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RESEARCH

Mixtures of Milkweed Cardenolides Protect Monarch Butterflies against Parasites

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Abstract

Plants have evolved a diverse arsenal of defensive secondary metabolites in their evolutionary arms race with insect herbivores. In addition to the bottom-up forces created by plant chemicals, herbivores face top-down pressure from natural enemies, such as predators, parasitoids and parasites. This has led to the evolution of specialist herbivores that do not only tolerate plant secondary metabolites but even use them to fight natural enemies. Monarch butterflies (*Danaus plexippus)* are known for their use of milkweed chemicals (cardenolides) as protection against vertebrate predators. Recent studies have shown that milkweeds with high cardenolide concentrations can also provide protection against a virulent protozoan parasite. However, whether cardenolides are directly responsible for these effects, and whether individual cardenolides or mixtures of these chemicals are needed to reduce infection, remains unknown. We fed monarch larvae the four most abundant cardenolides found in the anti-parasitic-milkweed *Asclepias curassavica* at varying concentrations and compositions to determine which provided the highest resistance to parasite infection. Measuring infection rates and infection intensities, we found that resistance is dependent on both concentration and composition of cardenolides, with mixtures of cardenolides performing significantly better than individual compounds, even when mixtures included lower concentrations of individual compounds. These results suggest that cardenolides function synergistically to provide resistance against parasite infection and help explain why only milkweed species that produce diverse cardenolide compounds provide measurable parasite resistance. More broadly, our results suggest that herbivores can benefit from consuming plants with diverse defensive chemical compounds through release from parasitism.

Keywords Tri-trophic · Phytochemistry · Cardenolides · Parasitism · Danaus · Milkweed

Introduction

Plants produce toxic secondary metabolites to defend themselves against herbivores and pathogens. Oftentimes, secondary metabolites from a single class of compounds (e.g. alkaloids) occur in complex mixtures within plant tissues (Romeo et al. 1996). The role of phytochemical mixtures within plants has been debated widely, and several evolutionary hypotheses have been developed to explain this common trait through an anti-herbivore lens (Berenbaum and Zangerl 1996; Ehrlich and Raven 1964; Jones et al. 1991; Speed et al. 2015; Whitehead et al. 2021). Most prominently, the synergy hypothesis states that multiple secondary metabolites act non-additively against herbivores and plant pathogens, thus providing a benefit over single compounds deployed at the same concentration (Dyer et al. 2003; Richards et al. 2016). Synergies between phytochemicals can affect herbivores through numerous mechanisms, and understanding their relative importance is a growing area of research (Leckie et al. 2016; Whitehead et al. 2021; Whitehead and Bowers 2014).

The role of phytochemical mixtures has been mostly studied in the context of detrimental effects to herbivores. However, herbivores may hypothetically also benefit from mixtures of plant toxins, either through dilution (reduced

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concentration of any one compound) or through interactions with the third trophic level. Many specialist herbivores sequester secondary metabolites, making them toxic and bitter-tasting to predators (Opitz and Müller 2009). Indeed, herbivores can also self-medicate with secondary metabolites, gaining resistance against parasites and pathogens (de Roode and Hunter 2019; Lefèvre et al. 2010; Singer et al. 2009; Smilanich et al. 2011). Although plant species identity and the amount of secondary metabolites consumed are known to influence interactions between herbivores and their natural enemies (Singer et al. 2009; Smilanich et al. 2018; Sternberg et al. 2012; Tao et al. 2016), no work to date has isolated the effects of specific phytochemical mixtures in a tri-trophic context. Here, we address how the composition and concentration of a group of secondary metabolites alter interactions between an herbivore and its protozoan parasite.

The monarch butterfly (*Danaus plexippus*) specializes on milkweeds, mostly *Asclepias* spp., as larval food. Milkweeds produce toxic cardenolides, and monarchs have evolved the ability to sequester cardenolides to deter predators (Brower and Moffitt 1974). Monarchs can also use high-cardenolide plants to reduce infection by their protozoan parasite *Ophryocystis elektroscirrha* (Lefèvre et al. 2010). However, milkweed species vary greatly in their total concentration and diversity of cardenolide compounds, and differences between milkweed species have been shown to alter monarch performance in numerous ways, including growth, oviposition, sequestration, and parasite resistance (Agrawal et al. 2021; Sternberg et al. 2012; Tao et al. 2016). Nonetheless, remarkably little work has been done with purified cardenolides, and we still lack a thorough understanding of their consequences either in isolation or in mixtures. By feeding purified cardenolide compounds to caterpillars, either alone or in mixture, we test the specific role of individual cardenolides and their mixtures on resistance to *O. elektroscirrha*. We hypothesize that we can replicate milkweed-mediated resistance to parasites by dosing monarch caterpillars with the most abundant cardenolides isolated from *A. curassavica.* Moreover, we predict that if there is a minimum concentration of cardenolides or a specific cardenolide responsible for parasite resistance, we should find lower parasite spore loads in monarchs dosed with cardenolides of certain concentrations or identity.

