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Does chemistry make a difference? Milkweed butterfly sequestered cardenolides as a defense against parasitoid wasps

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Abstract

Plant allelochemicals have important roles in plant defense as well as ecological and co-evolutionary dynamics within tri-trophic systems of plants, herbivores, and natural enemies. Milkweed butterflies represent a model system for chemical ecology because they sequester cardenolides semi-proportionally to the concentration in their host plants, yet little is known about the role of sequestered cardenolides in interactions with invertebrate natural enemies. We experimentally tested the preference and performance of two species of parasitic wasps (*Pteromalus cassotis* Walker and *Pteromalus puparum* Linnaeus) on milkweed butterfly pupae (monarchs, *Danaus plexippus* Linnaeus, and *Euploea core* Cramer) reared on plants to contain variable concentrations of sequestered cardenolides. We measured host survival and parasitoid reproductive success to determine whether greater concentrations of herbivore-sequestered plant toxins provide a defensive benefit or influence parasitoid success. We found that *P. puparum* was unable to develop from monarchs, regardless of toxicity. Monarchs containing more cardenolides (those fed *Asclepias curassavica*) were more likely to survive encounters with *P. cassotis* than those containing fewer cardenolides (fed *Asclepias incarnata*), but only because this parasitoid was less likely to attack more toxic monarchs. Once attacked, host toxicity had no effect on the likelihood of monarch survival nor the emergence of parasitoids. Host toxicity affected parasitoid performance in more subtle ways, however, decreasing *P. cassotis* brood size and survival to adulthood. When attacking cardenolide-free *E. core* pupae, *P. cassotis* reproduced successfully, but *P. puparum* did not, suggesting that milkweed butterflies may employ other defenses against parasitoids, perhaps in addition to cardenolides.

Keywords Danainae · Pteromalidae · Apocynaceae · Tri-trophic interaction · Pupal parasitoid · Monarch butterfly

Introduction

Plant allelochemicals have long been recognized for their influences on multitrophic interactions between plants, herbivores, and natural enemies (Dethier 1954; Price et al. 1980). These toxins are evolutionarily selected for in response to herbivory and can, at least initially, provide the plant a refuge of relatively "enemy free space" (Jeffries and Lawton 1984; Berryman and Hawkins 2006; Murphy et al. 2014). Over time, their defensive function may be diminished by the subsequent evolution of detoxification or tolerance traits by lineages of insect herbivores (Ehrlich and Raven 1964). In a co-evolutionary twist, many specialized insect herbivores have evolved to sequester and redeploy plant allelochemicals in their own defense (Duffey 1980; Blum 1981; Nishida 2002; Opitz and Müller 2009). Across a variety of taxa, herbivore-sequestered plant toxins have been found to deter or harm many classes of natural enemies, including vertebrate and invertebrate predators (Rowell-Rahier and

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Pasteels 1992), insect parasitoids (Duffey et al. 1986; Ode 2006), and pathogens (de Roode et al. 2008; Gowler et al. 2015).

Milkweeds (*Asclepias* spp., Apocynaceae: Asclepiadoideae) and monarch butterflies (*Danaus plexippus* Linnaeus, Lepidoptera: Nymphalidae: Danainae: Danaini, hereafter "monarchs") are a classic model system for chemical ecology, as monarch larvae sequester cardenolides from milkweed host plants and retain these chemicals as adults (Parsons 1965; Brower 1984; Malcolm 1995). Cardenolides are bitter tasting steroids that inhibit neural and cardiac ion channels in most animals. Monarchs and several other specialized insect herbivores of milkweeds, however, demonstrate insensitivity to these chemicals due to convergently evolved point mutations in the gene encoding the α-subunit of the sodium–potassium ATPase (Holzinger and Wink 1996; Dobler et al. 2011, 2015). Monarch cardenolide concentrations increase monotonically with host plant concentrations, but they are especially efficient at sequestering from low-cardenolide plants (Malcolm 1995). Whether milkweed butterflies are negatively affected by their consumption of cardenolides remains an area of active research (Petschenka and Agrawal 2015; Tao et al. 2016; Decker et al. 2018).

The defensive role of monarchs' sequestered cardenolides is better understood in their interactions with vertebrate than invertebrate natural enemies. Studies of monarch toxicity to bird predators show that variation in cardenolides leads to a "palatability spectrum," where butterflies containing fewer cardenolides are more readily consumed and greater quantities of tissue must be consumed to induce an emetic response (Brower et al. 1967, 1968; Roeske et al. 1976; Dixon et al. 1978). Variation in monarch toxicity has since been examined for several other vertebrate predators and, in most cases, these animals exhibit negative effects of cardenolide consumption and learn to avoid monarchs (Brower and Fink 1985; Glendinning 1993). An exception is the black-headed grosbeak, which is insensitive to cardenolides and causes considerable mortality at monarch overwintering sites in Mexico (Fink and Brower 1981). A diverse suite of invertebrates (e.g. ants, soldier bugs, ladybeetles, spiders) consume monarch eggs, larvae, and pupae in the field, but the effects of cardenolides have been studied for very few of these species (Zalucki and Kitching 1982; Prysby 2004; Oberhauser et al. 2015). Foraging paper wasps, *Polistes dominula* (Christ), generally prefer monarch larvae raised on lower toxicity milkweeds, although the relationship seems to also depend upon the host plant species compared (Rayor 2004). Also, wild-collected Chinese mantises, *Tenodera sinensis* (Saussure), have been found to remove the gut contents of monarch larvae before consuming the body, a behavior not performed while handling non-toxic Lepidoptera (Rafter et al. 2013). Interestingly, while the types of cardenolides in the gut and body differed, the overall concentrations did not (Rafter et al. 2013), and a follow-up study found that mantises suffered no apparent acute or long-term consequences of consuming monarch larvae (Rafter et al. 2017).

Like many other herbivorous insects, a primary source of mortality for monarchs is insect parasitoids (Hawkins et al. 1997; Oberhauser et al. 2017). At least seven species of tachinid flies have been reared from wild-collected monarch larvae in North America, though *Lespesia archippivora* (Riley) is the most common and thoroughly studied (Oberhauser et al. 2017). Milkweed species and relative cardenolide concentrations have been found to influence monarch-tachinid interactions, though not always as expected. Hunter et al. (1996) found that while the cardenolide content of host plants did not influence the likelihood of parasitism by tachinid flies, the number of adult flies produced per host was inversely related to host plant cardenolide concentration, suggesting that cardenolides influence maternal investment, survivorship of fly larvae, or both. Similarly, Oberhauser et al. (2015) demonstrated that penetration of a host does not always result in successful parasitism and that larvae reared on the more toxic host plants were marginally more likely to survive encounters with *L. archippivora* than larvae reared on less-toxic species.

Another parasitoid of monarchs is the wasp *Pteromalus cassotis* (Walker) (Hymenoptera: Pteromalidae: Pteromalinae). This gregarious, pupal endoparasitoid was first recorded from a monarch by Gillette (1888), though detailed studies of this interspecific interaction have only recently begun. Observations of these species interacting have been rare, perhaps because monarchs are cryptic and usually pupate away from host plants. Nine butterfly species (mostly nymphalids) have been recorded as hosts for *P. cassotis*, though none as frequently as the monarch (Muesebeck et al. 1951; Peck 1963; Burks 1975, 1979; California Academy of Sciences Entomology General Collection Database 2015; Noyes 2017). Oberhauser et al. (2015) conducted field experiments in the northern US using monarchs reared on either *A. syriaca* (low cardenolides) or *A. curassavica* (high cardenolides) and showed that pupae reared from less-toxic plants were more likely to be parasitized (87% vs. 60%). Whether this difference was due to differences in parasitoid preference, host survival, or both is unknown. Parasitism of monarchs by *P. cassotis* has been documented in multiple locations across the eastern US, revealing significant variation in brood sizes, sex ratios, and apparent survival (Stenoien et al. 2015).

