding on chemically defended host plants often have developed metabolic detoxification resistance to cope with phytotoxins (Després et al. 2007). The

overproduction of detoxification proteins, such as esterases and cytochrome P450 monooxygenases, have been documented for a number of insect species (Kasai et al. 1998). This phenomenon has prompted the exploration of cross-resistance between plant allelochemicals and insecticides. For example, swallowtail butterflies, *Papilio glaucus canadensis* [Lepidoptera: Nymphalidae], have evolved resistance to phenolic glycosides in the leaves of host plants via elevated esterase activity, but when challenged with pyrethroid insecticides, the increased activity had no effect on pyrethroid toxicity (Lindroth 1989). Recent work has shown changes in expression of some detoxification genes in monarch caterpillars after feeding on different milkweed species (Tan et al. 2019). We did not observe any evidence for cross resistance with ouabain and bifenthrin at the concentrations tested in this study.

The bifenthrin concentrations used in this study have been shown to be field-relevant following aerial applications of the formulated product Brigade-2EC (Krueger et al. 2021). We mimicked a KCl exposure where bifenthrin and KCl would be applied simultaneously. In cotton, where potassium fertilization is imperative, tank mixes of pyrethroids and potassium have been shown to be compatible and not interfere with pyrethroid efficacy (Oosterhuis 2002). Fertilizer applications may increase in the future to counteract nutrient limitations and mitigate drought resiliency in the face of increasing temperatures and eroding soils. The habitat requirements for restoring the monarch population (i.e., 1.8 billion stems) can only be met if milkweed stems are planted on agricultural working lands (Thogmartin et al. 2017). Therefore, it is imperative to

understand the implications of increased potassium, and other agricultural product, inputs to build monarch habitat for 50-year candidate conservation agreements (USFWS 2020).

Here, we report no significant interactions of ouabain and KCl on bifenthrin sensitivity at the concentrations tested in this study. An understanding of interacting agricultural inputs on monarch growth and survival is important for managing their critical habitats in the Midwest US. To assess these interactions, we first need to understand the effects of milkweed-specific cardenolides on monarch physiology and, in turn, the implications for monarch insecticide toxicity. Future studies should explore these exposure combinations to provide a better understanding of monarch resiliency, such as oviposition, foraging, and fecundity, in changing landscapes when faced with multiple stressors.

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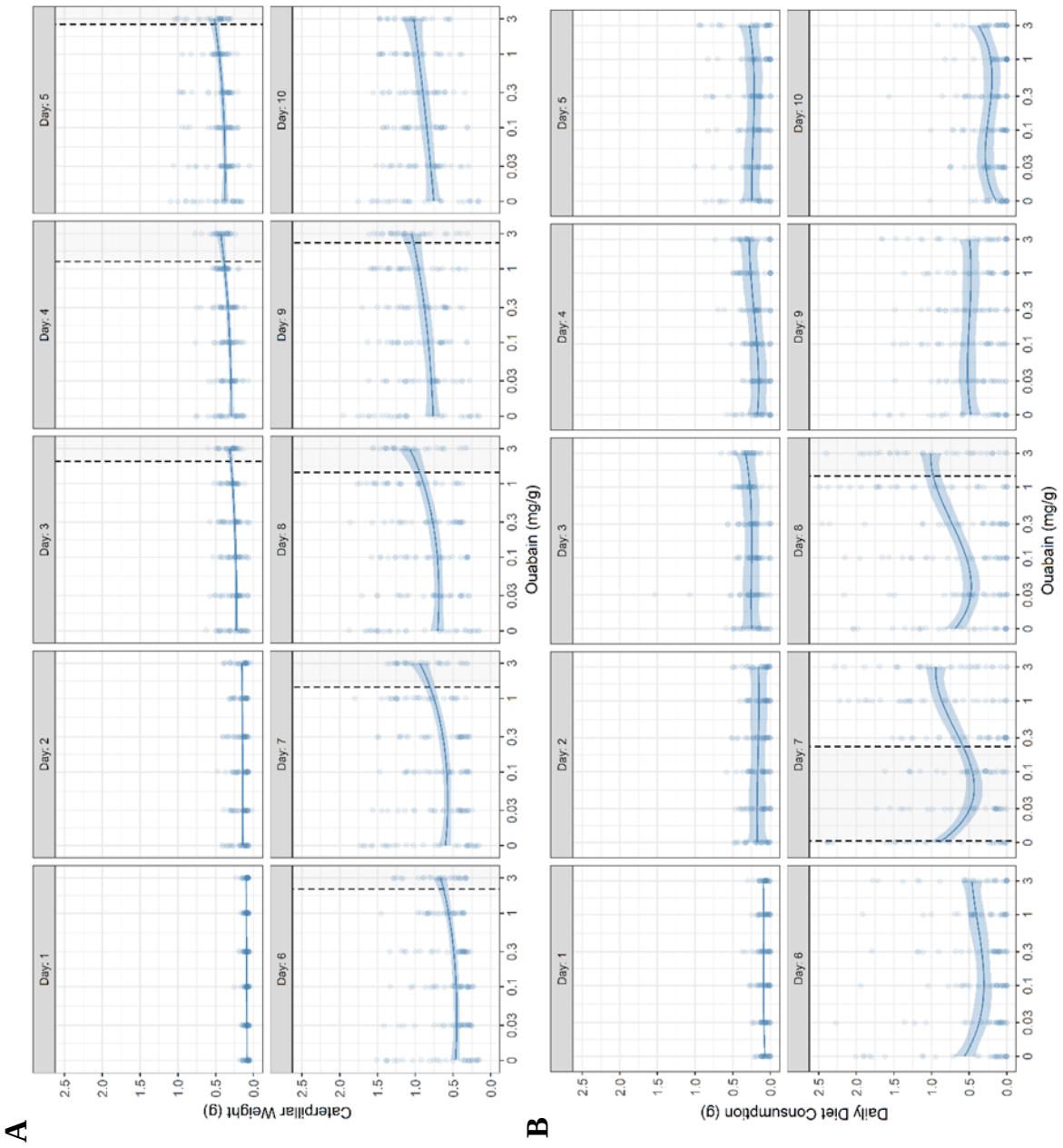

Figure 3.1 Caterpillar weight (A) and daily diet consumption (B) throughout ouabain exposure. Estimates the linear mixed model output are represented by the connecting line and 95% confidence intervals are shown as shading around the line. Response is plotted on a logarithmic scale on the ouabain axis but axis labels are converted to linear scale. Gray shading indicates significant ($p < 0.05$) differences from the control.

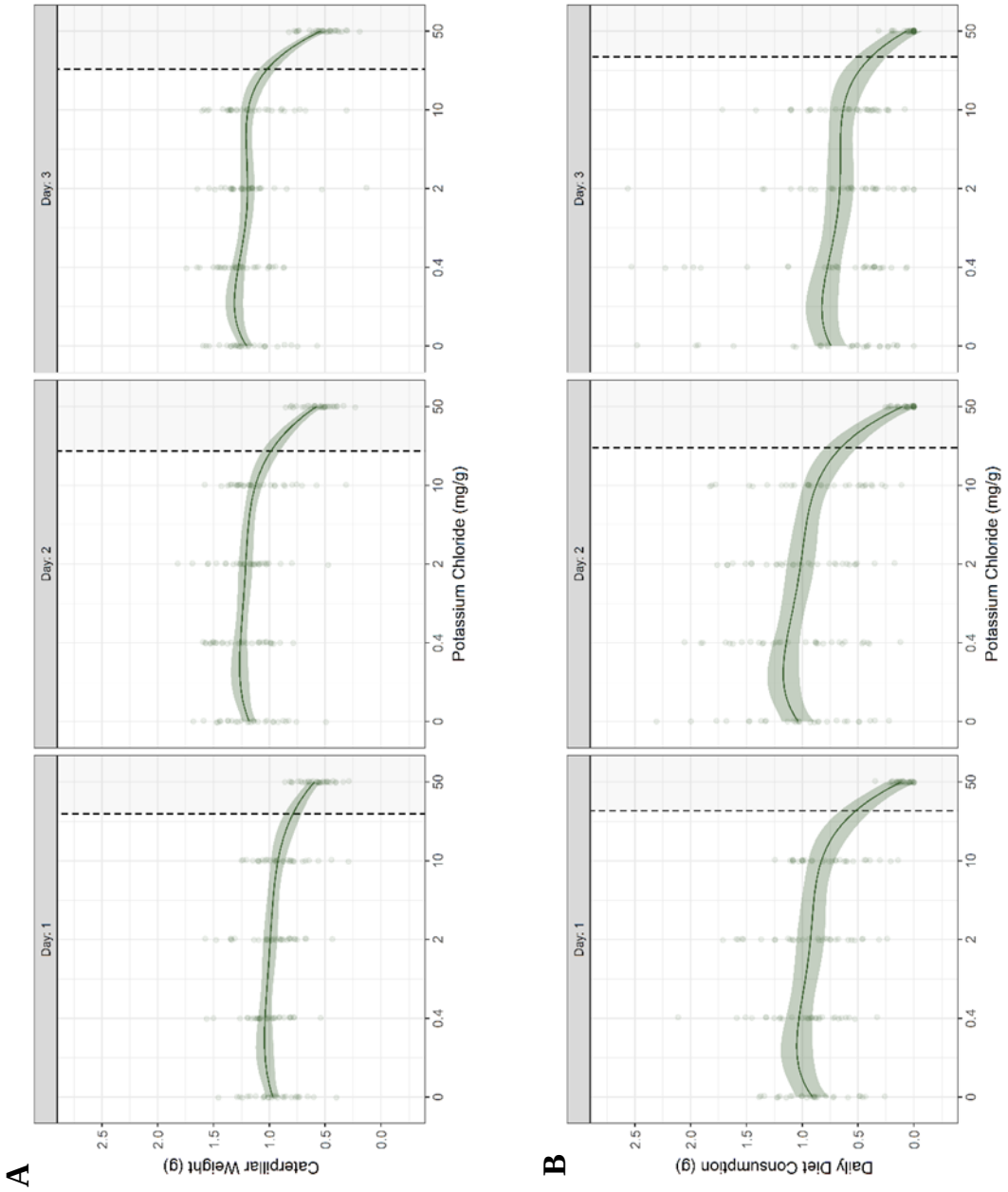

Figure 3.2 Caterpillar weight (A) and daily diet consumption (B) throughout potassium chloride exposure. Estimates the linear mixed model output are represented by the connecting line and 95% confidence intervals are shown as shading around the line. Response is plotted on a logarithmic scale on the potassium chloride axis but axis labels are converted to linear scale. Gray shading indicates significant ($p < 0.05$) differences from the control.