Methods

Background on Monarch-Parasite-Milkweed System

Monarchs become infected with *O. elektroscirrha* after larvae ingest dormant parasite spores, which are typically transmitted onto eggs and milkweed leaves by infected adult monarchs (Mclaughlin and Myers 1970). Spores lyse in the gut and parasite cells penetrate the intestinal wall and infect the larval hypoderm. After pupation, rapid replication of parasite cells occurs in the hypoderm of the pupae, and parasite spores form around developing adult scales. Finally, when infected adult monarchs emerge, their bodies are covered in dormant *O. elektroscirrha* spores, and spores are especially concentrated on their abdomens, thus further facilitating transmission of spores during mating and oviposition. Previous studies have shown negative fitness effects of parasite infection on adult lifespan, adult body mass, mating success and flight and migration ability (Altizer and Oberhauser 1999; Babalola et al. 2022; Bradley and Altizer 2005; de Roode et al. 2007; Kendzel et al. 2023), with greater spore loads (infection intensity) associated with greater fitness effects (de Roode et al. 2008b, 2009).

The first evidence of host-plant derived parasite resistance in monarch butterflies was found using the tropical milkweed *Asclepias curassavica*, which has approximately 20 unique cardenolide compounds and a high total concentration (de Roode et al. 2008a). Previous work has shown that a typical concentration of cardenolides in *A. curassavica* is approximately 1-3 mg/g dry weight (de Roode et al. 2008a; Roode et al. 2011b; Sternberg et al. 2012; Tao et al. 2016). In contrast, the swamp milkweed *Asclepias incarnata* has only a few cardenolides found in extremely low concentrations, and which have low toxicity. In the study by de Roode et al. (2008a), all monarchs were inoculated with a dose of ten *O. elektroscirrha* spores, and monarchs reared on *A. curassavica* were less likely to become infected and experienced significantly lower parasite spore loads when infected, compared to monarchs reared on *A. incarnata*. Further work showed that feeding *A. curassavica* to monarch caterpillars prior to and on the day of inoculation with parasite spores can greatly reduce parasite spore load in adulthood, as well as increasing lifespan compared to infected caterpillars that were reared on the swamp milkweed, *A. incarnata.* However, if monarchs became infected on *A. incarnata*, feeding them *A. curassavica* after infection establishment did not reduce parasite infection (de Roode et al. 2011a). This indicates that medicinal milkweeds interfere with the establishment of parasites in the host, as opposed to subsequent parasite growth. Accordingly, in the current study, we manipulated caterpillar diets by feeding them cardenolides prior to and/or during parasite establishment.

Cardenolides

Cardenolides are steroidal compounds that inhibit Na^+/K^+-ATPase, an enzyme that is vital for animal cells to maintain ion gradients; however, due to three amino acid substitutions, the monarch Na^+/K^+-ATPases are highly resistant to cardenolides, requiring extremely high concentrations of cardenolides to inhibit the enzyme (Karageorgi et al. 2019).

When compared to porcine Na^+/K^+-ATPases, which are representative of vertebrates' sodium-potassium pumps, monarch Na^+/K^+-ATPases are 50 to 100 times more resistant to cardenolides (Agrawal et al. 2021).

Our experiments consisted of feeding milkweed leaf disks with purified cardenolides pipetted onto the leaf's surface to caterpillars. Cardenolides were isolated from *A. curassavica* plant tissue by A.A.A and A.P.H. at Cornell University in Ithaca, NY, and then resuspended in 100% food-grade ethanol by repeatedly vortexing and sonicating the solutions before each use. Briefly, cardenolides were isolated from *A. curassavica* seeds, leaves, and latex using standard phase separation techniques and fraction collection using an Agilent 1260 Preparatory HPLC. Compound identity and purity (>90%) were confirmed using LC-MS or NMR as described previously (Agrawal et al. 2021; Agrawal and Hastings 2023). The four most abundant cardenolide compounds naturally occurring in *A. curassavica* were used for these experiments: uscharidin isomer 2 (U), frugoside (F), calactin (C), and voruscharin (V). Leaf disks for the cardenolide treatments were prepared by pipetting a total of 12 µL of cardenolides suspended in 100% food-grade ethanol directly onto a 3 mm diameter *A. incarnata* leaf disk and allowing the ethanol to evaporate, leaving only the cardenolides on the leaf disk surface. Leaf disks for the ethanol treatment were prepared by pipetting a total of 12 µL of 100% food-grade ethanol directly onto an *A. incarnata* leaf disk and allowing the ethanol to evaporate. To prevent the solutions from running off the leaf disks, the 12 µL volume was pipetted in two rounds of 6 µL of solution, allowing the ethanol to evaporate between the first and second rounds.

Experimental Design

Experiment 1: Effects of Cardenolide Mixtures on Monarch Resistance to Parasites

Experiment 1 was conducted in June of 2021. Monarchs used in this experiment were lab-reared, outbred grand-progeny of wild-caught monarchs collected in St. Marks, FL in November 2020 and overwintered in the lab. *O. elektroscirrha* spores were collected at the same time from a wild-caught monarch and re-established in lab-reared monarchs to supply viable spores.