Pteromalus puparum (Linnaeus) (Hymenoptera: Pteromalidae: Pteromalinae), another gregarious pupal parasitoid and close relative of *P. cassotis*, was once released around the world as a biocontrol agent of the *Brassica* crop pests, *Pieris rapae* and *P. brassicae* (Moss 1933; Lasota and Kok 1986; Barron et al. 2003; Benson et al. 2003). However, *P. puparum* is no longer released since it has been found to

utilize at least 48 lepidopteran hosts, including many nymphalid butterflies (Muesebeck et al. 1951; Peck 1963; Noyes 2017). While monarchs have not been reported as a host utilized by *P. puparum*, this wasp has been observed attacking monarch pupae in the wild, though the frequency of their interactions is unknown. Ramsay (1964) described unsuccessful attacks of monarchs by *P. puparum* in New Zealand, which resulted in host death and failure of the wasps to develop. The identity of the host plant and number of observed parasitism attempts were not recorded however, so it remains possible that monarchs' apparent unsuitability for *P. puparum* is context-dependent. Because monarch pupae are rarely observed, collected, and reared from the field, we paired these species under laboratory conditions in order to more conclusively determine whether monarchs could serve as a suitable host for *P. puparum*.

Despite monarchs' status as a model organism for chemical ecology and as a species of conservation concern (Stenoien et al. 2016; Semmens et al. 2016), very little is known about the role of sequestered cardenolides in their interactions with parasitic wasps. Here, we tested whether sequestered cardenolides aid in defense against *P. puparum* and *P. cassotis*. Because monarch butterfly larvae sequester cardenolides in concentrations that correlate with those of the host plant, we reared larvae on two species of milkweeds known to be relatively high and low in total cardenolide concentration (Malcolm 1995). We measured host survival, parasitoid foraging behaviors, and several other performance metrics including brood size, survival to adulthood, and potential maternal effects related to host chemistry. We hypothesized that: (1) greater concentrations of herbivore-sequestered plant toxins would increase butterfly survival against both species of parasitic wasp, (2) both species of wasp would be less likely to attack the more toxic hosts, and (3) parasitism of more toxic hosts would result in decreased offspring performance, as measured by the rate of successful parasitism, brood size, survival to adulthood, development time, adult lifespan, and fecundity in the next generation.

We also sought to determine whether either parasitoid might be negatively affected by the mere presence of cardenolides. Unfortunately, because monarchs efficiently sequester from low-cardenolide host plants, it is not feasible to rear pupae devoid of cardenolides (Brower et al. 1967; Malcolm 1995). Instead, we tested the ability of both wasp species to develop in *Euploea core* Cramer (Nymphalidae, Danainae, Danaini), a milkweed butterfly found in South Asia and Australia. Like monarchs, *E. core* larvae ingest cardenolides from Apocynaceae host plants. Unlike monarchs, their midgut epithelium is impermeable to cardenolides, so they do not retain plant-derived cardenolides as pupae or adults (Malcolm and Rothschild 1983; Petschenka and Agrawal 2015). Although no species of Pteromalidae have been reported from *E. core* hosts (Noyes 2017), this does not preclude the possibility, as many parasitoids have been found to develop from novel hosts in laboratory and natural settings (Harvey et al. 2012).

We expected that if sequestered cardenolides are the sole mechanism preventing *P. puparum* from developing in monarch hosts, then *P. puparum* should be able to reproduce in *E. core* hosts. Although monarchs and *E. core* are closely related, it is possible that their suitability to these parasitoids might vary for reasons independent of the presence or absence of sequestered cardenolides. Therefore, if *P. puparum* can reproduce in *E. core* but not monarch hosts, this would suggest, but not prove, that cardenolides may be the mechanism by which monarchs are unsuitable hosts. If *P. puparum* is incapable of parasitizing *E. core*, non-cardenolide mechanisms must be responsible for this incompatibility. Because rearing cardenolide-free monarchs is difficult or impossible (Brower et al. 1967; Malcolm 1995), we view this comparative approach as a logical, though imperfect, first step toward understanding the role of sequestered cardenolides in milkweed butterflies' defense against parasitic wasps.

Materials and methods

Butterflies

Monarch colonies were established each summer (2013–2016) from wild-caught butterflies in Minnesota and maintained in mesh cages in a greenhouse. We allowed monarch butterflies to oviposit on host plants, then randomly assigned eggs to one of two milkweed species, either *Asclepias incarnata* Linnaeus or *Asclepias curassavica* Linnaeus (both Apocynaceae: Asclepiadoideae). We reared monarch larvae in the greenhouse at densities of 1–15 larvae per potted host plant, with lower densities as larvae matured until they reached the fifth stadium. We then transferred individual larvae to clear ventilated 500 ml plastic containers in the lab and fed them fresh plant clippings. Day length was approximately L16:D8 h and temperatures ranged from 18 to 32 °C in the greenhouse and 20–23 °C in the lab.

Euploea core was shipped as pupae from Australia (first generation offspring from wild-caught adults) and maintained for one generation in growth chambers (2016, per specifications of USDA APHIS permit # P526-160112-040). To encourage normal mating behaviors, we hand fed each *E. core* adult 1 mg of monocrotaline (a pyrrolizidine alkaloid) dissolved in 25–40 µl of 20% honey-water two to four times during their lifetime and allowed females to oviposit onto *Nerium oleander* Linnaeus (Apocynaceae: Apocynoideae) host plants. We reared *E. core* larvae on *N. oleander* in growth chambers at densities of 2–6 larvae per potted host plant until they reached the third stadium.

We then transferred individual larvae to clear ventilated 500 ml plastic containers and fed them fresh clippings of the same host plant species. Day length was L16:D8 h and temperatures ranged from 20 to 22 °C.

Plants

Host plants were grown from seed in individual pots in a greenhouse and fertilized biweekly. Monarch larvae were fed either *A. incarnata* or *A. curassavica*, which are morphologically similar plants with very different cardenolide profiles. Mean cardenolide concentrations for these plants have previously been determined: *A. incarnata* = 14 and *A. curassavica* = 1055 µg 0.1 g^{-1} dry weight of leaf tissue (Malcolm 1990). *A. incarnata* seeds were collected from naturally occurring plants in Minnesota, and *A. curassavica* seeds were purchased from OutsidePride.com. *A. incarnata* grows across much of the Eastern US and is often used by ovipositing monarchs (Ladner and Altizer 2005; Pocius et al. 2018). *A. curassavica*, which was introduced to the US and is often planted in gardens, is used by ovipositing monarchs throughout much of their North American range (Batalden and Oberhauser 2015). Petschenka and Agrawal (2015) found that, of eight milkweeds tested, monarch larvae reared on *A. incarnata* had the lowest concentration of hemolymph cardenolides and those reared on *A. curassavica* had the highest concentration (< 0.01 and > 0.21 µg $µl^{-1}$, respectively). In this same study, *E. core* did not have detectable levels of hemolymph cardenolides, regardless of host plant (Petschenka and Agrawal 2015).

We reared *E. core* on *Nerium oleander* Linnaeus (Apocynaceae: Apocynoideae) because it is known to develop on this cardenolide-rich plant under lab and field conditions (Malcolm and Rothschild 1983; Rahman et al. 1985). *N. oleander* seeds were purchased from a grower in Florida via Ebay.com.

Parasitoids

Laboratory populations of both parasitoid species were established from field-infected hosts. The *P. puparum* colony originated from *P. rapae* pupae placed in the field in Roseville, MN in September 2013. The *P. cassotis* colony consisted of wasps from monarch pupae collected in four US states: Oklahoma (October 2013), Georgia (January 2014), Minnesota (August 2015 and September 2016), and Florida (July 2016). Both colonies of parasitoids were maintained in a laboratory with natural and supplemented light (L16:D8) at 20–23 °C and variable humidity.

Pupal cardenolide measurements

To verify whether the cardenolide content of monarch pupae varied as expected based on their larval host plant, we randomly chose four monarch pupae reared on each host plant and freeze-dried them for measurement of total cardenolide content (FreeZone Cascade Benchtop Freeze Dry System; Labconco Corp.). Each pupa was analyzed using high-performance liquid chromatography (HPLC) at Western Michigan University. Analyses were performed using the method of Wiegrebe and Wichtl (1993) on a Waters gradient HPLC system with WISP autosampler, 600E pump, 996 diode array detector and Millennium^{32}® chromatography software. The reverse-phase elution gradient was acetonitrile:water at 1.2 ml min^{-1} at 40 °C, with 20% acetonitrile at start, 32% after 35 min., 40% after 45 min., 50% after 55 min., then back to 20% at 61 min., and 20% at 65 min., on a 250-4 LiChroCART® RP-18 column packed with LiChrospher® 100, 5 µm (E. Merck) with a 10 mm guard column. Sample injections were 20 µl, run for 65 min and separated by 10-min equilibration at 20% acetonitrile.