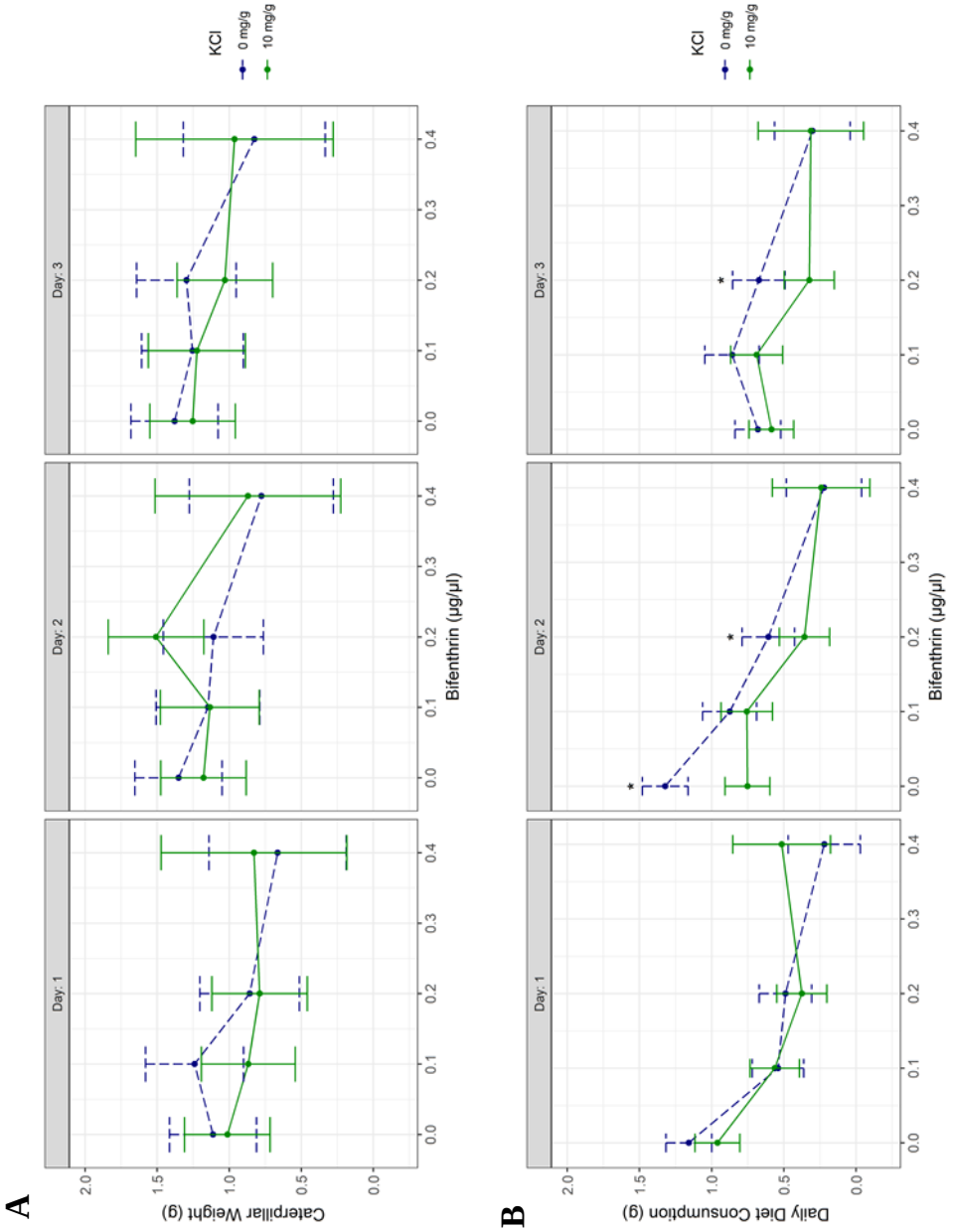

Figure 3.3 Caterpillar weight (A) and diet consumption (B) with KCl and bifenthrin exposure. Symbols depict average with upper and lower 95% confidence intervals. Asterisks denote significant ($p < 0.05$) differences between 0 KCl and 10 mg/g KCl.

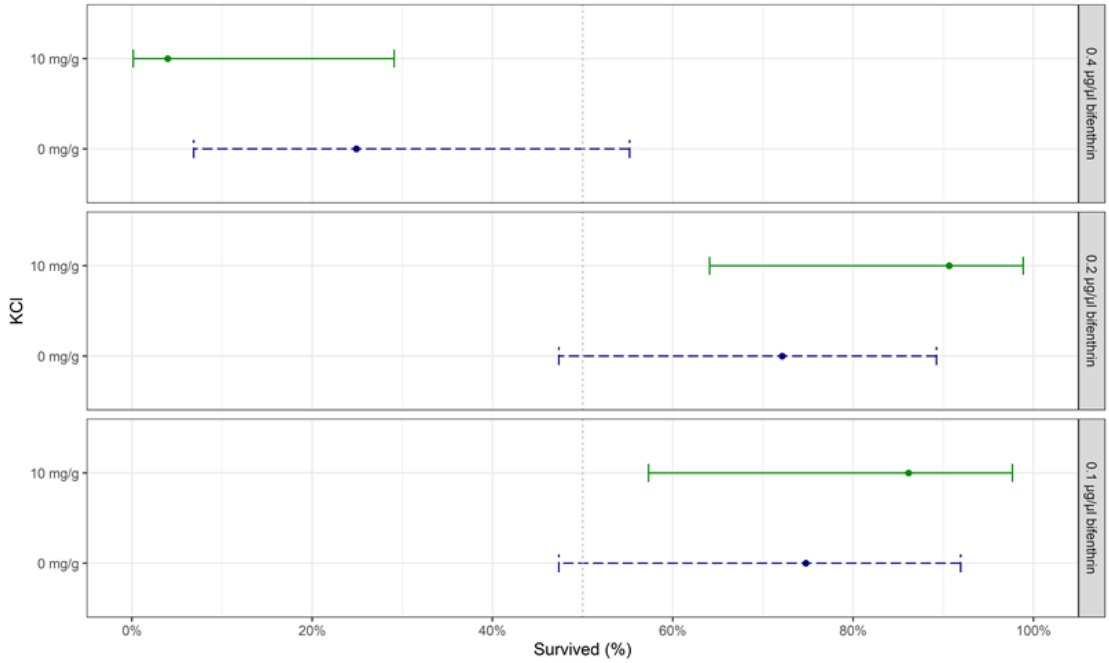

Figure 3.4 Survival of fifth-instars 72-h following topical bifenthrin treatment with and without KCl exposure. Symbols depict average with upper and lower 95% confidence intervals. No significant ($p < 0.05$) differences in survival between caterpillars exposed to 0 KCl or 10 mg/g KCl were observed.