Mated females were provided *A. incarnata* for oviposition. As soon as eggs hatched, caterpillars were moved with a fine paint brush to fresh *A. incarnata* or *A. curassavica* plants, depending on treatment. Groups of 25 caterpillars were randomly assigned to each of nine treatment groups (see below for details on each treatment). Within each treatment group, caterpillars were reared on live plants for two days (Fig. 1). On day 3, caterpillars were moved to a petri dish with a leaf disk dosed with ten parasite spores; depending on treatment, leaf disks were also dosed with 12 µL of ethanol or cardenolides dissolved in ethanol. *O. elektroscirrha* spores (from a parasite lineage originally obtained from a wild-caught butterfly in St Marks, FL in 2021 and referred to in our lab as 21P19) were manually deposited onto the leaf disk following evaporation of ethanol or ethanol/cardenolide mixes. See Fig. 1 for a general overview of experimental procedures, and Figure S1 for additional details.

All caterpillars were given up to 48 h to consume their leaf disk. Caterpillars that did not consume their full leaf disk were excluded from the experiment. After finishing their leaf disk, each caterpillar was moved to a mature potted *A. incarnata* or *A. curassavica* plant (depending on treatment group), kept in an 11.4 cm diameter x 61 cm tall clear plastic tube closed off at the top with a net. Caterpillars were allowed to eat ad libitum until pupation, approximately seven days later. Pupae were glued onto the lids of plastic solo cups. Starting on day 5 post-pupation, pupae were checked daily for signs of parasitism, as seen by discoloration of the pupal case, and then scored from 0 (no signs of infection) to 5 (severely infected) based on the methods described in de Roode et al. 2009 (referred to from hereon as 'pupal score'). Pupal scores were converted to approximate spore counts using previously established relationships between pupal scores and spore numbers (de Roode et al. 2009).

Caterpillars in the *A. incarnata* treatment consumed *A. incarnata* throughout the entire experiment (see Fig SI1 for experimental design). Caterpillars in the *A. curassavica* treatment consumed *A. incarnata* for two days post-hatching, received *A. curassavica* on the day of inoculation with *O. elektroscirrha*, and then consumed *A. incarnata* for the rest of their life; this treatment tests for the effect of only an acute dose of *A. curassavica* at the time of inoculation. Caterpillars in the *A. curassavica*-all-life treatment consumed *A. curassavica* throughout their entire life. Caterpillars in the cardenolide treatments consumed *A. incarnata* for two days post-hatching and received an *A. incarnata* leaf disk dosed with parasites and a mixture of four cardenolides (uscharidin isomer 2, frugoside, calactin, and voruscharin) at varying concentrations: 0.5, 1.0, 3.0 and 6.0 mg/g dry leaf mass. These concentrations were chosen because they reflect a natural range of moderate to high milkweed cardenolide concentrations that correlate with reductions in parasite infection (Tao et al. 2016). Finally, caterpillars in the ethanol treatment received leaf disks with parasite spores and ethanol; they were reared on *A. incarnata* for their entire life.

Experiment 2: Effects of Individual Cardenolides on Resistance to Parasites

Experiment 1 indicated that a mixture of the four cardenolides at a concentration of 3.0 mg/g reduced parasite