Cardenolides were detected at 218.5 nm and identified by their symmetrical spectra between 205 and 235 nm and a λ_{max} of between 214 and 224 nm (Malcolm and Zalucki 1996). Cardenolide concentration for each peak (µg/0.1 g sample DW) was calculated from a calibration curve with the external cardenolide standards digitoxin and *Calotropis procera* (Sigma). Only cardenolide peaks reported by Millennium software as consistently pure were considered for analysis.

We did not measure the cardenolide content of *E. core* pupae because previous studies have detected zero-sequestered cardenolides in pupae of this species (e.g., Malcolm and Rothschild 1983; Petschenka and Agrawal 2015).

Experimental protocols

All experiments were conducted from September 2013 to December 2016. After removing frass and unconsumed plant matter, trials were conducted in the same containers in which the larvae were fed during their final stadium. Within 24 h of pupal ecdysis (shedding of the final larval exoskeleton, revealing the pupa), hosts were exposed to one naïve female wasp (mean days = 0.27, SD = 0.41). In other experiments, monarch pupae have proven susceptible to parasitism by *P. cassotis* for several days following pupal ecdysis (Stenoien, unpublished). *Pteromalus* wasps practice sib-mating immediately upon emergence from their host, so females were assumed to have mated prior to their use in trials. Wasps were 1–25 days old (mean days since emergence from natal host = 7.6, SD = 5.43) and provided with a ~ 1 cm^3 sponge soaked in 20% honey-water. All trials lasted 2–4 days (mean = 2.67, SD = 0.41). In all cases, the natal host (host

from which the wasp emerged) of *P. puparum* was *P. rapae*, and in most cases, the natal host of *P. cassotis* was a monarch (92% from monarchs, 6.2% from *P. rapae,* and 1.8% from *Colias philodice*). In total, 497 monarchs were exposed to *P. cassotis* (249 fed *A. incarnata*, 248 fed *A. curassavica*), 161 were exposed to *P. puparum* (88 fed *A. incarnata*, 73 fed *A. curassavica*), and 188 were unexposed controls, used to determine background rates of pupal mortality (96 fed each host plant).

During all trials, at least three observations, each at least 6 h apart were made to determine whether the wasp was standing on the host (median and mode = 6 observations per trial). Observations of a wasp crawling onto and then off the host within 30 s were considered 'off'. When observed closely, a wasp standing on a host typically had her ovipositor inserted into the host, though this was not always discernible due to the wasp's orientation. Observations of wasps on hosts usually led to verified oviposition in typical host species, in which either wasps emerged or were detected via dissection, so we considered any wasp viewed in contact with a host to have attempted oviposition. As an example, 83.4% of trials in which *P. cassotis* was seen on a monarch host resulted in verified oviposition, while only 17.1% of trials in which *P. cassotis* was not seen on the host resulted in verified oviposition.

Upon removal of the wasp, most pupae (91%) were weighed, then reattached to the container by tying a string around the cremaster (a hardened and hooked abdominal protrusion) and taping the string to the inside of the lid. Due to occasional time constraints, a subset of pupae was not weighed, and were left undisturbed. Containers were placed on a lab bench at approximately 20–23 °C, where they were exposed to natural light and supplemented with artificial light to achieve a photoperiod of 16L:8D. We recorded the date of emergence of butterflies or wasps, as well as the number and sex of all emerged wasps. If neither host nor parasitoids had emerged after 30 days, we dissected hosts to determine the cause of death. The result of each trial was either (1) the butterfly eclosed, (2) the butterfly died due to an unknown cause, (3) the butterfly died with unsuccessful parasitoids inside, or (4) the butterfly was successfully parasitized (live parasitoids emerged). Hosts from which any wasps emerged were also dissected to determine the proportion of parasitoids that died as visible larvae, pupae, or adults (including sex) inside of the host. Therefore, when reporting the mean proportion of emerged wasps per brood, all hosts known to be parasitized are included, regardless of whether any wasps emerged successfully. We could not account for wasp eggs or larvae that did not develop to a stage visible under a dissecting microscope, but penultimate and ultimate (second and third) instar larvae, pupae, and adult wasps inside hosts are easily distinguished. Thus, we cannot know with certainty the total number of eggs oviposited into a host and hosts that died for unknown reasons may have contained eggs or early-stage larvae. Due to unbiased technical errors, we are missing brood size, sex, and wasp-survival data for 41 of the 497 *P. cassotis*-monarch trials which resulted in successful parasitism (26 fed *A. incarnata*, 15 fed *A. curassavica*).

Based on the observed behavior of the wasp and the trial outcome, each trial was classified as either successful, unsuccessful, or no attempt of parasitism. Trials in which adult wasps emerged were scored as 'successful', regardless of whether the wasp was observed on the host. 'Failed' outcomes include trials in which the wasp was seen on the host, but no offspring emerged, plus any trials in which we found dead offspring inside of the host, regardless of whether the wasp was seen in contact with the host. Trials for which we never saw the wasp in contact with the host that resulted in either an emerged butterfly or a pupa that died due to an unknown cause were scored as 'no attempt.'

A subset of these classifications may sometimes be inaccurate. If any trials in which the wasp was not seen on the host resulted in unobservable failed parasitism (no wasp development), this would cause us to underestimate the rate of failure and overestimate the rate of not attempting parasitism. If, however, any trials which appeared to be 'failed attempts' resulted in the parasitoid deciding not to oviposit despite being observed on the host, this would result in the opposite mischaracterization: overestimating the rate of failed parasitism attempts, while underestimating the rate of not attempting parasitism. We generated multiple estimates of the frequency of each type of error and found no evidence that either type of error occurred more frequently than the other (Supplemental Information).

Pteromalus cassotis frequently successfully parasitized monarch hosts, allowing us to compare fecundity (total brood size and female brood size), survival to adulthood, development time based on host diet, and lifespan based on host diet. We also measured whether the host diet affected emerging parasitoids' fecundity by including only those trials in which the mother was an offspring from an earlier trial in the dataset (148 of the 367 *P. cassotis*-monarch trials in which parasitism was attempted). This allowed us to test for the effect of the mother's diet (maternal effect) while controlling for the effect of the focal wasp's host's diet. For all of these comparisons, we restricted the dataset to include only those trials that used a maternal *P. cassotis* wasp which had been reared from monarch host, in case maternal effects related to host species affect performance.

To test the effect of host diet on *P. cassotis* lifespan, we randomly chose 20 females from two *A. incarnata*-fed monarch hosts and two *A. curassavica*-fed monarch hosts that had emerged at known times on the same day. Each wasp was maintained in a 40-dram polystyrene tube with no food or water in a growth chamber (L12:D12, 18 °C). We checked

the survival of each wasp in the morning and evening (at approximately 7:00 AM and 7:00 PM) and randomized location within the chamber daily. All wasps that had died overnight were ascribed a time of death of 2:00 AM and those that died during the day were ascribed a time of death of 2:00 PM (approximate midpoints between observations).

We exposed *E. core* pupae to each species of parasitoid (10 trials with *P. puparum*, 11 trials with *P. cassotis*, and 9 unexposed controls). We then compared trial outcomes and parasitoid performance for all host-parasitoid combinations.

Statistical analyses

The concentrations of monarchs' sequestered cardenolides between diet treatments were compared using a Wilcoxon rank sum test, and a Fisher exact test was used to determine whether host plant affected rates of background pupal mortality in unexposed controls. The mean mass of monarch pupae reared on each host plant were compared using a *t* test.

We used mixed effects binomial logistic regressions (GLMMs) with logit link functions in R (R Core Team, version 3.3.3 2017) to test for effects of host plant on the likelihood of monarchs reaching adulthood when facing either parasitoid, the likelihood of oviposition by both parasitoids, the likelihood of monarchs reaching adulthood when attacked by either parasitoid, and the likelihood of *P. cassotis* success when attacking monarchs. In each, we included wasp age, pupa age, and pupa mass as fixed effects and the month-year in which trials were conducted as a random effect. In the model testing monarch survival when attacked by either parasitoid, we also included an interaction term for the effects of wasp species and wasp age, in case aging led to different outcomes for each parasitoid species.

For all analyses of the fate of attacked butterflies and wasp performance, we excluded trials in which parasitism was deemed 'not attempted' to avoid confounding preference with performance. We did not differentiate between failed attempts that resulted in wasp death, host death without evidence of wasps inside, or butterfly emergence.