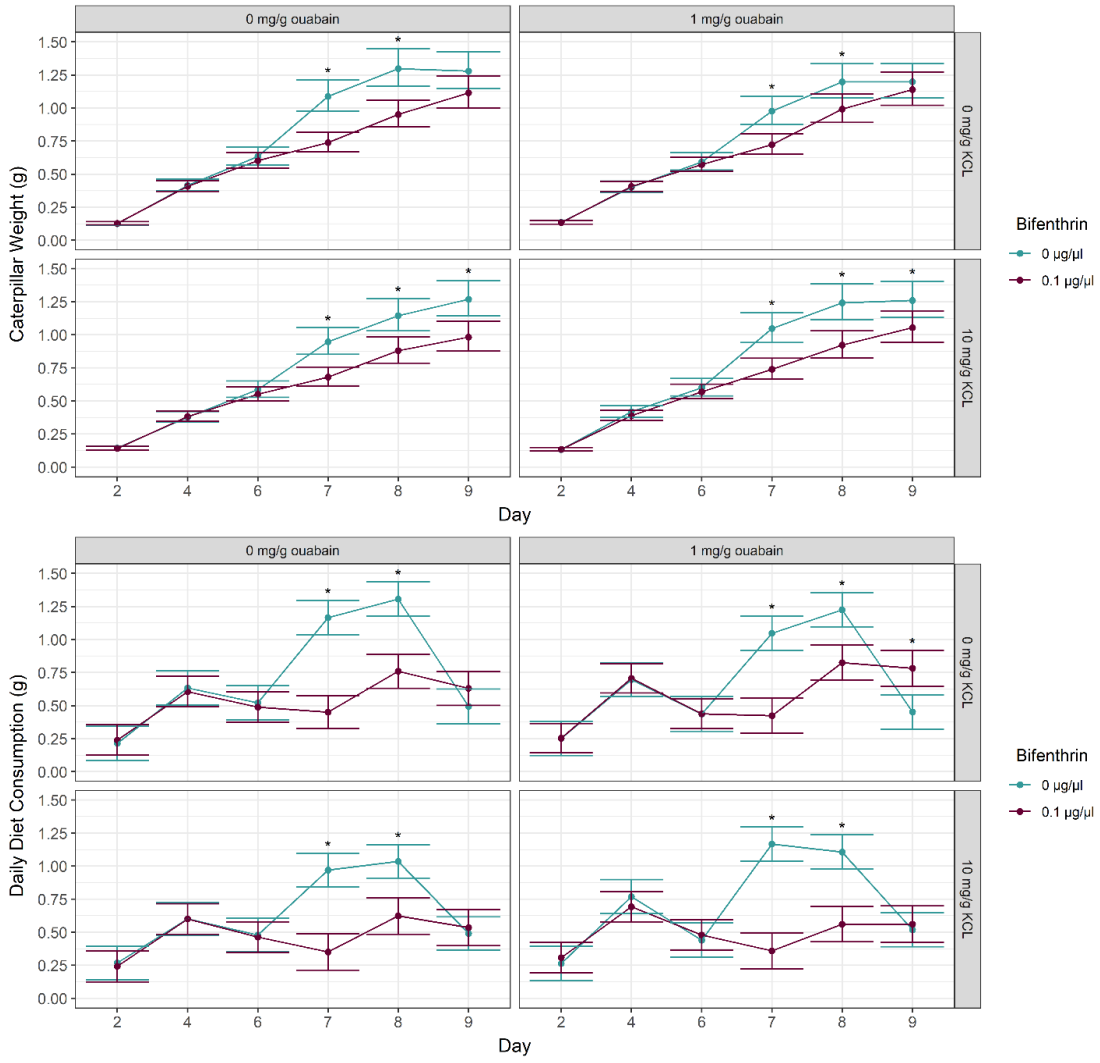

Figure 3.5 Caterpillar weight (A) and diet consumption (B) with combinations of ouabain, KCl and bifenthrin. Symbols depict average with upper and lower 95% confidence intervals. Asterisks denote significant ($p < 0.05$) differences between 0 bifenthrin and 0.1 µg/µl bifenthrin.

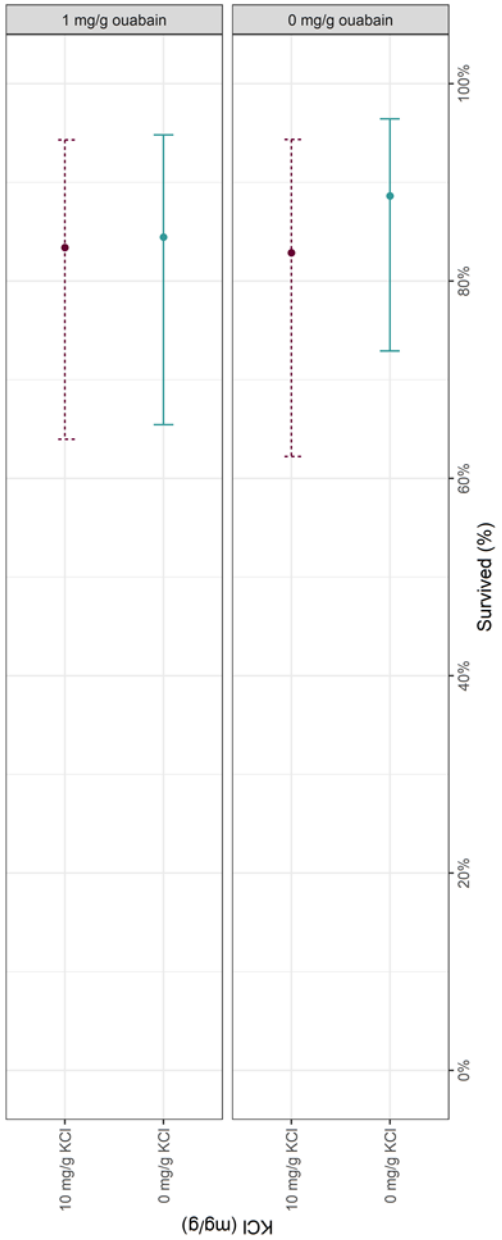

Figure 3.6 Survival of fifth-instars 72-h after treatment with 0.1 µg/µl bifenthrin and combinations of ouabain and KCl. Symbols depict average with upper and lower 95% confidence intervals. Caterpillars were exposed to ouabain from third instar (day 0) through the duration of the experiment. Caterpillars were exposed to KCl at fifth instar day 6-10 and treated with bifenthrin on day 6.

CHAPTER 4 : MILKWEED SPECIES AFFECT DETOXIFICATION ENZYME ACTIVITY AND EXPRESSION IN MONARCH CATERPILLARS

This chapter is currently under preparation for publication in Pesticide Biochemistry and Physiology A. J. Krueger, L. C. Rault, E. A. Robinson, T. J. Weissling, A. M. Vélez, and T. D. Anderson "Milkweed species affect detoxification enzyme activity and expression in monarch caterpillars"

1. Introduction

The monarch butterfly (*Danaus plexippus)* has co-evolved with milkweed (*Asclepias* sp.) [Gentianales: Apocynaceae] and has developed physiological mechanisms to cope with cardenolide defenses of the plant. The reduction of milkweed stems throughout the US, specifically the US Midwest, is implicated in the decline of the monarch butterfly population (Pleasants and Oberhauser, 2013). The remaining milkweed stems are primarily confined to the margins of agricultural fields which, in turn, forces monarchs to concentrate near row-crop production (Thogmartin et al. 2017). Thogmartin et al. (2017) estimate an additional 1.8 billion milkweed stems are needed to recover the eastern monarch population.

There are 73 native milkweed species in North America that monarchs can utilize during the breeding season (Agrawal et al., 2012). Monarch caterpillars and other herbivorous insects that feed on milkweed are exposed to insecticidal cardenolide defenses of milkweeds. Cardenolides are secondary plant metabolites within a sub-class of cardiac glycosides derived from triterpenoids with broad-spectrum insecticidal activity (Agrawal et al., 2012). These compounds target Na^+/K^+-ATPase of the nervous system, where they reversibly bind to the α-subunit, locking it in a phosphorylated conformation, and disrupting ion translocation and nerve function (Dobler et al., 2011; Horisberger,

2004). There are *ca*. 500 identified cardenolide derivatives with diverse structural conformations (Agrawal et al., 2012; Schönfeld et al., 1985). Milkweed species vary in their cardenolide composition and concentration (Agrawal et al., 2012; Brower et al., 1982; Frick and Wink, 1995; Seiber et al., 1983). However, monarchs have evolved resistance to cardenolides via modifications of the Na^+/K^+-ATPase (Holzinger and Wink, 1996). Monarchs and other cardenolide-insensitive butterfly species possess a single mutation of glutamine (Q) to valine (V) at the 111 position in the ouabain binding site of the Na^+/K^+-ATPase, however monarchs have two additional mutations of alanine (A) to serine (S) and asparagine (N) to histidine (H) at the 119 and 122 positions, respectively (Karageorgi et al., 2019; Petschenka et al., 2013). The combination of these three mutations has been associated with the ability of monarchs to sequester cardenolides (Petschenka and Agrawal, 2015). However, the mechanisms of cardenolide sequestration in monarchs are still largely unknown. In both the salicin-sequestering poplar leaf beetle (*Chyrosmela populi*) and the cardenolide-sequestering dogbane beetle (*Chrysocus auratus*), ATP-binding cassette (ABC) transporters have been implicated as the active carrier the sequestration of these respective compounds (Kowalski et al., 2020; Strauss et al., 2013). Little is known about ABC transporters in monarchs and the potential role they play in cardenolide sequestration in Lepidoptera.

Insects interacting with chemically defended host plants often have evolved metabolic detoxification resistance to cope with phytotoxins (Després et al., 2007). The overproduction of esterases and cytochrome P450 monooxygenases in response to these phytotoxins have been documented for a number of insect species (Kasai et al., 1998).

This phenomenon has prompted exploration of cross-resistance between plant allelochemicals and insecticides. For example, swallowtail butterflies, *Papilio glaucus canadensis*, have evolved resistance to phenolic glycosides in the leaves of host plants via elevated esterase activity, but when challenged with two different pyrethroids, the increased activity had no effect on pyrethroid toxicity (Lindroth, 1989). For monarchs, there is little information on metabolic detoxification enzyme activities and the potential for cross-resistance or impact of cardenolide resistance on these enzymes. Cytochrome P450 monooxygenases are involved in cardenolide metabolism in monarchs (Agrawal et al., 2012). The potential for cross-resistance or impact of cardenolide resistance on detoxification enzymes has largely remained unexplored. Recent work has shown changes in expression of detoxification genes after monarch caterpillars feed on different milkweed species (Tan et al., 2019). Furthermore, Krishnan et al. (2020) documented a significantly higher LD_{50} for imidacloprid when caterpillars fed on tropical milkweed compared to common milkweed. There is relatively little information on how milkweed species can affect detoxification mechanisms in monarchs. However, understanding this potential host-plant interaction could have significant implications for monarch-insecticide interactions near agriculture.

Pyrethroid insecticides are commonly used to control insect pests in corn and soybean across the US Corn Belt (Ragsdale et al., 2011). These broad-spectrum insecticides are acutely neuro-toxic, targeting the voltage-gated Na+ channel and disrupting neurological function (Clements and May, 1977). Because of their acute toxicity, pyrethroids have been used to control a variety of insect outbreaks. The timing

of soybean aphid outbreaks and subsequent foliar applications of pyrethroids often occur when monarch caterpillars are present on the landscape (Bradbury et al., 2017). Pyrethroid toxicity and exposure data shows monarchs developing in field margins 0-10 m from the field may be adversely affected by foliar applications during the breeding season (Krishnan et al., 2020; Krueger et al., 2021; Olaya-Arenas and Kaplan, 2019). While foliar applications of pyrethroids pose the greatest risk to monarchs in field margins, many Lepidoptera pests these applications target have evolved resistance to pyrethroids. Pyrethroid-resistant populations of *Helicoverpa armigera* are reported to overproduce esterases for the rapid detoxification of many commonly used pyrethroids (Young et al., 2005). Yang et al. (2005) has also documented increased activity and investment in cytochrome P450 monooxygenase in resistant *H. armigera* populations. Across many insect orders, the over expression of glutathione *S*-transferases can reduce pyrethroid cytotoxicity (Ketterman et al., 2011) and protect from pyrethroid-induced oxidative stress (Vontas et al., 2001). (Vontas et al., 2001). If cardenolides in milkweed have increased expression and production of detoxification enzymes in monarchs, it is possible monarch caterpillars would be better able to metabolize pyrethroid insecticides.