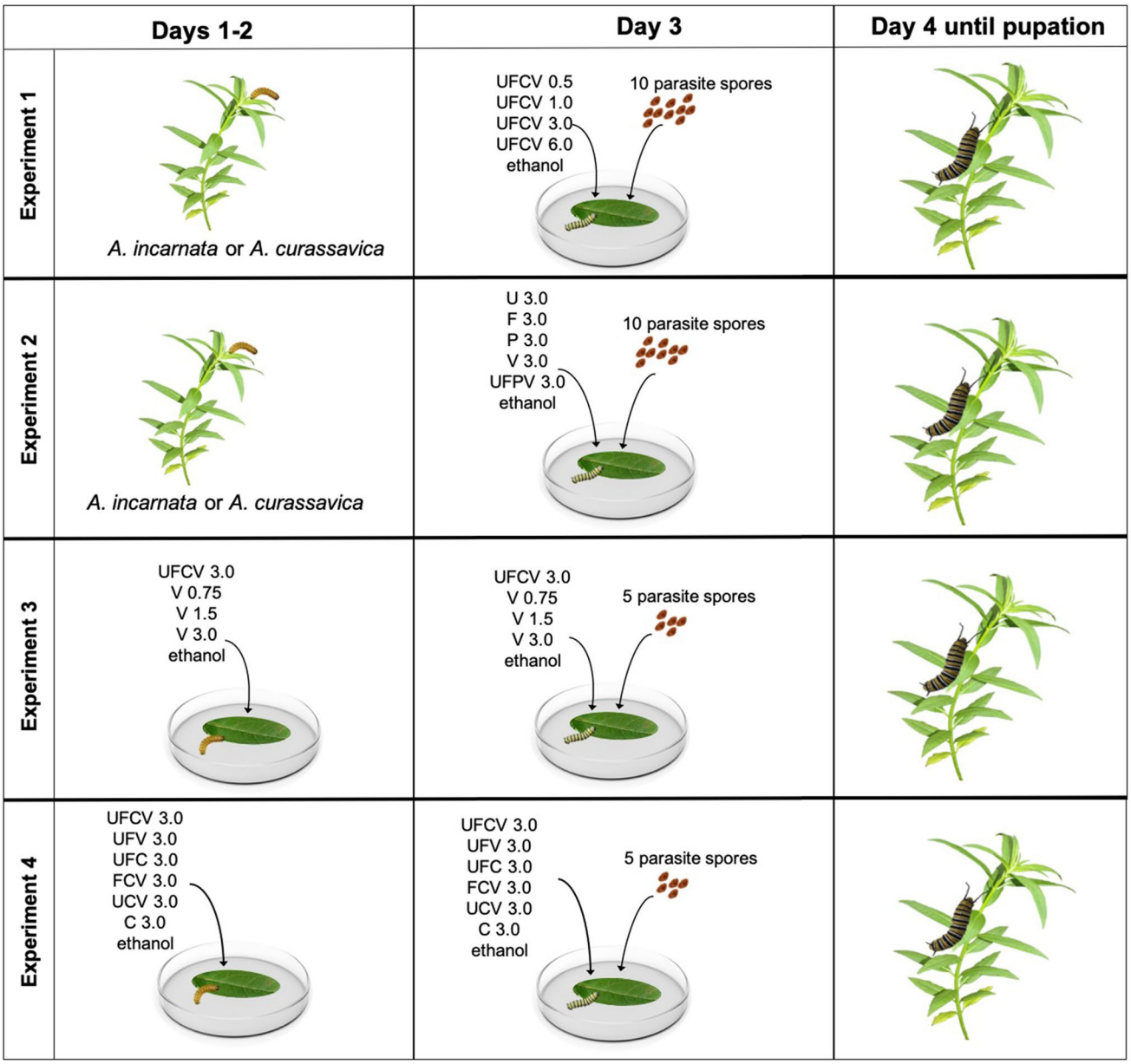

Fig. 1 Experimental design of all four experiments. Generic overview of treatments within experiments. Caterpillars were reared for 2 days on a live plant (Experiments 1, 2) or in a petri dish with a milkweed leaf disk with or without ethanol/ethanol + cardenolides (Experiments 3,4). Cardenolide notations are as follows: U, uscharidin; F, frugoside; C, calactin; V, voruscharin; P, calotropin. Caterpillars were then inoculated with parasites deposited on a leaf disk with or without ethanol/ethanol + cardenolides on day 3. Upon completion of their leaf disk with parasites, they were transferred to a live plant for the remainder of their larval development

infection (Fig. 2a). We next investigated whether the individual compounds making up the mixture could reduce parasite infection on their own. Experiment 2 was conducted in July of 2021 with a new cohort of monarch caterpillars and followed the same procedures as described in Experiment 1 but varied by cardenolide treatments (Fig SI1). To test for the effect of individual cardenolides, the four cardenolides from the mix in Experiment 1 were dosed individually to caterpillars on the day of inoculation with ten *O. elektroscirrha* spores. The concentration of each cardenolide solution was 3.0 mg/g dry leaf mass, following up on the results from Experiment 1. However, this experiment used calotropin (P), an enantiomer of calactin, in place of calactin. The *A. incarnata, A. curassavica, A. curassavica*-all-life, and ethanol treatments were also included as controls.