Counts of total brood size and female brood size for *P. cassotis* were analyzed using hurdle models implemented via the pscl package (Jackman 2015) in R. Hurdle models allow the analysis of non-parametric count data that include an overabundance of zeroes (Mullahy 1986). These models use a binomial distribution for the zero versus positive portion of the model and a negative binomial distribution for the count portion of the model. Survival to adulthood of wasps and butterflies was separately analyzed using binomial distributions with a logit link function. Finally, a linear model was applied to the wasp lifespan data with host plant as a fixed effect and brood identity as a random effect. These models were performed on a subset of trials in which the focal wasp was reared from a monarch. Each of these models included the host plant, pupal mass, time since pupal ecdysis, and wasp age as covariates. The model of survival to adulthood also included the total number of emerged and detectable unemerged wasps as a covariate, in case maternal preferences affected the number of offspring invested in a host, which could positively or negatively affect the survival of the brood. The model of wasp development time also included the total number of wasps and sex ratio of the brood, as these variables have been found to influence developmental rates in other gregarious parasitoids (Milonas 2005; Reudler and van Nouhuys 2018). Finally, the model of monarch host diet effects on wasp fecundity included the focal wasp's developmental environment (*A. incarnata* vs. *A. curassavica*-reared host) as an interaction term with the focal host's diet. For all hurdle models, we used Tukey-adjusted pairwise comparisons of least square means via the lsmeans package (Lenth 2016) to determine whether differences between host plant treatments were significant.

Because the monarch and *E. core* hosts tested in our studies were reared and tested under different laboratory conditions, we do not report any statistical comparisons of their suitability for these parasitoids. Instead, we filtered our monarch trial data to be similar to our *E. core* trial data, keeping only trials with wasps aged 1–12 days and pupae within 0.25 days of pupal ecdysis. For each host-parasitoid combination, we report the frequencies of trial outcomes. For those host-parasitoid combinations resulting in successful parasitism, we also provide descriptive statistics of emerged wasps, emerged wasps per gram of host, the proportion of wasps surviving to adulthood, and sex ratio.

Results

Monarch pupal characteristics related to host plant

Cardenolide concentrations were significantly greater in monarch pupae reared on *A. curassavica*, the high cardenolide plant (mean = 7.21, range 3.73–11.33 µg per 0.1 g of host mass) than those reared on the low-cardenolide plant, *A. incarnata* (mean = 0.81, range 0.22–1.35 µg per 0.1 g of host mass) (Wilcoxon Rank Sum Test, W = 16, $p = 0.029$). These differences indicate that the host plant treatment resulted in distinct amounts of cardenolides sequestered into the pupal stage.

Background mortality of unexposed control pupae was unrelated to host plant (Fig. 1, $n_{A.c.} = 4/88$, $n_{A.i.} = 6/90$, Fisher Exact Test, $p = 0.75$). On average, monarchs reared on *A. curassavica* were slightly heavier than those reared on *A. incarnata* (Mean $\pm$ $SE_{A.c.} = 1.186 \pm 0.01$ g, Mean $\pm$ $SE_{A.i.} = 1.141 \pm 0.01$ g, $t(769.44) = 3.1099$, $p = 0.00194$).

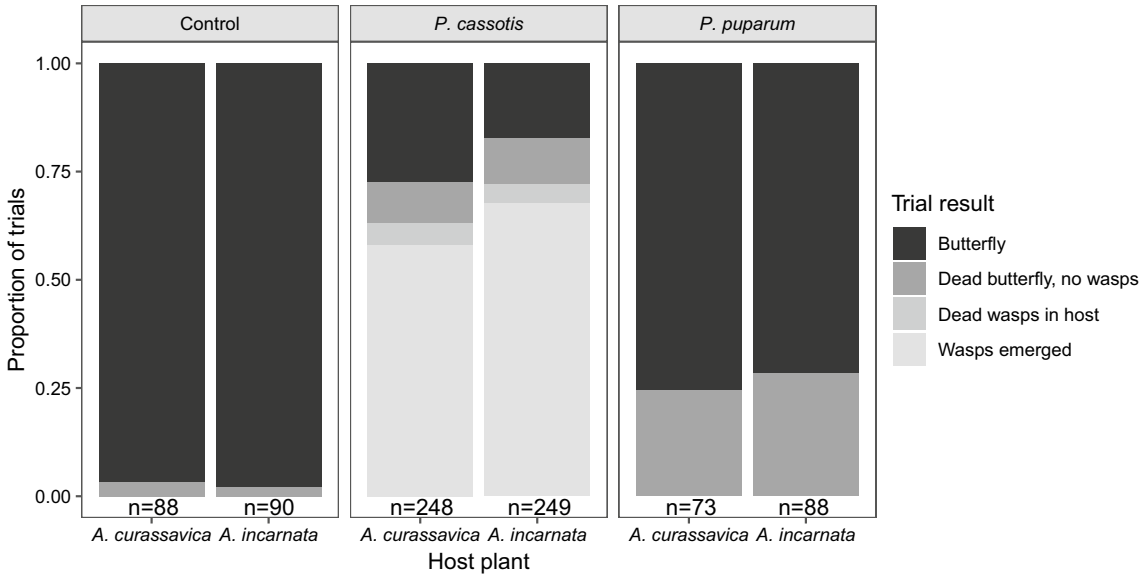

Fig. 1 Results of all monarch trials, regardless of wasp behavior, when exposed to either one *P. cassotis* female, one *P. puparum* female, or no wasps (control). Monarchs were reared on either high (*A. curassavica*) or low (*A. incarnata*) cardenolide plants. Trials either resulted in the eclosion of the butterfly ("Butterfly"), the butterfly's death due to an unknown cause ("Dead butterfly, no wasps"), the butterfly's death with unsuccessful parasitoids inside ("Dead wasps in host"), or the butterfly's death due to successful parasitism ("Wasps emerged"). Total sample sizes are indicated below each bar. Monarchs reared on *A. curassavica* were significantly more likely to survive trials with *P. cassotis* than monarchs reared on *A. incarnata*

Are monarchs that contain more sequestered cardenolides more likely to survive encounters with parasitoids?

When considering all trials, regardless of whether the wasp was observed on the host, monarchs reared on the high-toxicity milkweed were more likely than those reared on the low-toxicity milkweed to survive encounters with *P. cassotis* (Fig. 1 and Table 1A, $n_{A.c.}=68/248$, $n_{A.i.}=43/249$, GLMM, $p=0.018$). When monarchs were exposed to *P. puparum*, host plant had no effect on the proportion of hosts surviving these encounters (Fig. 1 and Table 1B, $n_{A.c.}=55/73$, $n_{A.i.}=63/88$, GLMM, $p=0.830$).

Is the likelihood of parasitoid oviposition affected by sequestered cardenolide concentration?

Pteromalus cassotis females were more likely to attempt parasitism of monarchs reared on the low-toxicity milkweed (Fig. 2 and Table 1C, $n_{A.c.}=188/248$, $n_{A.i.}=212/249$, GLMM, $p=0.009$). Host plant did not, however, affect the likelihood that *P. puparum* females attempt parasitism (Fig. 2 and Table 1D, $n_{A.c.}=34/73$, $n_{A.i.}=37/88$, GLMM, $p=0.772$).

When parasitism is attempted, are monarchs that contain more sequestered cardenolides more likely to survive attacks by either parasitoid?

Contrary to expectations, when considering only trials in which parasitism was attempted, monarchs' larval diet had no effect on the likelihood of host survival when paired with either parasitoid (Fig. 3, Table 1E, and Table 1F, *P. cassotis*: $n_{A.c.}=13/188$, $n_{A.i.}=11/212$, *P. puparum*: $n_{A.c.}=20/34$, $n_{A.i.}=17/37$, GLMM, *P. cassotis* $p=0.572$, *P. puparum* $p=0.870$). Monarchs were more likely to survive attacks by *P. puparum* than *P. cassotis* (Fig. 3 and Table 1G, GLMM, $p<0.001$). Although attempted parasitism of monarchs by *P. puparum* were never successful, these attempts led to the mortality of 47.9% hosts, compared to 5.3% unexposed controls ($n_{\text{exposed to } P.p.}=34/71$, $n_{\text{control.}}=10/188$).

Does the performance of either wasp species depend on monarchs' larval diet?