The aim of this study was to compare the toxicity of the pyrethroid insecticide bifenthrin to fifth-instar caterpillars feeding on tropical (*A. curassavica*) or swamp (*A. incarnata*) milkweed. Tropical and swamp milkweed differ in both total cardenolide concentration and content, which allows for comparative responses of detoxification mechanisms following exposure to field-realistic concentrations of cardenolides. Further, differential detoxification gene expression has already been documented in the second-

instar stage of monarchs feeding on these two species (Tan et al., 2019). The average cardenolide content reported for tropical and swamp milkweed is 3.3 and 0.5 µg/mg dry weight, respectively (Rasmann and Agrawal, 2011). Fifth-instar caterpillars were topically treated with either acetone (solvent control) or bifenthrin (in acetone) and monitored for 24 h. After 24 h, caterpillar mortality, detoxification enzyme activity, and detoxification gene expression were quantified to assess the effects of milkweed species and bifenthrin exposure to monarch caterpillars. We specifically focused on the activity and expression of select esterases, glutathione *S*-transferases, cytochrome P450s, and ABC transporters given their relevance in both cardenolide metabolism and pyrethroid detoxification. Here, we report significant differences in detoxification enzyme activity and expression between caterpillars feeding on different milkweed species.

2. Materials and Methods

2.1 Test organism

Monarch caterpillars were sourced from a colony maintained in the Department of Entomology at the University of Nebraska as described by Krueger et al. (2021). Briefly, the eggs were collected daily, stored at 16 °C up to 1-week post-collection and moved to room temperature for each experiment. Neonates hatched within 2-3 d and were placed on leaves of either swamp or tropical milkweed. Caterpillars were maintained on leaves through the fifth-instar stage for each experiment.

2.2 Milkweed plants

Milkweed seeds were purchased from Prairie Nursery (Westfield, WI). Common and swamp milkweed were cold stratified for one week at 16 °C. Seeds were planted in standard greenhouse soil and fertilized with nitrogen, phosphorous, potassium fertilizer

(NPK, 20:60:20) at 4-, 8- and 12-weeks post emergence. Leaves were removed from plants, washed in a 10% (v:v) bleach solution and air dried. Leaf petioles were clipped just prior to provisioning and wrapped in a wet cotton ball to maintain leaf rigidity.

2.3 Experimental setup

Monarch caterpillars on each milkweed species were maintained in 3 groups of 20 from first to third-instar stage in custom made vented collection pans. At the third-instar stage, a total of 30 individual caterpillars were moved to individual 8 oz. plastic cups (Lake Forest, IL) and provisioned with leaves *ad libitum* until reaching the fifth-instar stage.

2.3.1 Bifenthrin exposure

Bifenthrin (CAS# 82657-04-3, 99.5%) was purchased from Chem Service Inc. (West Chester, PA) and stored at room temperature. Stock solutions were prepared in acetone (Sigma-Aldrich, St. Louis, MO). One-day old fifth instar caterpillars were randomly assigned to control or bifenthrin treatments within each milkweed species. Caterpillar were treated with either acetone as the solvent control or 0.1 µg/µl bifenthrin. A 1 µL aliquot was applied to the dorsal prothorax, between the anterior tentacles, of each caterpillar. Mortality was recorded 24h post-treatment and surviving caterpillars were frozen in liquid nitrogen and stored for enzyme activity and gene expression analyses. Each experiment was repeated in triplicate.

2.4 Dissections

Five caterpillars from each treatment in each experiment were removed from -80ºC for enzyme activity assays. Caterpillars were placed on ice where the hemolymph was collected and the head was dissected from the body. The head was stored in a 1.5-ml

microcentrifuge. The internal organs were not dissected from the body. The last two pairs of abdominal prolegs were clipped with dissecting scissors and hemolymph was collected and transferred to a 1.5-ml microcentrifuge tube containing a few crystals of phenylthiourea (PTU) (CAS# 103-85-5, Sigma-Aldrich, St. Louis, MO) to prevent melanization as described by Wongkobrat and Dahlman (1976). After a minimum of 100 µL hemolymph was collected, the caterpillar body was placed in a 1.5-ml microcentrifuge tube. All dissected tissues (head, hemolymph, body) were immediately frozen at –80 ºC for enzyme assays.

2.5 Detoxification enzyme activity assays

Measurements for all colorimetric enzyme activity assays were conducted using a SpectraMax i3x multimode microplate reader (Molecular Devices, Inc., Sunnyvale, CA). Detoxification enzyme activities are reported for individual caterpillars. Total protein in each sample preparation was determined using bicinchoninic acid assay with bovine serum albumin as a standard and measurements conducted at 560 nm (Smith et al. 1985).

2.5.1 Chemicals

Acetone, α-naphthyl acetate (α-NA), fast blue B salt (O-dianisidine, tetrazotized), sodium dodecylsulfate (SDS), α-naphthol, reduced glutathione, and 1-chloro-2, 4-dinitro-benzene (CDNB) acetone, 7-ethoxycoumarin (7-EC), β-nicotinamide adenine dinucleotide phosphate (reduced β-NADPH), oxidized glutathione, glutathione reductase, acetonitrile, TRIZMA-base, bicinchoninic acid solution, bovine serum albumin and cupric sulfate were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.5.2 General esterase activity

General esterase activity of monarch caterpillar hemolymph was determined using α-NA as the substrate according to the methods described by van Asperen (1962) with modifications by O'Neal et al. (2019). Additional sample preparation steps were required for monarch hemolymph samples. Hemolymph was thawed and centrifuged at 10,000 x g for 10 min at 4ºC to remove PTU crystals from the sample. Centrifugation and PTU removal were necessary to avoid interference with esterase, GST and protein colorimetric assays. A PTU blank was prepared using PTU crystals in 500 µl nanopure water and centrifuged along with the hemolymph samples. Samples and the PTU blank were then diluted 100-fold in ice-cold 0.1 M sodium phosphate buffer at pH 7.8. Aliquots of 15 µl of diluted hemolymph or diluted PTU blank were added to clear microplate wells with 135 µl or 0.3 M α-NA. The microplate was then incubated for 30 min at 37ºC. After incubation, the reaction was stopped by adding 50 µl of fast blue B in 5% SDS solution. The microplate was then left at room temperature for 15 minutes to allow color to develop before the absorbance was read at 600 nm. A standard curve was prepared using α-naphthol to quantify the amount of hydrolytic product in each sample. Estimated mean specific activity (µmol/min/mg of total protein) and upper and lower confidence limits are reported for each treatment.

2.5.3 Glutathione S-transferase Activity

Glutathione *S*-transferase (GST) activity of monarch caterpillar hemolymph was determined according to O'Neal et al. (2019) using CDNB as the substrate. The same 100-fold dilution used for esterase activity assays was used to quantify GST activity with

the 100-fold diluted PTU solution as a blank. Aliquots of 20 µl of diluted hemolymph or diluted PTU blank were added to microplate wells. A reaction mix was prepared with 10 mM reduced glutathione and 150 mM CDNB and 180 µl of reaction mix were added to each well. The change in absorbance for each sample was recorded at 340 nm for 10 min at 1 min intervals. Estimated mean specific activity (µmol/min/mg) and upper and lower confidence limits are reported for each treatment.

2.5.4 Cytochrome P450 activity

Cytochrome P450 activity of monarch caterpillars was determined in isolated microsomes according to the method of O'Neal et al. (2019) using 7-EC as a substrate. Microsomes were isolated using the microsome isolation kit (ab206995) from Abcam (Cambridge, United Kingdom) to improve the detection of enzyme activity. Due to their size, monarch body samples were cut in half and homogenized in 1 ml of supplied homogenization buffer with protease inhibitor. Samples were centrifuged for 10,000 x g for 15 min at 4ºC and supernatant from each half caterpillar was combined to reconstitute the individual sample. Manufacturer instructions were followed for the remaining isolation steps. Isolated microsomes were resuspended in 150 µl of supplied storage buffer with protease inhibitor.

Cytochrome P450 activity was then quantified from isolated microsomes as follows. Aliquots of 20 µl of isolated microsomes or 20 µl of storage buffer with protease inhibitor were added to a black microplate. A reaction mixture was prepared with 50 mM 7-EC and 62.5 mM reduced β-NADPH and 80 µl was added to each well. The microplate was placed in a shaking incubator for 1 hour at 30 ºC shaking at 400 rpm.

After 1 hour, the plate was removed and 10 µl of 100 mM oxidized glutathione and 1.0 U glutathione reductase were added to each microplate well and incubated for 15 min at 37ºC. The plate was then removed and 120 µl of 50% (v:v) acetonitrile in 50 mM TRIZMA-base buffer were added to each well to stop the reaction. Fluorescence was read at 465 nm while exciting at 390 nm. Estimated mean relative fluorescence units (RFU/mg total protein) and upper and lower confidence limits are reported for each treatment.

2.5.5 Protein quantification

Protein was quantified for each hemolymph and isolated microsome samples following methods described by (Smith et al., 1985). For quantification of hemolymph samples, 20 µl of 100-fold diluted hemolymph or 100-fold diluted PTU blank were added to each well of a clear microplate. For quantification of isolated microsome samples, 20 µl of isolated microsome or 20 µl of storage buffer with protease inhibitor were added to each well. A reaction mix was prepared with 4% cupric sulfate solution and bicinchoninic acid and 180 µl was added to each well. The microplate was incubated at 37ºC for 30 minutes and set at room temperature for 5 minutes before absorbance was measured at 560 nm. A standard curve was prepared using bovine serum albumin to quantify protein concentrations for each sample.