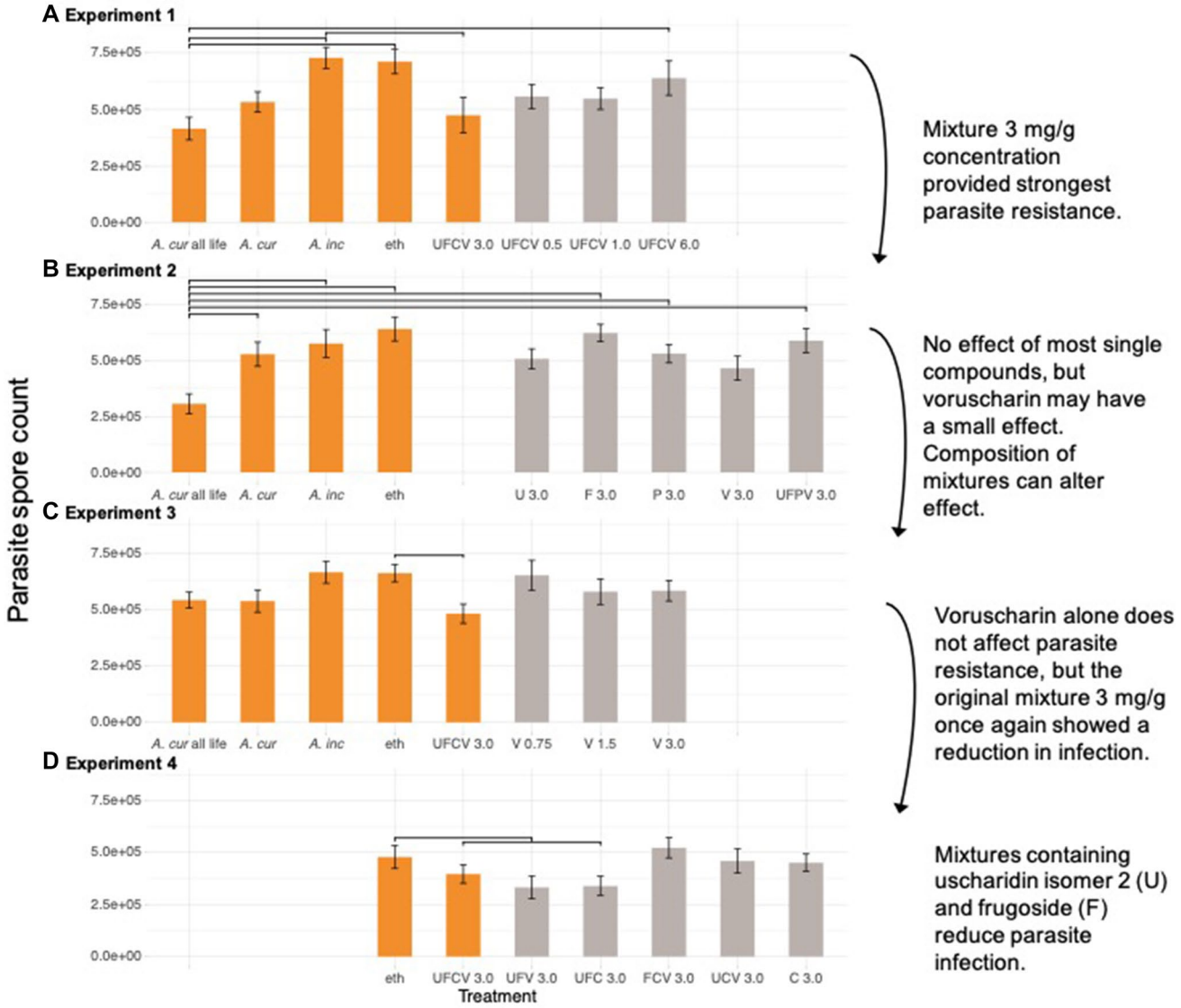

Fig. 2 Effects of dietary cardenolide treatments on parasites in monarch butterflies. Experiment 1: Spore counts of monarchs infected with *O. elektroscirrha* and dosed with a gradient of concentrations of a mixture of the four most abundant cardenolides in *A. curassavica* (*A. cur*): uscharidin isomer 2 (U), frugoside (F), calactin (C), and voruscharin (V). Experiment 2: Spore counts of infected monarchs dosed singly with the same four cardenolides as Experiment 1 at a concentration of 3.0 mg/g, except calotropin (P) (an enantiomer of calactin) was used in place of calactin. Experiment 3: Spore counts of infected monarchs dosed with a gradient of voruscharin and the original mixture from Experiment 1 at 3 mg/g. Experiment 4: Spore counts of infected monarchs dosed with calactin, one of four unique mixtures of three cardenolides, or the mixture of all four cardenolides. All treatments were 3 mg/g. Bars show mean spore counts ± SE. Brackets show statistically significant pairwise differences ($P < 0.05$) using Tukey's post hoc test. Orange bars indicate controls or the UCFV mixture repeated across most experiments, whereas gray bars were unique to specific experiments. The notations to the right of the graphs show the main finding from each experiment that influenced the design of the following experiment

Experiment 3: Effects of Voruscharin on Resistance to Parasites

Because Experiment 2 indicated that the only cardenolide that may have some individual effect was voruscharin (Fig. 2b), we next investigated how varying concentrations of this specific cardenolide affected parasite infection. Experiment 3 was conducted in May of 2022. Monarchs used in this experiment were lab-reared, outbred grand-progeny of wild-caught monarchs collected in St. Marks, FL in October 2021 and overwintered in the lab. This experiment followed the same procedures as Experiment 1, except that monarchs in this experiment were given cardenolide and ethanol treatments two days prior to and on the day of inoculation because consuming *A. curassavica* both before and during parasite inoculation increases parasite resistance

(Fig SI1)(de Roode et al. 2011a). Additionally, each group started with 40 caterpillars, and caterpillars were dosed with five *O. elektroscirrha* spores (from parasite lineage 22P13) rather than ten, with an aim to obtain more variation in the proportion of monarchs that become infected (lower doses result in lower infection probability (de Roode et al. 2007). Following the results of Experiment 2, the treatments in this experiment were the 3.0 mg/g mixture from Experiment 1 (uscharidin isomer 2, frugoside, calactin, and voruscharin) and a gradient of voruscharin alone: 0.75, 1.5, and 3.0 mg/g, as this cardenolide is highly toxic and appeared to have a potential effect on parasite infection. The *A. incarnata, A. curassavica, A. curassavica*-all-life, and ethanol treatments were also included as controls.