Pteromalus puparum never successfully parasitized monarch hosts, regardless of host plant, nor even developed to a larval stage discernible via dissection (Fig. 3). Therefore,

Table 1 Model outputs for mixed effects binomial logistic regressions of trial outcomes

A. Monarch survival when exposed to *P. cassotis*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−3.750	0.845	−4.440	< 0.001*
Host plant: *A. incarnata*	−0.565	0.239	−2.364	0.018*
Wasp age (days)	0.062	0.021	2.941	0.003*
Pupa mass (grams)	1.710	0.627	2.727	0.006*
Pupa age (days)	0.679	0.422	1.609	0.108
B. Monarch survival when exposed to *P. puparum*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−0.639	1.141	−0.560	0.576
Host plant: *A. incarnata*	0.093	0.430	0.215	0.830
Wasp age (days)	−0.057	0.026	−2.214	0.027*
Pupa mass (grams)	1.831	1.070	1.712	0.087
Pupa age (days)	1.311	0.729	1.798	0.072
C. Likelihood of oviposition into monarch hosts by *P. cassotis*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	2.973	0.863	3.446	< 0.001*
Host plant: *A. incarnata*	0.655	0.250	2.617	0.009*
Wasp age (days)	−0.0712	0.022	−3.258	0.001*
Pupa mass (grams)	−0.802	0.643	−1.248	0.212
Pupa age (days)	−1.004	0.442	−2.273	0.023*
D. Likelihood of oviposition into monarch hosts by *P. puparum*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−0.522	1.049	−0.498	0.619
Host plant: *A. incarnata*	−0.111	0.383	−0.290	0.772
Wasp age (days)	0.0345	0.0226	1.526	0.127
Pupa mass (grams)	0.124	0.950	0.131	0.896
Pupa age (days)	−1.158	0.596	−1.942	0.052
E. Monarch survival when parasitism was attempted by *P. cassotis*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−4.618	1.557	−2.966	0.003*
Host plant: *A. incarnata*	−0.243	0.430	−0.565	0.572
Wasp age (days)	0.034	0.040	0.851	0.395
Pupa mass (grams)	1.416	1.162	1.218	0.223
Pupa age (days)	0.219	0.743	0.295	0.768
F. Monarch survival when parasitism was attempted by *P. puparum*				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−1.031	1.805	−0.571	0.568
Host plant: *A. incarnata*	0.113	0.688	0.163	0.870
Wasp age (days)	−0.010	0.040	−2.505	0.012*
Pupa mass (grams)	1.774	1.640	1.081	0.280
Pupa age (days)	1.586	1.260	1.259	0.208
G. Monarch survival as related to wasp species				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	−4.996	1.256	−3.967	< 0.001*

Table 1 (continued)

G. Monarch survival as related to wasp species				
	Estimate	Std. Error	*z*-value	*p*-value
Wasp: *P. puparum*	4.507	0.720	6.259	< 0.001*
Wasp age (days)	0.035	0.040	0.882	0.3776
Pupa mass (grams)	1.545	0.933	1.656	0.0976
Pupa age (days)	0.548	0.596	0.920	0.3578
P. puparum * Wasp age	−0.137	0.056	−2.453	0.0142*
H. *Pteromalus cassotis* success when attempting parasitism of monarch hosts				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	0.366	0.920	0.398	0.690
Host plant: *A. incarnata*	0.288	0.271	1.060	0.289
Wasp age (days)	−0.036	0.025	−1.416	0.157
Pupa mass (grams)	1.001	0.705	1.420	0.156
Pupa age (days)	−0.207	0.504	−0.410	0.682

In all models, month–year was included as a random effect

Asterisks indicate $p < 0.05$

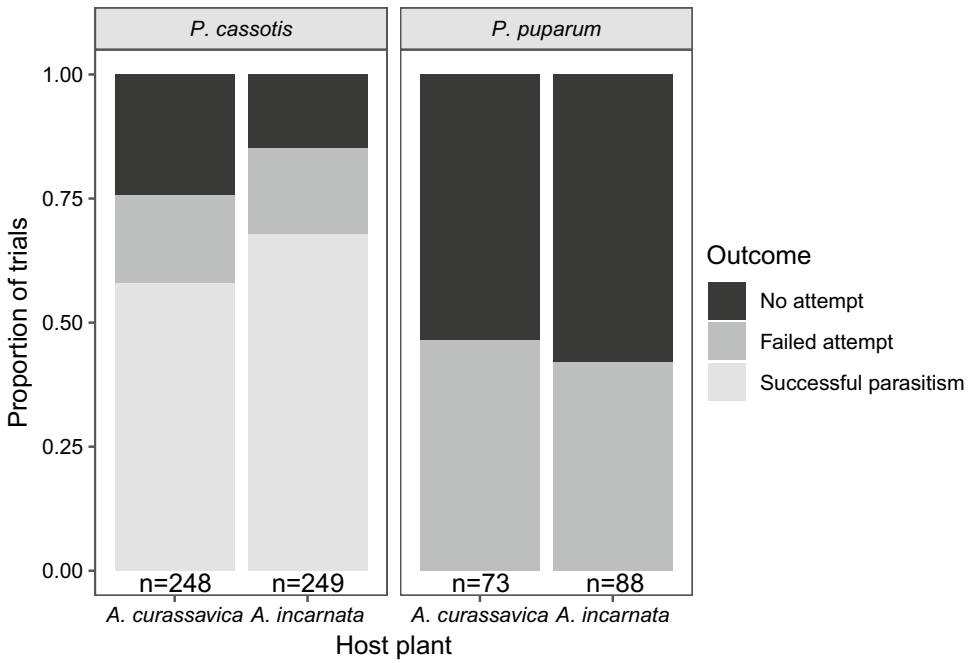

Fig. 2 Oviposition behavior and success or failure of both parasitoids when paired with monarchs reared on either high (*A. curassavica*) or low (*A. incarnata*) cardenolide plants. Total sample sizes are indicated below each bar. *P. cassotis* was significantly more likely to attempt parasitism of hosts reared on *A. incarnata*

under the conditions tested, the performance of *P. puparum* is unrelated to monarch diet.

Although host diet had no effect on the overall success rate of attempted parasitism by *P. cassotis* wasps (Fig. 3 and Table 1H, $n_{A.c.} = 44/188$, $n_{A.i.} = 43/212$, GLMM, $p = 0.289$), this species performed better in hosts reared on *A. incarnata* than on *A. curassavica* as measured by several offspring performance metrics. Broods reared from *A. incarnata*-fed hosts (low toxicity) were significantly more numerous when considering all offspring (Fig. 4a, Table 2A), as well as when considering only female offspring (Fig. 4b, Table 2B). Brood size metrics derive from both preference- and performance-related processes (maternal investment decisions and survival, respectively). Therefore, to control for maternal preferences in our analysis of wasps' survival to adulthood, we included the combined total of emerged and dead wasps as an estimate of total maternal investment. Using this metric, we found that *P. cassotis* invests more offspring, on average, in *A. incarnata*-fed hosts (mean ± SE = 72.1 ± 3.55) than *A. curassavica*-fed hosts (mean ± SE = 60.9 ± 3.47)

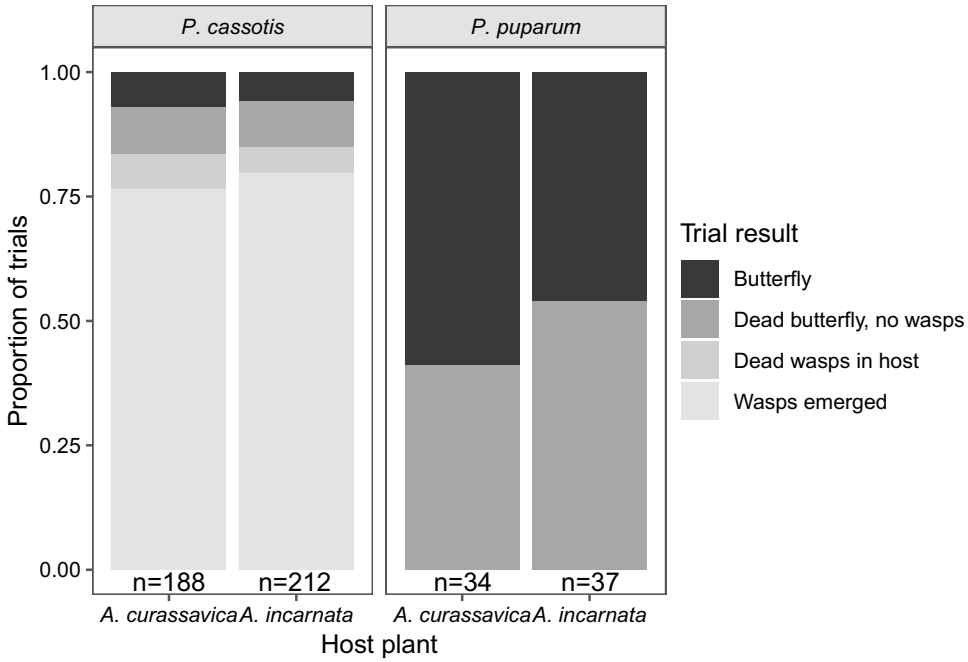

Fig. 3 Outcome of monarch trials in which parasitism was attempted. Trials either resulted in the eclosion of the butterfly ("Butterfly"), the butterfly's death due to an unknown cause ("Dead butterfly, no wasps"), the butterfly's death with unsuccessful parasitoids inside ("Dead wasps in host"), or the butterfly's death due to successful parasitism ("Wasps emerged"). Total sample sizes are indicated below each bar. Monarch host plant had no effect on the likelihood of butterfly or wasp emergence when paired with either parasitoid