2.6 Gene expression analysis

2.6.1 RNA extraction

RNA was extracted from fifth-instar caterpillars using a Qiagen RNeasy Maxi Kit (Valencia, CA). Following the RNA extraction, 1 µg of RNA per 20 µL reaction volume was reverse transcribed using a iScript cDNA reverse transcription kit (BioRad, Hercules,

CA) and stored at –20 ºC. The resulting cDNA was diluted 10-fold for quantitative-reverse transcriptase polymerase chain reaction (qRT-PCR).

2.6.2 Quantitative-reverse transcriptase PCR

Primer pairs for the qRT-PCR were designed using Primer3 (Rozen and Skaletsky, 2000) from genes identified by Tan et al. (2019) as differentially expressed in swamp and tropical milkweed. Sequences, approximate amplicon size and primer efficiencies are reported in Appendix 6. Primers were synthesized by Sigma Aldrich (St. Louis, MO). The qRT-PCR experiments followed the methods and PCR protocol outlined in Rault et al. (2019b). Briefly, qRT-PCR was conducted with a Biorad CFX Connect Real-Time System using iTaq Universal SYBR® Green Supermix (Biorad, Hercules, CA). Two housekeeping genes, 28S ribosomal RNA (28S) and elongation factor 1α (EF1a), were selected from Pan et al.(2015). However, 28S expression was affected by bifenthrin treatment so EF1a was the only housekeeping gene used in the analysis.

2.7 Data analysis

All statistical analyses were conducted in R 4.0.1 (R Core Team, 2020). All figures were generated using the estimates obtained using the estimated marginal means (emmeans) package (Lenth, 2020) from the model outputs and the ggplot2 package (Wickham, 2016) for plotting. For survival and enzyme activity analyses, residuals were plotted using qq-plots and used to test assumptions of normality. For gene expression analyses, trace plots were used to assess model fit.

2.7.1 Survival analysis

The proportion of surviving caterpillars was analyzed for bifenthrin treatments on swamp and tropical milkweed using a generalized linear mixed model assuming a binomial distribution with a cumulative log-log link function using the glm function in the stats package (R Core Team, 2020). Experimental replicate was included as a fixed block effect in the model. The model was only run for bifenthrin treated caterpillars since no mortality was observed in the acetone-treated caterpillars on either milkweed species. Differences in survival following bifenthrin treatment were tested between milkweed species.

2.7.2 Enzyme activity analyses

Detoxification enzyme activities were analyzed using a linear mixed model assuming a normal distribution using the lm function in the stats package (R Core Team, 2020). The model for each enzyme, included experimental replicate as a fixed block and bifenthrin, milkweed species, and the interaction of bifenthrin and milkweed species as treatment factors. From each model, estimated mean enzyme activity was compared between 1) bifenthrin-treated and solvent-treated caterpillars on tropical milkweed, 2) bifenthrin-treated and solvent-treated caterpillars on swamp milkweed, and 3) solvent-treated caterpillars on tropical and swamp milkweed, using the emmeans package (Lenth, 2020) to obtain pairwise t-tests of interest. Residual and quantile-quantile plots were used to assess model fit and test assumptions of normality.2.7.3 Gene expression analyses

2.7.3 Gene Expression Analysis

Gene expression data was analyzed using Bayesian methods in R using the MCMC.qpcr package (Matz et al., 2013). The Bayesian analysis provides key advantages

for data sets with larger variability (Rault et al., 2019a). A Markov Chain Monte Carlo is run to fit a Poisson-lognormal generalized linear mixed model (GLMM) to the gene molecular counts for EF1a, esterase, ABC, GST20, GST21, and CYP. Molecule counts could be calculated by the model using primer efficiency values and the measured C*t* values for each gene and treatment combination. Fixed effects for milkweed, bifenthrin, and their interaction are estimated with EF1a as the control gene with the control prior variance specified with an allowed average fold-change of 1.2. Effective sample sizes and trace plots were used to evaluate model fit.

3. Results

3.1 Survival

The results of caterpillar survival after 24-h treatment with bifenthrin are shown in Fig. 4.1. No mortality was observed for the solvent-treated caterpillars from each of the milkweed species. Furthermore, the survival of caterpillars from swamp and tropical milkweed was not significantly different after treatment with bifenthrin ($z = -0.723$; $p = 0.47$).

3.2 General esterase activity

The results of caterpillar general esterase activity are shown in Fig. 4.2A. Caterpillars feeding on swamp milkweed and treated with 0.1 µg/µl bifenthrin had significantly lower general esterase activity following a 24-h treatment of bifenthrin ($t = 3.44$; $df = 54$; $p = 0.0011$). There was a significant decrease in general esterase activity in bifenthrin-treated caterpillars compared to solvent-treated caterpillars feeding on tropical milkweed ($t = 2.78$, $df = 54$; $p = 0.0075$). The solvent-treated caterpillars feeding on

tropical milkweed had significantly lower general esterase activity compared to solvent-treated caterpillars feeding on swamp milkweed (t = 4.81; df = 54; $p < 0.001$).

3.3 Glutathione S-*transferase activity*

The results of caterpillar glutathione *S*-transferase activity are shown in Fig. 4.2B. Caterpillars feeding on swamp milkweed and treated with 0.1 µg/µl bifenthrin had significantly higher glutathione *S*-transferase activity compared to solvent-treated caterpillars (t = 2.72; df = 54; $p = 0.0089$). Bifenthrin-treated caterpillars feeding on tropical milkweed did not have significantly different glutathione *S*-transferase activity compared to solvent-treated caterpillars (t = 1.86; df = 54; $p = 0.068$). There was a marginally significant difference in glutathione *S*-transferase activity for solvent-treated caterpillars feeding on swamp and tropical milkweed (t = -1.91; df = 54; $p = 0.062$).

3.4 Cytochrome P450 activity

The results of caterpillar cytochrome P450 activity are shown in Fig. 4.2C. There were no significant differences in cytochrome P450 activity between solvent- and bifenthrin-treated caterpillars feeding on swamp (t = 0.83; df = 37; $p = 0.41$) or tropical (t = -1.33; df = 37; $p = 0.19$) milkweed. However, there was a significant difference in cytochrome P450 activity between solvent-treated caterpillars feeding on tropical and swamp milkweed (t = -2.20; df = 37; $p = 0.034$).

3.5 Gene expression analysis

The results of gene expression for caterpillars feeding on tropical and swamp milkweed and treated with bifenthrin are shown in Fig. 4.3. The expression of esterase was significantly higher in solvent-treated caterpillars feeding on swamp milkweed compared to solvent-treated caterpillars feeding on tropical milkweed (+1.62, $p = 0.0058$)

without bifenthrin treatment. The expression of esterase was significantly higher for bifenthrin-treated caterpillars compared to solvent-treated caterpillars feeding on tropical milkweed (+1.18, $p = 0.037$). The expression of GST20 was significantly lower in solvent-treated caterpillars feeding on swamp milkweed compared to solvent-treated caterpillars feeding on tropical milkweed (-1.11, $p = 0.0012$). The expression of GST20 was significantly lower for bifenthrin-treated caterpillars compared to solvent-treated caterpillars feeding on tropical milkweed (-0.75, $p = 0.025$). There were no significant differences in GST21 or CYP450 expression for caterpillars treated with bifenthrin and feeding on tropical or swamp milkweed. The expression of ABC transporter was significantly higher in solvent-treated caterpillars feeding on swamp milkweed compared to solvent-treated caterpillars feeding on tropical milkweed (+1.03, $p = 0.022$).

4. Discussion

This study provides the first evidence of differential detoxification enzyme activities and the expression of select detoxification gene transcripts in monarch caterpillars treated with the pyrethroid insecticide bifenthrin and feeding on tropical and swamp milkweed. The toxicity of bifenthrin to caterpillars was not significantly different for individuals feeding on tropical and swamp milkweed. However, the general esterase and glutathione *S*-transferase activities were significantly different for caterpillars feeding on tropical and swamp milkweed. Furthermore, a differential expression of esterase and glutathione *S*-transferase genes was observed for caterpillars treated with bifenthrin. Tan et al. (2019) report a transcriptomic approach for observing the differential expression of detoxification genes in second-instar monarch caterpillars. While the study provides valuable insight for the expression of detoxification genes potentially affected by

cardenolides in milkweed, it is limited to a transcriptome profile with no confirmation for the observed differences in expression using qRT-PCR (Tan et al., 2019). Here, we confirm the differential expression of select detoxification transcripts, in fifth-instar caterpillars treated with a sub-lethal concentration of bifenthrin and feeding on tropical and swamp milkweed.

Tropical and swamp milkweed did not significantly affect the survival of bifenthrin-treated caterpillars despite significant differences in detoxification enzyme activity and expression. Previous studies exploring cross-resistance in swallowtails (*Papilio glaucus canadensis*) report significant differences in both esterase and GST enzyme activity were associated with increased levels of phenolic glycosides (Lindroth, 1989). Furthermore, swallowtails challenged with the ester-containing insecticides, malathion and permethrin, were not observed to have a significant survival advantage when feeding on diets higher in phenolic glycosides.

Detoxification enzyme activity significantly differed between solvent- and bifenthrin-treated caterpillars feeding on both swamp and tropical. General esterase activity was significantly reduced in caterpillars feeding on swamp milkweed whereas GST activity was significantly increased for caterpillars feeding on tropical milkweed. Increased detoxification enzyme activity has been associated with insecticide resistance in other Lepidoptera (Vontas et al., 2001; Yang et al., 2005). Interestingly, general esterase activity from solvent-treated caterpillars feeding on either milkweed species are significantly higher than previously reported for other Lepidoptera (Achaleke et al., 2009; Lai et al., 2011; Mukherjee, 2003). Swallowtails that adapted to a diet composed of

ester-containing phenolic glycosides represented the highest reported activity levels across Lepidoptera (Lindroth, 1989). However, swallowtail general esterase activity was still had 5-fold lower than what has been reported here in monarchs.