Experiment 4: Effects of Cardenolide Mixture Compositions on Resistance to Parasites

While Experiment 3 showed that voruscharin alone does not reduce parasite infection, it did verify the finding in Experiment 1 that the mixture of the originally used four cardenolides (uscharidin isomer 2, frugoside, calactin, and voruscharin) reduces parasite infection (Fig. 2c). We therefore carried out another experiment with a new cohort of caterpillars, in which we created different mixes of these cardenolides to determine whether all four are needed, or whether a subset mixture can also reduce infection. Experiment 4 was conducted in June of 2022 and followed the same procedures as Experiment 3 (Fig SI1). To investigate if a certain composition of the cardenolide mixture is necessary for parasite resistance, we tested multiple combinations of cardenolides from the mixture used in Experiment 1 by subtracting one of the four cardenolides for each combination. This resulted in four unique mixtures of three cardenolides in addition to the mixture of all four cardenolides. We also included a calactin-only treatment (since calactin had yet to be tested individually) and an ethanol treatment as a control. All cardenolide treatments were 3.0 mg/g.

Statistical Analysis

Analyses were performed using R Version 4.1.2. To determine if the proportion of monarchs that became infected varied between treatments, we used generalized linear models (GLM) with a quasibinomial error distribution, and treatment as the explanatory variable. We minimized the models and used model comparisons (using the R command anova) between models with and without treatment as the explanatory variable to assess its significance using χ^2 tests (Crawley 2012). One-way analyses of variance (ANOVA) were performed to compare the effect of treatment on spore counts. As above, significance of treatment as an explanatory variable was assessed by term removal followed by model comparison using the anova command in R (using F tests). Tukey's post hoc tests were performed for pairwise comparisons between treatment groups. Note that we included monarchs that did not become infected in calculating mean spore counts; thus, spore counts combine both infection rate and infection intensity into a single measure of infection.

Since the ethanol and 3.0 mg/g cardenolide mixture containing uscharidin isomer 2, frugoside, calactin, and voruscharin treatments were repeated in three of the four experiments, we combined spore count data for those two treatments from Experiments 1, 3, and 4. We carried out an ANOVA with experiment, treatment and their interaction as the explanatory variables, then minimized models and used model comparisons to assess significance of each term (as above). Assumptions were checked by analyzing the normality of model residuals.

Results

Experiment 1: Cardenolide Mixture of Intermediate Concentration Reduces Parasite Infection

A total of 165 out of 200 monarchs survived to adulthood, and 157 monarchs (95%) became infected. Treatment significantly affected the proportion of monarchs that became infected ($\chi^2_{7,156}$ = -15.41, $P < 0.001$). Treatments also varied in spore count (Fig. 2a; $F_{7,\ 156} = 4.244$, $p < 0.001$). Tukey's HSD test for multiple comparisons showed that caterpillars that were fed *A. curassavica* for their entire life suffered ~ 43% lower parasite counts than those reared on *A. incarnata* (Fig. 2A; $P = 0.001$). Caterpillars fed *A. curassavica* for their whole life also suffered lower spore counts than those fed ethanol only on day 3 (Fig. 2A; $P = 0.006$), and those fed the 6.0 mg/g cardenolide mixture ($P = 0.024$). Additionally, caterpillars that consumed the 3.0 mg/g cardenolide mixture experienced ~ 35% lower parasite counts than those reared on *A. incarnata* ($P = 0.039$) (Fig. 2A). All other comparisons were non-significant (Table SI1).

Experiment 2: Individual Cardenolides Do Not Strongly Reduce Parasite Infection, but Voruscharin May Play a Role

A total of 191 out of 225 monarchs survived to adulthood, and 182 monarchs (95%) became infected. Treatments did not vary in the proportion of monarchs that became infected ($\chi^2_{8,182}$ = -9.69, $P = 0.085$), but they did vary in spore counts ($F_{8,\ 182} = 4.14$, $P < 0.001$). Tukey's HSD test for multiple comparisons showed that caterpillars reared on *A. curassavica* for their entire life experienced 20–40% lower parasite counts than those reared on *A. incarnata* (Fig. 2B; $p = 0.003$), those inoculated on *A. curassavica* (Fig. 2;

$P = 0.047$), and those inoculated with ethanol ($P < 0.001$), calotropin ($P = 0.048$), frugoside ($P < 0.001$), and the cardenolide mixture ($P = 0.004$) (Fig. 2B). All other comparisons were non-significant (Table SI2).

Experiment 3: A Concentration Gradient of Voruscharin Does Not Reveal a Large Effect of this Chemical

A total of 298 out of 340 monarchs survived to adulthood, and 274 monarchs (92%) became infected. Cardenolide treatment had no effect on the proportion of monarchs that became infected ($\chi^2_{7,287} = -7.85$, $P = 0.37$), but treatments varied in spore counts (Fig. 2C; $F_{7, 287} = 2.52$, $P = 0.015$). Tukey's HSD test for multiple comparisons showed that monarchs that were fed the 3.0 mg/g cardenolide mixture reduced spore counts by ~27% compared to those fed ethanol only (Fig. 2C; $P = 0.048$) and also tended to be ~27% lower than those fed *A. incarnata* ($P = 0.070$) (Fig. 2C). All other comparisons were non-significant (Table SI3).