(t(329.95) = − 2.25, $p = 0.025$). We also found that larger broods of *P. cassotis* experienced higher survival, on average, than smaller broods, regardless of host plant (Table 2C). Therefore, it could be that *P. cassotis* wasps experience higher survival on less-toxic hosts not because of differences in host chemistry, but because of positive density-dependence within broods, combined with the tendency to oviposit more eggs into *A. incarnata*-reared hosts. However, even after accounting for differences in maternal investment related to host diet, broods reared from *A. incarnata*-fed hosts had significantly higher survival than broods reared from *A. curassavica*-fed hosts (Fig. 4c, Table 2C).

Host diet did not affect parasitoid development time; *P. cassotis* wasps from both types of hosts emerged as adults approximately 14–15 days following oviposition (Fig. 4d, Table 2D). Regarding potential delayed effects of host plant on wasp performance, there was no detectable effect of host diet on the adult lifespan of wasp offspring, as *P. cassotis* females from both types of hosts survived with no food or water for approximately 7.5 days (Fig. 4e, Table 2E). There was, however, a maternal effect related to wasps' developmental environment; females reared from *A. incarnata*-fed hosts produced significantly larger broods than females reared from *A. curassavica*-fed hosts, even when controlling for the diet of the hosts those females would later attack (Fig. 4f, Table 2F).

There were strong and consistent effects of several covariates between models. In general, older host pupae were better defended against parasitism, while younger wasps, more recently eclosed hosts, and larger hosts were predictive of increased wasp performance (Tables 1 and 2). In addition, most of the significant effects in the hurdle models were detected in the count portion, rather than the binomial portion of the model, indicating that wasp success as a binary variable (whether *any* wasps survived to emergence) is less affected by host diet than continuous measures of wasp success (Table 2A–C, F).

Does the performance of either parasitoid differ in E. core, a host which does not sequester cardenolides into the pupal stage

By testing both parasitoids' ability to attack a cardenolide-free relative of monarchs, *E. core*, we could begin to discern whether the mere presence of cardenolides might prevent *P. puparum* from successfully parasitizing monarchs or hinder the performance of *P. cassotis* when attacking monarchs. Ultimately, the performance of *P. puparum* on *E. core* was indistinguishable from its performance on monarchs. In all ten trials in which *P. puparum* females attempted parasitism of *E. core*, they were unsuccessful in producing offspring or developing to a larval stage discernible upon dissection (Fig. 5).

The performance of *P. cassotis* seems to be approximately equivalent, or perhaps better, on *E. core* compared to monarch hosts. Our ability to compare these hosts is limited because we were only able to test a small number of *E. core* hosts in a different laboratory than that used for monarchs. The likelihood of successfully attempted parasitism was similar between hosts (*E. core*: 8/11 = 73%, monarchs: 222/277 = 80%). When attempting parasitism, *P. cassotis*' surviving brood size was larger, on

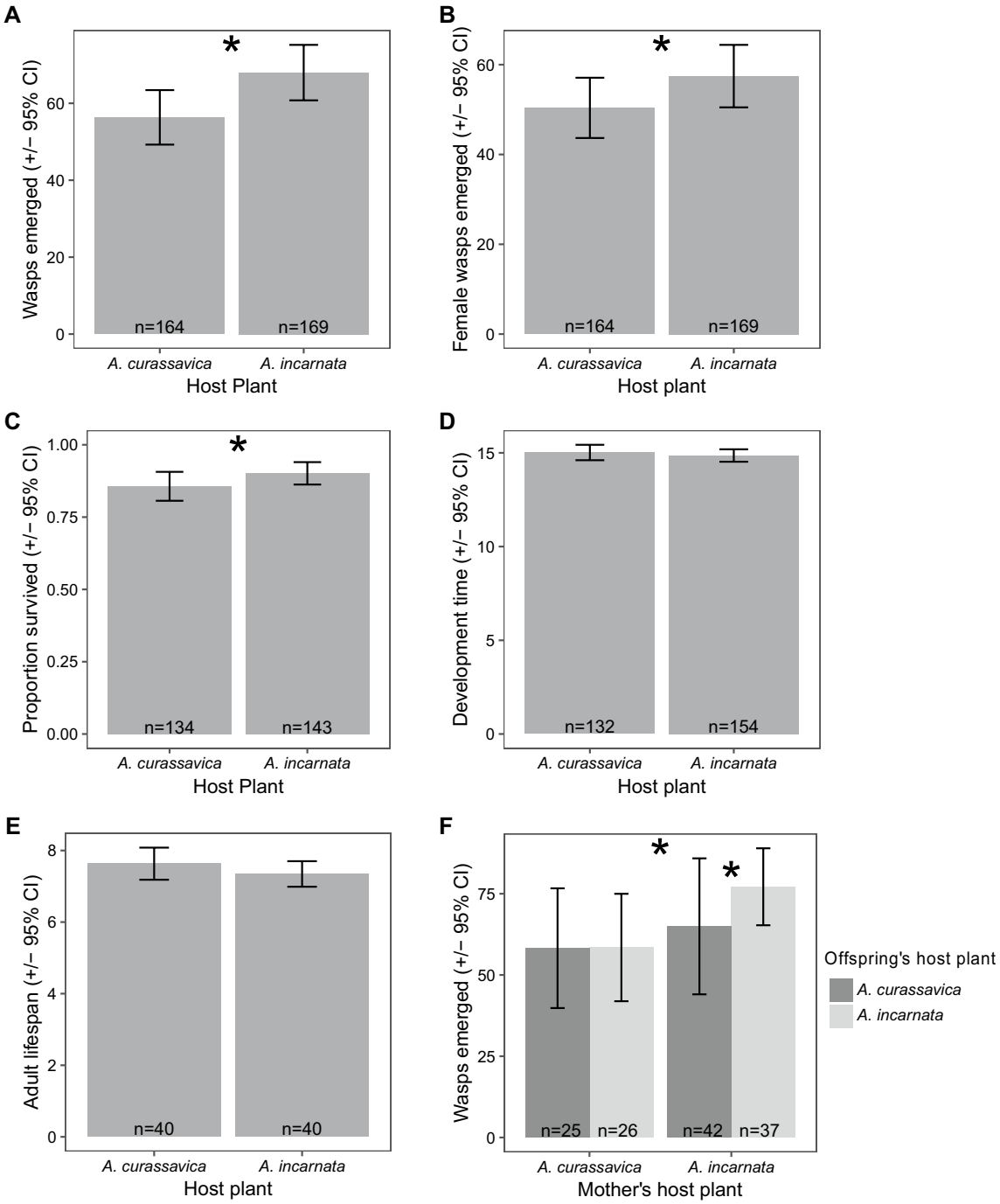

Fig. 4 Effects of monarch host plant on *P. cassotis* performance. Mean ± 95% confidence interval for **a** total brood size, **b** females per brood, **c** proportion of wasps successfully emerged from host, **d** developmental time to adulthood, **e** mean lifespan when provided no food or water, and **f** brood size based on focal and parental host diet. Significant differences are indicated by (*). Total sample sizes are indicated below each bar. For test statistics and full model results, see Table 2

average, from *E. core* hosts (mean ± $SD_{E.c.}$ = 75.6 ± 54.3; mean ± $SD_{D.p.}$ = 63.4 ± 47.2), even though *E. core* pupae weighed less than monarchs (mean ± $SD_{E.c.}$ = 0.79 ± 0.09 g; mean ± $SD_{D.p.}$ = 1.18 ± 0.20 g). As a result, the difference in the mean number of offspring per gram of host was even greater (mean ± $SD_{E.c.}$ = 93.6 ± 68.1; mean ± $SD_{D.p.}$ = 52.9 ± 39.3). Rates of survival to adulthood in each host species were not markedly different (mean ± $SD_{E.c.}$ = 0.94 ± 0.08; mean ± $SD_{D.p.}$ = 0.89 ± 0.25). Finally, *P. cassotis* had, on average, more