Expression of detoxification genes on control caterpillars differed between swamp and tropical milkweed. Previous transcriptome analysis of second instars on swamp and topical milkweed showed significant differences in CYP450 and GST gene expression, with several genes upregulated on swamp and others upregulated on tropical milkweed (Tan et al., 2019). Interestingly, the authors also documented 8 ABC-transporters differentially expressed in swamp milkweed relative to tropical milkweed, with all 8 genes upregulated in swamp. Our results with one of these 8 ABC-transporter genes in the G-subfamily show a similar trend between milkweed species. Genes belonging to the ABCG family have been associated with detoxification in a number of insect species (Wu et al., 2019). Interestingly, members of the ABCB and ABCC subfamilies have been identified as an active carrier involved in cardenolide sequestration (Kowalski et al., 2020; Strauss et al., 2013). Future work should explore how expression of ABC-transporter genes belonging to these subfamilies compares across caterpillars developing on different milkweed species. Tan et al. (Tan et al., 2019) also observed one esterase enzyme significantly upregulated in second instar caterpillars feeding on tropical milkweed compared to swamp milkweed. This same gene appeared to show the opposite trend in fifth instar caterpillars, with higher esterase expression on swamp milkweed compared to tropical milkweed. Esterase enzymes are involved in many different physiological processes (Montella et al., 2012). There are two potential matches for this

gene in BlastX as either a juvenile hormone esterase or a carboxyl/choline esterase enzyme. It is possible this esterase is related to growth metabolism rather than detoxification however, this would not explain the significant increase in expression following bifenthrin treatment on tropical milkweed.

Gene expression significantly differed between control caterpillars and bifenthrin treated caterpillars on tropical milkweed but not swamp milkweed. These differences showed the opposite trend of gene expression data for esterase or GST. Other studies comparing responses to different host plant species in grasshopper have linked trends in enzyme data with those in gene expression data for both GSTs and CYP450s (Huang et al., 2017). This disconnect in gene expression and enzyme activity could be related to different half-lives of the proteins (Hargrove and Schmidt, 1989) or other GST or esterase genes may be responsible for the significant differences in enzyme activity. Further, there could be interplay of expression of multiple esterase, GST, or CYP450 genes not quantified in this study.

Swamp and tropical milkweed species have been used for multiple studies to compare physiological responses and behavior of milkweed specialists with different cardenolide exposures (Martel and Malcolm, 2004; Tan et al., 2019; Zalucki et al., 1990). For habitat restoration, swamp milkweed is a more suitable species for plantings in the US Midwest. Tropical milkweed is non-native to the Midwest and adverse effects on adult migration have been reported (Faldyn et al., 2018). However, tropical milkweed is used to maintain many laboratory monarch colonies (e.g., Krishnan et al., 2020; Krueger et al., 2021) and is relevant in southeastern breeding habitat. Understanding how

detoxification enzymes differ between milkweed species will be important for evaluating the risk of insecticides across different host plants.

Here, we report significant differences in detoxification enzyme activity and expression in caterpillars on swamp versus tropical milkweed. Overall, there was no significant difference in survival, suggesting while physiological differences occur, pyrethroid sensitivity will not differ on these milkweed species at this dose. Milkweed species selection for monarch habitat restoration must offer adult monarchs a preferred oviposition site, sufficient biomass to support larval development, and the highest likelihood of survival. Data presented here suggest there are physiological differences between caterpillars on swamp and tropical milkweed. Further research should explore these physiological consequences in the context of detoxification on other milkweed species relevant for conservation. Optimizing habitat for monarchs will be key for maximizing conservation benefits of the 1.8 billion stems planted and ensuring resiliency for the monarch population.

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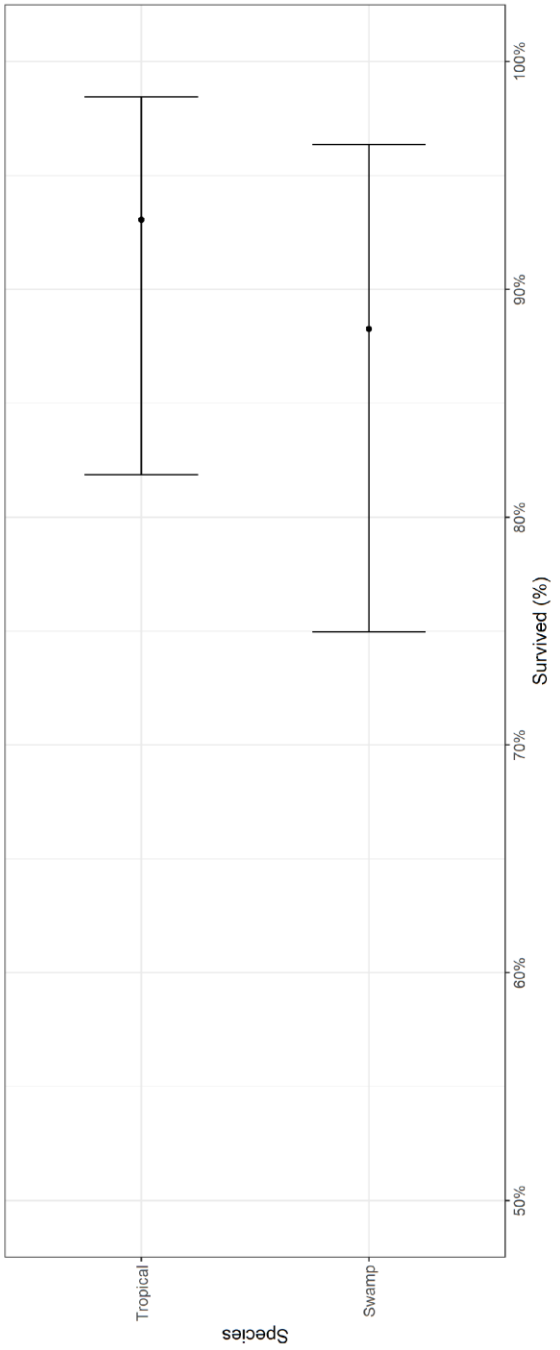

Figure 4.1 Survival of caterpillars feeding on swamp or tropical milkweed with bifenthrin treatment. Percent survival modeled with glm on caterpillars exposed to 0.1 µg/µl bifenthrin and feeding on swamp (n = 39) or tropical (n = 45) milkweed. Points represent estimated mean percent survival, error bars depict upper and lower confidence limits.

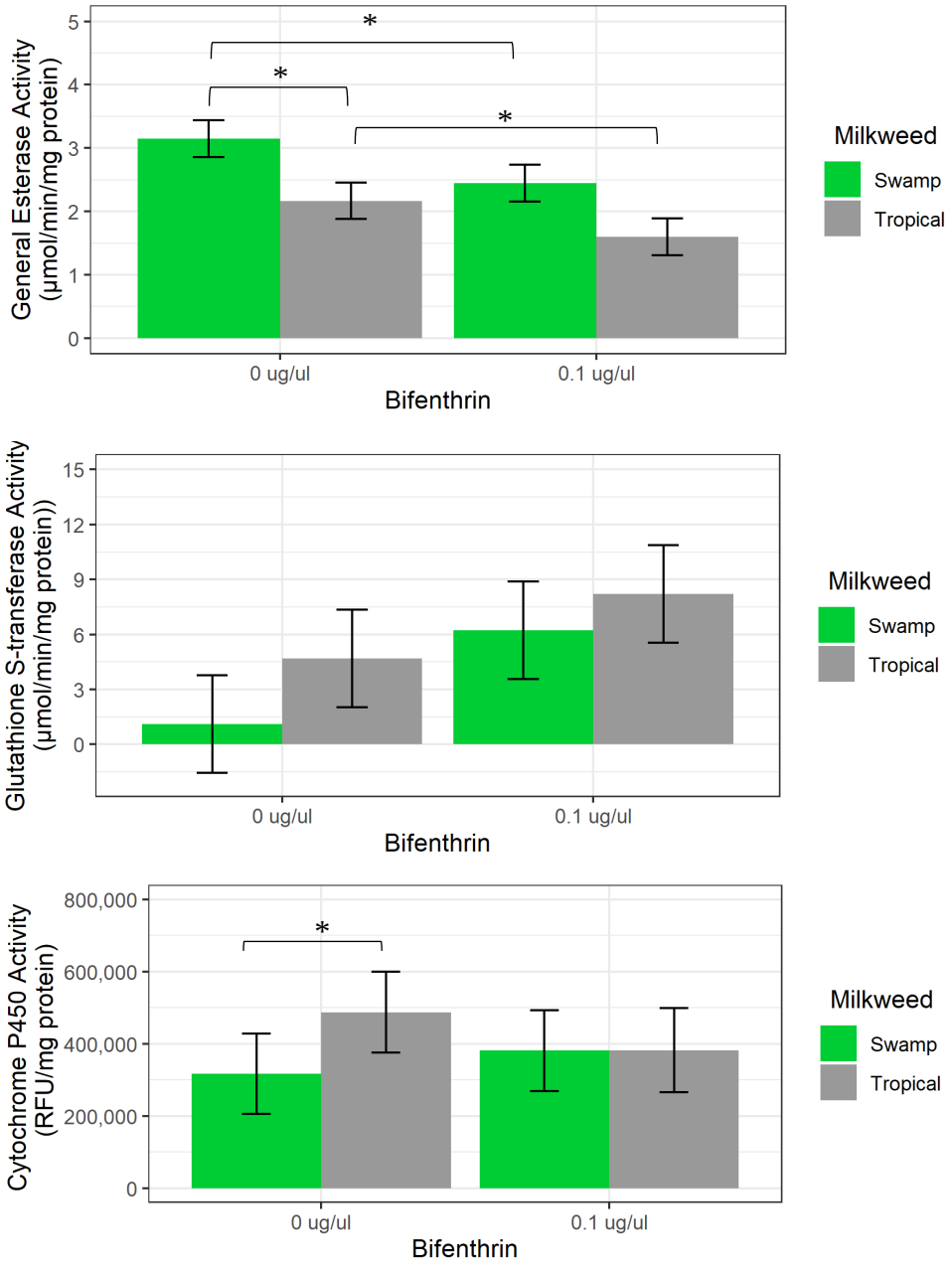

Figure 4.2 General esterase, glutathione *S*-transferase, and cytochrome P450 activities for caterpillars feeding on swamp or tropical milkweed with and without bifenthrin treatment. Bars represent estimated mean specific activity for esterase (n=15), glutathione *S*-transferase (n=15) and cytochrome P450 (n=11), error bars depict the upper and lower confidence limits for each estimate. Activity was compared between 0 and 0.1 µg/µl bifenthrin treatments for swamp and tropical milkweed, and between 0 bifenthrin on swamp and tropical milkweed. Brackets represent the statistical comparison tested and asterisks represent significant differences ($p < 0.05$) between treatments.