Experiment 4: Mixtures Containing Particular Cardenolides Reduce Parasite Infection the Most

A total of 249 out of 280 monarchs survived to adulthood, and 210 monarchs (84%) became infected. Cardenolide treatment had no effect on the proportion of monarchs that became infected ($\chi^2_{6,241} = -9.63$, $P = 0.15$). Cardenolide treatments tended to vary in spore counts (Fig. 2D; $F_{6, 241} = 1.932$, $P = 0.0763$), with cardenolide treatments that contained both uscharidin isomer 2 and frugoside appearing to have lower spore counts. When restricting the analysis to the mixtures that contained both uscharidin isomer 2 and frugoside and the ethanol control, caterpillars fed these cardenolide mixtures indeed experienced lower parasite counts by ~25% compared to those fed ethanol only (Fig. 2D and Table SI4; $F_{1,138} = 4.39$, $P = 0.038$). All other comparisons were non-significant (Table SI5).

Cardenolide Mixture in Experiments 1, 3 and 4

The same cardenolide mixture containing all four cardenolides at a concentration of 3.0 mg/g was used in Experiments 1, 3, and 4 (note that in Experiment 2, the mixture was different because calotropin replaced calactin in that experiment). Because this mixture reduced or tended to reduce infection in all three experiments compared to the ethanol-only control, we further analyzed the medicinal properties of this mixture by carrying out an analysis of variance in which we compared the spore counts of monarchs reared on this mixture compared to monarchs fed ethanol alone. This analysis confirmed that across the three experiments, the four-cardenolide mixture significantly reduced parasite infection by ~26% (Fig SI2; $F_{1, 196} = 14.8$, $P < 0.001$); there was no interaction between treatment and experiment ($F_{2, 196} = 1.05$, $P = 0.352$). When we minimized the model to include only the fixed effects of treatment and experiment, we found a significant effect of treatment ($F_{1, 198} = 14.8$, $P < 0.001$) and a significant effect of experiment ($F_{1, 198} = 5.6$, $P < 0.001$);

Discussion

Our results show that mixtures of isolated milkweed cardenolides at intermediate concentrations, but not individual cardenolides, can reduce parasite loads in monarch butterflies. There has been a lot of debate over why plants produce mixtures of secondary metabolites, and research has largely focused on how these chemical cocktails function to fight off herbivores. Ever since the seminal work by Fraenkel (Fraenkel 1959) and Ehrlich and Raven (Ehrlich and Raven 1964), scientists have interpreted plant secondary chemicals as key drivers in the coevolution and diversification of plants and insect herbivores (Agrawal and Zhang 2021). Thus, as insects attacked plants, plants evolved chemical defenses, to which insects evolved resistance, and so on, spurring divergent evolution in plants and specialist insects. While originally evolving to deter herbivores, many specialist insects have coopted specific defense compounds to locate host plants (Bruce et al. 2005) and promote oviposition (Tsuchihara et al. 2009).

The anti-herbivore effects of phytochemical synergies have been the focus of many studies (Berenbaum and Zangerl 1993; Macel et al. 2005; Scott et al. 2002). For example, Richards et al. (2010) dosed a generalist herbivore, *Spodoptera frugiperda*, and a specialist herbivore, *Eois nympha*, over a concentration gradient of four amides isolated from two plants within the *Piper* genus (*P. cenocladum* and *P. imperial*). Individual amides had a mildly toxic effect on both the generalist and specialist, and toxicity tended to increase with increasing concentrations of individual amides. However, the mixture of four amides greatly reduced survivorship of the generalist herbivore, greatly increased parasitoid success attacking the specialist herbivore, and these effects became stronger with increasing concentrations of the mixture (Richards et al. 2010). Another study experimentally manipulating *Piper* amides in the same two herbivore species found a mixture of three amides increased larval mortality, and mixtures of two amides dramatically increased larval development time, thus increasing vulnerability to predators and parasitoids (Dyer et al. 2003). These two experiments highlight the importance of both composition and concentration of plant secondary metabolites on herbivore fitness.

Despite these advances, less work has been done to understand how mixtures of secondary metabolites can benefit

herbivores through release from top-down pressures, such as predation and parasitism. To test how secondary metabolites benefit specialist herbivores, we inoculated monarch larvae with *O. elektroscirrha* spores while also exposing them to cardenolides isolated from *A. curassavica*, a milkweed species that provides monarchs with resistance to *this* parasite when consumed in tandem with spores. As our results demonstrate, cardenolide mixtures, but not individual cardenolides, provide protection against infection, suggesting that cardenolides may act synergistically. Thus, while plants may have evolved complex phytochemical mixtures as protection against herbivores, these mixtures also benefit co-evolving herbivores.