Table 2 Model outputs for brood-level and latent effects of monarch host plant on *P. cassotis* performance

A. Total wasps emerged				
	Estimate	Std. Error	*z*-value	*p*-value
Count model				
(Intercept)	4.447	0.053	84.426	< 0.001*
Host plant: *A. incarnata*	0.083	0.014	5.782	< 0.001*
Pupa age (days)	− 0.087	0.026	− 3.389	< 0.001*
Wasp age (days)	− 0.034	0.002	− 21.708	< 0.001*
Pupa mass (grams)	0.101	0.040	2.505	0.0123*
Zero hurdle model				
(Intercept)	− 0.625	0.940	− 0.665	0.506
Host plant: *A. incarnata*	0.398	0.276	1.444	0.149
Pupa age (days)	0.145	0.504	0.288	0.773
Wasp age (days)	− 0.031	0.027	− 1.172	0.241
Pupa mass (grams)	1.603	0.731	2.192	0.028
B. Total females emerged				
	Estimate	Std. Error	*z*-value	*p*-value
Count model				
(Intercept)	4.289	0.057	74.805	< 0.001*
Host plant: *A. incarnata*	0.067	0.0155	4.320	< 0.001*
Pupa age (days)	− 0.012	0.029	− 0.428	0.669
Wasp age (days)	− 0.031	0.002	− 18.426	< 0.001*
Pupa mass (grams)	0.1534	0.043	3.548	< 0.001*
Zero hurdle model				
(Intercept)	0.0730	0.881	0.083	0.934
Host plant: *A. incarnata*	0.1850	0.253	0.730	0.465
Pupa age (days)	− 0.634	0.441	− 1.436	0.151
Wasp age (days)	− 0.040	0.025	− 1.612	0.107
Pupa mass (grams)	1.037	0.678	1.531	0.126
C. Proportion surviving to emergence				
	Estimate	Std. Error	*z*-value	*p*-value
(Intercept)	− 0.870	0.219673	− 3.960	< 0.001*
Host plant: *A. incarnata*	0.227	0.0598	3.800	0.001*
Pupa age (days)	− 0.842	0.092173	− 9.131	< 0.001*
Wasp age (days)	0.053	0.007137	7.450	< 0.001*
Pupa mass (grams)	0.460	0.158059	2.910	0.004*
Total wasps oviposited	0.034	0.001034	32.502	< 0.001*
D. Developmental time				
	Estimate	Std. Error	*t*-value	*p*-value
(Intercept)	14.560	0.979	14.875	< 0.001*
Host plant: *A. incarnata*	− 0.099	0.251	− 0.394	0.694
Pupa age (days)	0.479	0.440	1.087	0.278
Wasp age (days)	0.029	0.027	1.066	0.287
Pupa mass (grams)	1.466	0.707	2.073	0.039*
Total wasps emerged	− 0.022	0.004	− 6.407	< 0.001*
Proportion male offspring	2.020	0.480	4.205	< 0.001*

Table 2 (continued)

E. Natal host diet				
	Estimate	Std. Error	*z*-value	*p*-value
Count model				
(Intercept)	4.116816	0.078393	52.515	< 0.001
Mother's host: *A. incarnata*	0.189253	0.036619	5.168	< 0.001
Host plant: *A. incarnata*	0.070726	0.034251	2.065	0.0389
Pupa age (days)	0.07087	0.045368	1.562	0.118
Wasp age (days)	− 0.02325	0.002363	− 9.842	< 0.001
Pupa mass (grams)	0.275479	0.060926	4.522	< 0.001
Mother's host: *A. incarnata* * Host plant: *A. incarnata*	− 0.0614	0.046892	− 1.39	0.190
Zero hurdle model				
(Intercept)	− 1.70764	1.47138	− 1.161	0.246
Mother's host: *A. incarnata*	− 0.17352	0.69898	− 0.248	0.804
Host plant: *A. incarnata*	− 0.37495	0.61181	− 0.613	0.540
Pupa age (days)	− 0.54719	0.95337	− 0.574	0.566
Wasp age (days)	0.01053	0.05147	0.205	0.838
Pupa mass (grams)	2.53676	1.17629	2.157	0.0310
Mother's host: *A. incarnata* * Host plant: *A. incarnata*	2.00224	1.00196	1.998	0.0457

F. Adult lifespan				
	Estimate	Std. Error	*t*-value	*p*-value
(Intercept)	7.6316	0.4873	15.66	0.004
Host plant: *A. incarnata*	− 0.2872	0.6892	− 0.417	0.717

Asterisks indicate $p < 0.05$

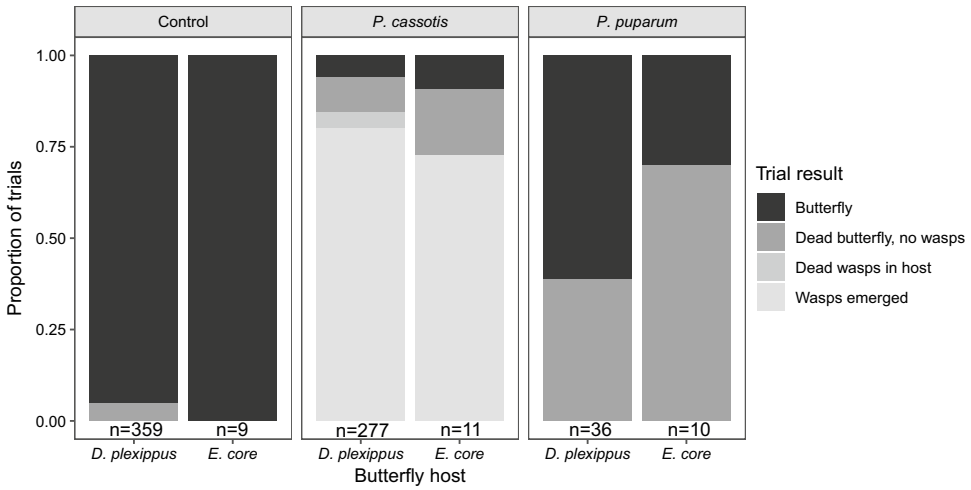

Fig. 5 Results of monarch and *E. core* trials in which parasitism was attempted. Trials either resulted in the eclosion of the butterfly ("Butterfly"), the butterfly's death due to an unknown cause ("Dead butterfly, no wasps"), the butterfly's death with unsuccessful parasitoids inside ("Dead wasps in host"), or the butterfly's death due to successful parasitism ("Wasps emerged"). Total sample sizes are indicated below each bar. Each species of parasitoid performed similarly on *E. core* as on monarchs, despite a lack of sequestered cardenolides in *E. core* hosts

female-biased sex ratios when attacking *E. core* than monarch hosts (mean ± $SD_{E.c.}$ proportion male = 0.05 ± 0.05; mean ± $SD_{D.p.}$ = 0.15 ± 0.25).

Discussion

The role of host plant chemistry is increasingly recognized as an important determinant of host susceptibility and parasitoid performance in plant-herbivore-parasitoid interactions (Ode 2013). We have conducted the first investigation of the defensive function of milkweed butterflies' sequestered cardenolides when experimentally exposed to two species of parasitic wasps. Our key findings were: (1) Monarchs were unsuitable hosts for *P. puparum* and were more likely to survive parasitism attempted by *P. puparum* than *P. cassotis*. (2) Overall, monarchs reared on *A. curassavica* (which resulted in higher levels of sequestered cardenolides) were more likely to survive encounters with *P. cassotis,* but only because these wasps were less likely to attempt parasitism of *A. curassavica*-fed hosts. (3) Within each parasitoid species, when parasitism was attempted, neither butterfly survival nor parasitoid success was influenced by host diet. (4) Although host diet did not affect whether *P. cassotis* wasps emerged from a host, attacks of *A. curassavica*-fed hosts led to smaller broods with lower survival and smaller broods in the next generation. Finally, (5) *P. puparum* was unable to parasitize cardenolide-free *E. core* hosts, while *P. cassotis* was able to develop normal broods from *E. core*, a novel host.