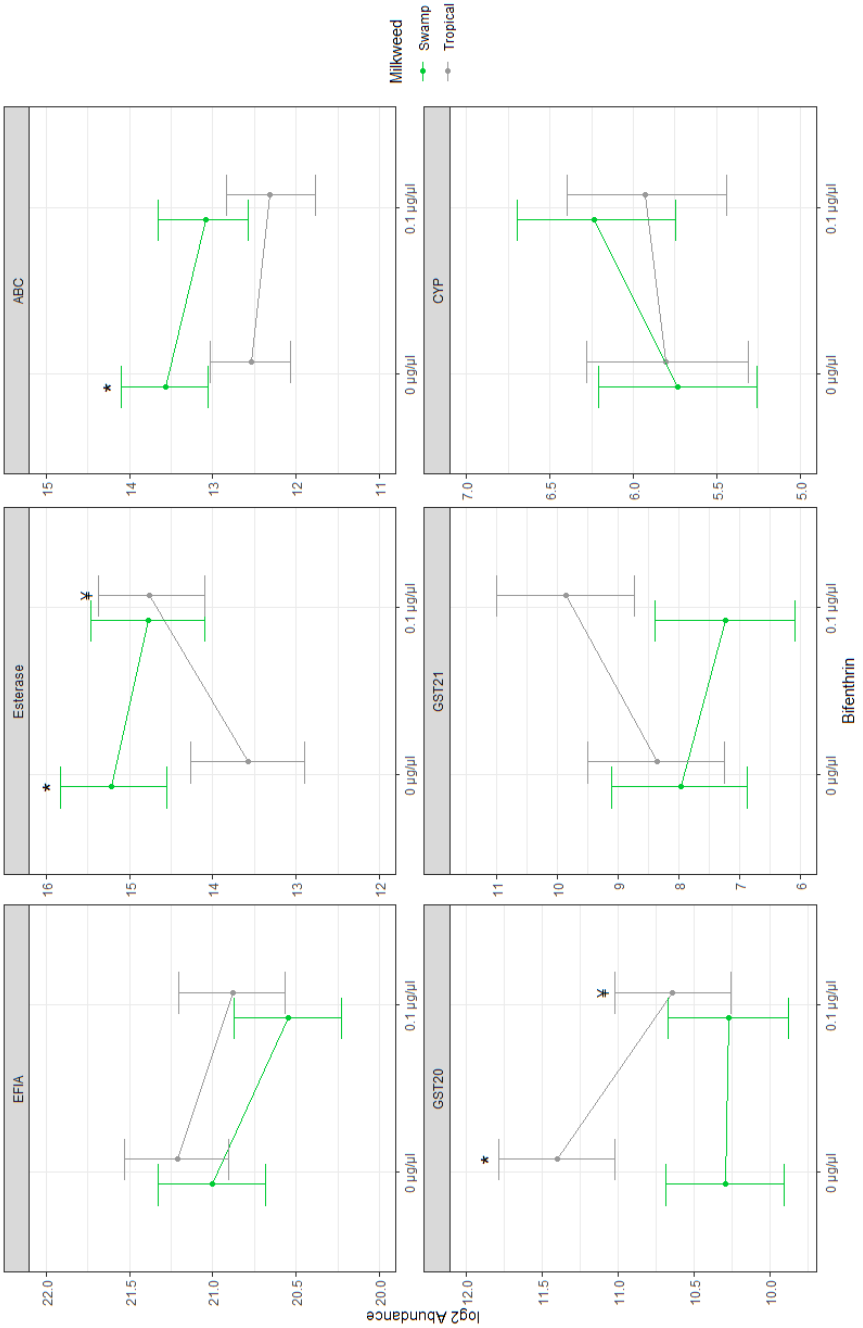

Figure 4.3. Transcript abundance of caterpillars feeding on swamp or tropical milkweed with and without bifenthrin treated. Points represent estimated mean transcript abundance (n=7), error bars represent the upper and lower credible intervals. Gene abbreviations are as follows: ATP-binding cassette transporter (ABC), cytochrome P450 (CYP), elongation factor 1-α (EF1a), glutathione *S*-transferase 20 (GST20) and 21 (GST21). Asterisks denote significant differences from the 0 µg/µl treatment on swamp milkweed, daggers (¥) denote significand differences from the 0 µg/µl treatment on tropical milkweed.

CHAPTER 5 : CONCLUSIONS

Understanding the risk of habitat bordering agricultural landscapes is critical for informing monarch conservation efforts in the U.S. Midwest. Foliar applications of insecticides likely pose the greatest risk to developing monarch caterpillars. Pyrethroid insecticides are one of the most commonly used modes of action in the U.S. Midwest, hence, understanding the lethal and sub-lethal effects of different pyrethroids on monarch development will help inform this risk assessment. The SECOND CHAPTER of this dissertation investigated the acute contact toxicity of two pyrethroid insecticides and their effects on the growth and development of monarch caterpillars. The data presented are the first monarch toxicity data generated for bifenthrin and provide evidence of pyrethroid effects on growth and diet consumption for monarchs developing in habitat down-wind of aerial or ground applications.

With these effect thresholds characterized, it is important to determine what other chemical interactions may affect the sensitivity of monarchs to pyrethroid insecticides. The interaction of milkweed cardenolides has been largely overlooked in monarch toxicity studies. Yet, any interaction with cardenolides would have significant implications for monarch conservation. There is an additional interaction with potassium fertilizers that may affect either pyrethroid sensitivity or the interaction of cardenolides and pyrethroids. Exploring this potential three-way interaction, using single representative compounds, provides novel insights into more complex chemical dynamics in this habitat. The THIRD CHAPTER of this dissertation explored how additional agrochemical interactions in habitat bordering agriculture might affect the toxicity of

bifenthrin to monarch caterpillars. The acute effects of fertilizer, potassium chloride, and the chronic effects of the cardenolide, ouabain, on caterpillar growth and development were evaluated first on their own. Field realistic levels of KCl and ouabain were then used for subsequent combination exposures with bifenthrin. This work demonstrates the increased growth and development effects of higher polar cardenolide concentrations on monarch caterpillars and the potential adverse effects of elevated levels of potassium chloride. Furthermore, this work illustrates the complex interactions between monarchs and milkweed cardenolides with regard to understanding the sublethal effects of pesticides.

While single compounds are easier for testing a potential mechanistic interaction, each milkweed species contains a complex mixture of different cardenolides. Ultimately, understanding the effects of milkweed species on bifenthrin toxicity to monarch caterpillars has significant implications for conservation around agriculture. The FOURTH CHAPTER of this dissertation further explored the potential effects of milkweed cardenolides on bifenthrin toxicity by comparing the survival, detoxification enzyme activity, and detoxification gene expression of monarch caterpillars feeding on tropical and swamp milkweed species. Caterpillars fed on either swamp milkweed (low cardenolide), common milkweed (mid cardenolide), or tropical milkweed (high cardenolide) until the fifth-instar stage when they were topically treated with a sublethal concentration of bifenthrin. This work not only demonstrates the impact of milkweed species on monarch caterpillar physiology and detoxification, but also how this can affect insecticide detoxification capabilities.

Future Directions

There is relatively limited knowledge of the toxicology of monarch butterflies and dose response curves for different modes of action have only been characterized in the past two years. Moreover, there is a limited understanding of how milkweed species may affect monarch resiliency towards agrochemical stressors. It is necessary to better understand this monarch-milkweed interaction for the identification of milkweed cardenolide metabolism and sequestration mechanisms and how these mechanisms might affect the insecticide detoxification of monarchs. The varied cardenolide concentrations and compositions of the 73 different milkweed species makes this an even more interesting system to work with. Monarch caterpillars develop at different rates on different milkweed species, suggesting the energy devoted to growth is required to cope with other energetically demanding processes on some milkweed species but not others. If different milkweed species require caterpillars to spend more energy on digestion and/or cardenolide metabolism (e.g., hydrolyzation of non-polar cardenolides), there could be repercussions for insecticide detoxification. While ouabain was used for controlled cardenolide exposures, future work should focus on either non-polar cardenolides that are more toxic (e.g., digitoxin), or cardenolides commonly found in milkweed species (e.g., calotropin, calactin) if possible. Combinations of KCl and cardenolides did not show any evidence of antagonism. However, further exploration of KCl antagonism, particularly with non-polar cardenolides, may provide key insights into cardenolide sequestration mechanisms.

Laboratory insect colonies are not always representative of field populations. However, laboratory monarch colonies can be used to generate a wealth of information on many basic physiology questions, such as those previously mentioned. A laboratory colony also provides a more homogenous population to work with for initial studies exploring biological trends to further evaluate in a field population. Monarchs are one of the few candidate species for listing under the Endangered Species Act that can be grown in the laboratory. While maintaining a laboratory colony of monarchs has challenges, these colonies should be utilized to help understand monarch physiology and inform conservation practices.