Milkweed species vary greatly in their concentration and composition of cardenolide compounds with some species having over 20 unique compounds and others having only a few (Agrawal et al. 2012; Züst et al. 2019). Cardenolide compounds also vary in toxicity as less polar compounds can more easily pass through cellular membranes and often also have stronger inhibition of sodium potassium pumps (Agrawal et al. 2021; Petschenka et al. 2018). Monarchs have been found to saturate cardenolides in their tissues to a maximum of approximately 3 µg/mg of dry mass (Jones et al. 2019; Malcolm et al. 1989), potentially because higher concentrations are detrimental. Thus, although monarchs have high levels of tolerance to cardenolides compared to other animals, these toxins still negatively impact monarch growth, development and survival, especially when larvae consume high-cardenolide milkweed species, such as *A. curassavica* (Agrawal et al. 2021; Tao et al. 2016; Zalucki et al. 2001). Previous work has reported positive correlations between parasite resistance and milkweed species with high concentrations and diversities of cardenolides (Sternberg et al. 2012; Tao et al. 2016). Further work also revealed that latex, which contains high concentrations of cardenolides (Züst et al. 2019), isolated from high-cardenolide milkweed species can provide parasite resistance (Gowler et al. 2015). However, because latex contains other chemical compounds, such as cysteine proteases (Agrawal and Konno 2009), this past work did not definitively conclude that cardenolides are the mechanism responsible for reducing parasite infection. Using purified cardenolides and feeding them to monarchs, our study is the first to directly show that consuming cardenolides alone can provide parasite resistance to monarchs. Cardenolide compounds were dosed either singly or in mixtures, and the concentrations of these treatments ranged from 0.5 to 6 mg/g dry leaf mass, which spans the full spectrum of cardenolide concentrations that correlates with parasite resistance in previous work (Tao et al. 2016).

We found that no single cardenolide compound provided resistance to *O. elektroscirrha*, even for the highly toxic cardenolide voruscharin at the highest concentration (Fig. 2C). However, we did find that mixtures of cardenolide compounds at an intermediate concentration provided parasite resistance similar to that of caterpillars that consumed only *A. curassavica* (Fig. 2A, C, D). The composition of cardenolide mixtures appears to be critical for gaining meaningful resistance as well. Our results indicate that these compounds are synergistically interacting in a way that negatively impacts *O. elektroscirrha* growth or establishment. Further studies are needed to determine exactly which cardenolide compounds are responsible and how they interact with each other and the host and parasite, and based on our results from Experiment 3, uscharidin isomer 2 and frugoside would be good candidates for future work on this question. Thus, although we have demonstrated that cardenolides can provide resistance to parasites in monarchs, the mechanism by which this occurs is still unknown. While *O. elektroscirrha* does not share the Type II Na^+/K^+-ATPase that cardenolides act on in vertebrates, it does contain PMCA ATPases (Mongue et al. 2023), which are inhibited by plant phenolics, such as curcumin, in other systems (Zhou et al. 2021). Cardenolides could inhibit this enzyme in *O. elektroscirrha*; however, *A. curassavica* also contains multiple phenolic flavonol glycosides (found to be involved in oviposition stimulation) that could play a role but were not tested in our experiments (Haribal and Renwick 1996). Alternatively, cardenolides could indirectly alter resistance to parasites by modulating the gut microbiome or by changing the monarch immune response (Tan et al. 2019). Secondary metabolites have been shown to affect both of these factors and indirectly influence interactions with pathogens in other plant-herbivore systems (Harris et al. 2019; Lampert 2012; Laurentz et al. 2012; Smilanich et al. 2018).

While our study showed that mixtures of cardenolides are needed to provide protection against parasites, previous work has shown that single cardenolides can be effective as anti-predator defense in monarchs because only a single compound is needed to inhibit Na^+/K^+-ATPases (Agrawal et al. 2021). These contrasting patterns could have evolutionary ramifications for monarchs, because protection against different natural enemies may depend on varying concentrations and diversity of cardenolides. Indeed, other studies have suggested that trade-offs exist between anti-predator defense and anti-parasite defense when herbivores consume plants with high concentrations or diversity of secondary metabolites. The buckeye caterpillar (*Junonia* coenia), for example, mounts a weaker immune response (measured by encapsulation and melanization) when feeding on diets containing more than one iridoid glycoside, which may increase susceptibility to parasitoids; however, higher rates of sequestration of iridoid glycosides are beneficial against predators, such as stinkbugs (*Podisus maculiventris*), and also reduces viral load (Bowers and Stamp 1997; Muchoney et al. 2022; Richards et al. 2012; Smilanich et al. 2009). Opposing

results have also been found, where toxic secondary metabolites can enhance immune responses, such as that in the specialist moth *Heliothis subflexa* against its bacterial pathogen *Bacillus thuringiensis* (Barthel et al. 2016; Muller et al. 2015). Alternatively, toxic secondary metabolites could reduce the need for energetically costly immune responses when herbivores consume toxic plants to fight parasites or parasitoids (Parker et al. 2011). In the monarch system, previous work has shown that cardenolides may replace the role of immunity, as multiple immune genes were down-regulated in infected monarchs reared on *A. curassavica* compared to those reared on *A. incarnata* (Smilanich and Nuss 2019; Tan et al. 2019). However, the same study also found that numerous detoxification genes were upregulated in infected monarchs reared on *A. curassavica*, suggesting a potential energetic trade-off between detoxification and immune responses.

In summary, our study showed that the previously demonstrated medicinal effects of tropical milkweed for monarch butterflies can be explained by milkweed cardenolide mixtures. While much research has focused on how chemical diversity benefits plants in their protection from herbivores, our results suggest that these mixtures can also provide benefits to herbivores, by providing protection against infection.

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Data Availability All data presented in this manuscript is publicly available in GitHub repository:https://github.com/mhoogshagen/cardenolide-mixtures.

Declarations

Competing Interests The authors declare no competing interests.

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