Appendix 1. Sample size overview for experimental replicates for each exposure

1. Ouabain Treatments						
	NC	0.03	0.1	0.3	1	3
R1	10	10	10	10	10	10
R2	10	10	10	10	10	10
R3	10	10	10	10	10	10
TOTAL	**30**	**30**	**30**	**30**	**30**	**30**

2. KCl Treatments					
	NC	0.4	2	10	50
R1	9	9	9	9	9
R2	7	7	7	7	7
R3	5	5	5	5	5
R4	7	10	10	10	10
TOTAL	**28**	**31**	**31**	**31**	**31**

3. KCl + Bifenthrin								
	Untreated Diet				KCl Diet			
	SC	0.1	0.2	0.4	SC	0.1	0.2	0.4
R1	10	10	10	10	10	10	10	10
R2	8	8	8	8	9	9	9	9
R3	8	9	10	10	10	10	10	10
TOTAL	**26**	**27**	**28**	**28**	**29**	**29**	**29**	**29**

4. Ouabain + KCl + Bifenthrin								
	Untreated Diet		KCl Diet		Ouabain Diet		Oua + KCl Diet	
	SC	BIF	SC	BIF	SC	BIF	SC	BIF
R1	8	8	8	8	10	12	10	10
R2	10	12	10	12	9	10	9	10
R3	6	10	7	10	4	10	5	10
TOTAL	**24**	**30**	**25**	**30**	**23**	**32**	**24**	**30**

Appendix 2. ANOVA tables from caterpillar weight and diet consumption analyses for ouabain experiments

Caterpillar Weight: Model Selection				
Model	df	AIC	BIC	logLik
Full	37	162.3478	365.4147	-44.1739
Reduced	36	160.4659	358.0445	-44.233

Caterpillar Weight: Final Model				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	1580	2570.873	**0.00000**
experiment	2	174	23.70158	**0.00000**
logouabain_x	1	174	13.29347	**0.00035**
day	9	1580	457.7591	**0.00000**
I(logouabain_x^2)	1	174	0.467727	0.49494
basecat0	1	174	43.60872	**0.00000**
logouabain_x:day	9	1580	3.915577	**0.00006**
day:I(logouabain_x^2)	9	1580	1.757951	0.07167

Diet Consumption: Model Selection				
Model	df	AIC	BIC	logLik
Full	66	1603.575	1965.839	-735.788
Reduced	46	1598.179	1850.666	-753.089

Diet Consumption: Final Model				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	1572	558.4225	**0.00000**
experiment	2	173	0.393011	0.67562
logouabain_x	1	173	3.711473	0.05568
day	9	1572	40.83835	**0.00000**
I(logouabain_x^2)	1	173	5.088425	**0.02534**
I(logouabain_x^3)	1	173	0.194462	0.65978
basecat0	1	173	10.61339	**0.00135**
logouabain_x:day	9	1572	3.96228	**0.00005**
day:I(logouabain_x^2)	9	1572	2.723375	**0.00375**
day:I(logouabain_x^3)	9	1572	1.987918	**0.03720**

Appendix 3. ANOVA tables from caterpillar weight and diet consumption analyses for KCl experiments

Caterpillar Weight: Model Selection				
Model	df	AIC	BIC	logLik
Full	22	-496.495	-405.8	270.2474

Caterpillar Weight: Final Model				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	294	6336.728	**0.00000**
experiment	3	143	5.11628	**0.00216**
logkcl_x	1	143	182.7989	**0.00000**
day	2	294	129.3693	**0.00000**
I(logkcl_x^2)	1	143	119.9775	**0.00000**
I(logkcl_x^3)	1	143	10.92353	**0.00120**
I(logkcl_x^4)	1	143	6.530348	**0.01165**
basecat0	1	143	135.3774	**0.00000**
logkcl_x:day	2	294	22.87283	**0.00000**
day:I(logkcl_x^2)	2	294	16.13276	**0.00000**
day:I(logkcl_x^3)	2	294	6.329494	**0.00204**
day:I(logkcl_x^4)	2	294	3.449106	**0.03307**

Diet Consumption: Model Selection				
Model	df	AIC	BIC	logLik
Full	22	471.2009	561.8958	-213.6
Reduced	18	465.5392	539.7441	-214.77

Diet Consumption: Final Model				
Column1	numDF	denDF	F-value	p-value
(Intercept)	1	298	938.1506	**0.00000**
experiment	3	143	4.240271	**0.00664**
logkcl_x	1	143	126.5118	**0.00000**
day	2	298	21.44136	**0.00000**
I(logkcl_x^2)	1	143	62.80727	**0.00000**
I(logkcl_x^3)	1	143	5.894955	**0.01643**
I(logkcl_x^4)	1	143	3.949137	**0.04881**
basecat0	1	143	5.373216	**0.02187**
logkcl_x:day	2	298	2.896143	0.05679
day:I(logkcl_x^2)	2	298	2.020786	0.13436

Appendix 4. ANOVA Tables for KCL+Bifenthrin analyses

Caterpillar Weight				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	284	6.622185	**0.01058**
experiment	2	146	0.918053	0.40159
kcl	1	146	0.215271	0.64336
bifenthrin	3	146	1.713606	0.16677
day	2	284	1.939332	0.14570
basecat0	1	146	13.24515	**0.00038**
kcl:bifenthrin	3	146	0.563393	0.64001
kcl:day	2	284	0.034017	0.96656
bifenthrin:day	6	284	0.676921	0.66841
kcl:bifenthrin:day	6	284	0.955563	0.45573

Diet Consumption				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	284	95.06509	**0.00000**
experiment	2	146	14.21049	**0.00000**
kcl	1	146	3.12167	0.07935
bifenthrin	3	146	18.15681	**0.00000**
day	2	284	26.6295	**0.00000**
basecat0	1	146	1.559517	0.21373
kcl:bifenthrin	3	146	1.69148	0.17141
kcl:day	2	284	9.306582	**0.00012**
bifenthrin:day	6	284	7.977917	**0.00000**
kcl:bifenthrin:day	6	284	2.1478	**0.04821**

Survival				
Model Terms	npar	Sum Sq	Mean Sq	F value
experiment	2	6.64576	3.32288	3.32288
bifenthrin	2	11.51811	5.759057	5.759057
kcl	1	0.007135	0.007135	0.007135
basecat0	1	21.65864	21.65864	21.65864
bifenthrin:kcl	2	6.915922	3.457961	3.457961

Appendix 5. ANOVA Tables for Ouabain+KCL+Bifenthrin analyses

Caterpillar Weight				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	954	4261.256	**0.00000**
experiment	2	208	73.90893	**0.00000**
bifenthrin	1	208	42.79901	**0.00000**
day	5	954	1662.43	**0.00000**
ouabain	1	208	0.002932	0.95687
kcl	1	208	1.074022	0.30124
lnbasecat0_1000	1	208	105.6034	**0.00000**
bifenthrin:day	5	954	22.40271	**0.00000**
bifenthrin:ouabain	1	208	0.140182	0.70848
day:ouabain	5	954	0.435984	0.82360
bifenthrin:kcl	1	208	0.560787	0.45479
day:kcl	5	954	1.708305	0.12996
ouabain:kcl	1	208	0.095412	0.75772
bifenthrin:day:ouabain	5	954	0.270122	0.92952
bifenthrin:day:kcl	5	954	0.629798	0.67707
bifenthrin:ouabain:kcl	1	208	0.056686	0.81205
day:ouabain:kcl	5	954	2.275633	**0.04524**
bifenthrin:day:ouabain:kcl	5	954	0.774928	0.56786

Diet Consumption				
Model Terms	numDF	denDF	F-value	p-value
(Intercept)	1	952	3034.777	**0.00000**
experiment	2	208	23.67556	**0.00000**
bifenthrin	1	208	43.65991	**0.00000**
day	5	952	112.5098	**0.00000**
ouabain	1	208	1.912352	0.16818
kcl	1	208	1.369778	0.24319
basecat0_1000	1	208	5.716545	**0.01770**
bifenthrin:day	5	952	54.10652	**0.00000**
bifenthrin:ouabain	1	208	0.161868	0.68786
day:ouabain	5	952	1.265813	0.27649
bifenthrin:kcl	1	208	0.445791	0.50508
day:kcl	5	952	3.226955	**0.00677**
ouabain:kcl	1	208	1.017513	0.31428
bifenthrin:day:ouabain	5	952	0.350651	0.88198

bifenthrin:day:kcl	5	952	0.773483	0.56892
bifenthrin:ouabain:kcl	1	208	1.397297	0.23853
day:ouabain:kcl	5	952	0.647748	0.66330
bifenthrin:day:ouabain:kcl	5	952	0.736792	0.59595

Survival				
Model Terms	npar	Sum Sq	Mean Sq	F value
experiment	2	2.863647	1.431824	1.431824
ouabain	1	0.065627	0.065627	0.065627
kcl	1	0.112816	0.112816	0.112816
basecat6_1000	1	22.17215	22.17215	22.17215
ouabain:kcl	1	0.143299	0.143299	0.143299

Appendix 6. Sequences and relative amplicon size for monarch primers.

Gene	Forward primer	Reverse primer	Product length	Efficiency	Origin
EF1α	TGTCGCTTTCGTACCCATTT	CCTTCAGCCTTACCCTCTTTAC	114	106.3%	Pan et al.
Esterase	AGAAGCAGGACATGACCAGA	AACTGCGAAGGTTTGTGAGC	87	104.8%	Custom
GST20	GTGGCGTGCTAAATACCCTG	TTCGCCTTCTTCATCCCCTT	372	98.4%	Custom
GST21	TCCAACCAAACTACCGAGCT	TTGAGCGTGCTGACAGTAGA	255	94.1%	Custom
CYP	GTGCCCAAGACTTCGTTCAA	GTGAGACCGAAAACTGAGGC	1309	110.9%	Custom
ABC	CATAGCAACCATGGAAGCCC	AGTAGGATCACGTTTCGCCT	851	102.0%	Custom