Herbivores could gain direct or indirect defensive benefits by sequestering plant secondary compounds. We did not find evidence of a direct benefit of monarchs' sequestered plant toxins because survival was unaffected by diet when attacked by either parasitoid. We did, however, find a behaviorally mediated benefit of host plant in interactions between monarchs and *P. cassotis*, as these wasps were less likely to attempt parasitism of *A. curassavica*-fed hosts than *A. incarnata*-fed hosts (76% vs. 85%, respectively). This diet-related difference in attack rates led to differences in overall parasitism rates, as 63% of *A. curassavica*-fed hosts and 72% of *A. incarnata*-fed hosts produced wasps or were found with dead wasps inside. Using this same metric, Oberhauser et al. (2015) found that monarchs reared on *A. curassavica* in the lab and then placed in the field were less likely to be parasitized by *P. cassotis* than those reared on a less-toxic host plant, *Asclepias syriaca* (60% vs. 87%, respectively). Given our results, it is possible that host plant-dependent rates of parasitism in the field may also be due to parasitoid foraging behaviors, rather than differences in the rates of successful parasitism across host types. Indeed, others have found that host plant identity can affect parasitoid foraging preferences, ultimately influencing rates of parasitism of potential hosts (Feng et al. 2015; Reudler and van Nouhuys 2018). A potential adaptive explanation for *P. cassotis*' apparent preference for hosts reared on the less-toxic plant is that this parasitoid has evolved to perceive and prefer less-toxic hosts because sequestered toxins decrease offspring performance. Alternatively, *P. cassotis* females may be less likely to detect or recognize hosts reared on *A. curassavica*, perhaps because these parasitoids might have rarely encountered monarchs reared on this plant in their evolutionary history, as it is not native to monarchs' core breeding range (Batalden and Oberhauser 2015). Either mechanism could cause host plant-dependent rates of parasitism, independent of variation in the physiological suitability of hosts due to host plant.

Mortality rates for monarch eggs and larvae have been investigated in the lab and field by various research groups (Nail et al. 2015 and citations therein). The causes and frequency of monarch pupal mortality, however, are poorly understood because pupae are difficult to locate in the field. Monarch pupal mortality due to *P. cassotis* has been measured by placing groups of pupae in the field in the northern US, and via opportunistic collection of naturally occurring pupae in the southern US (Oberhauser et al. 2015; Stenoien et al. 2015). These studies have found rates of parasitism of 0–100%, with a great deal of temporal and spatial variability (Stenoien et al. 2015). More geographically extensive studies are needed to understand the population-level effects of pupal parasitoids on monarch mortality.

Although *P. puparum* had been anecdotally reported to unsuccessfully attack monarchs in the field (Ramsay 1964), our study is the first to verify the incompatibility of monarchs and *E. core* as hosts for this parasitoid. In addition to the experiments presented here, we have also exposed monarchs to up to five *P. puparum* females simultaneously and with *P. cassotis*, all of which resulted in failed parasitism by *P. puparum* (Stenoien, unpublished). Although attempted parasitism may be difficult to detect in the field, it could have important negative consequences for host and parasitoid populations (Abram et al. 2016; 2018).

Few studies have examined the roles of monarch diet on their palatability or nutritional quality for invertebrate natural enemies. We found that the consumption of more toxic host plants diminished the quality of monarchs as hosts for *P. cassotis* as measured by brood size, survival to emergence, and brood size in the next generation. The outcomes for *P. cassotis* coincide with a study of monarch parasitism by tachinid flies in which greater concentrations of dietary cardenolides did not affect the likelihood of successful parasitism or host death but resulted in decreased parasitoid brood size and survival (Hunter et al. 1996). Other parasitoids have occasionally been reported to reproduce in monarchs, including *Brachymeria ovata* (Halstead 1988) and *Trichogramma minutum* in North America (Peck 1963), as well as *Brachymeria lasus* in Australia (Zalucki and Freebairn

1982). Given the intensity with which monarch larvae across North America are monitored for parasitism by citizen scientist contributors to the Monarch Larva Monitoring Project, it seems likely that parasitism by *B. ovata* and *T. minutum* is truly rare, and not simply rarely observed (MLMP 2018). Still, it would be interesting to know whether monarchs' suitability for these parasitoids also varies with host plant.

Previous studies have attempted to determine the role of host plant chemistry in interactions between *Pteromalus spp.* and their hosts. Reudler and van Nouhuys (2018) sought to test whether host plants of iridoid glycoside-sequestering *Melitaea* butterflies affect *Pteromalus apum* performance. However, because concentrations within host pupae were similar, even when reared on plants that varied significantly in iridoid glycoside concentrations, they could not determine whether iridoid glycosides influence this parasitoid. The performance of *P. puparum* has been shown to be affected by the strain of host plant (*Brassica oleracea*) consumed by its *Pieris brassicae* hosts (Harvey et al. 2011). These plants vary in the concentrations of various glucosinolates, and the performance of *P. puparum* was negatively correlated with the concentration of at least one glucosinolate found in the plants tested, even though *P. brassicae* does not sequester these compounds (Harvey et al. 2011).

Like these previous studies, ours also failed to fully answer whether host plant chemistry influences interactions between *P. puparum* and potential hosts because *P. puparum* failed to develop in all hosts, including cardenolide-free *E. core*. Perhaps monarchs and *E. core*, which are in the same subfamily, have similar immunological defenses or endogenously produced unidentified cardioactive compounds (Rothschild et al. 1978; Malcolm and Rothschild 1983) which *P. puparum* cannot overcome.

Our finding that monarch cardenolides influence the success of *P. cassotis* contribute to a growing body of evidence that herbivore-sequestered plant toxins can negatively affect parasitoid performance (Barbosa et al. 1986; Ode 2006; Harvey et al. 2007a, b; Gols and Harvey 2009), but why some interspecific interactions are more affected than others remains an open question. In the case of these parasitoids, *P. puparum* has been reported to use at least 48 host species, while *P. cassotis* has been reported from only eight host species, most commonly monarch butterflies (Muesebeck et al. 1951; Peck 1963; Burks 1975; Burks 1979; California Academy of Sciences Entomology General Collection Database 2015; Noyes 2017). In addition to the frequency of monarchs in host records, a field study demonstrating positive correlations between monarch and *P. cassotis* population dynamics (Stenoien et al. 2015), and foraging studies conducted with other host species (Stenoien, unpublished) suggest that *P. cassotis* might be a specialist on monarchs (and perhaps other milkweed butterflies). If so, the evolutionary trade-offs hypothesis (MacArthur and Connell 1966; Levins 1968; Wilson and Yoshimura 1994; Asplen et al. 2012) might explain why *P. cassotis* can develop in milkweed butterflies containing sequestered or endogenously produced cardioactive compounds while *P. puparum* cannot. For parasitic organisms, this hypothesis predicts that specialists should be more likely to adapt to host-specific defenses than generalists (Gauld et al. 1992; Gauld and Gaston 1994). In support of this hypothesis, host records, field studies, and lab studies indicate that many parasitoids which attack toxic herbivores are specialists on these hosts (Bernays and Graham 1988; Stireman and Singer 2003; Harvey et al. 2005). Several experimental studies have also suggested that generalist parasitoids are often more susceptible to variation in host plant allelochemistry than specialist parasitoids (Campbell and Duffey 1979, 1981; Barbosa et al. 1991; El-Heneidy et al. 1988; Lampert et al. 2011; Reudler et al. 2011). We found the performance of the apparent specialist to be noticeably affected by variation in cardenolide concentrations within monarch hosts. The generalist, however, was unable to develop in any type of host, indicating that other mechanisms prevent the development of *P. puparum*, either independently or, perhaps, in conjunction with cardenolides.

A proximate explanation for *P. cassotis'* ability to parasitize monarchs is that they are able to minimize (e.g. excrete, sequester, degrade) the negative effects of cardioactive compounds. For example, *P. cassotis* might have a modified midgut or sodium–potassium pumps, like many specialized insect herbivores of Asclepiads (Després et al. 2007; Dobler et al. 2012). If so, such genetic modifications would represent convergent evolution in response to a plant toxin across multiple trophic levels.

Our study is the first to demonstrate the suitability of *E. core* as a host for *P. cassotis*. This novel host seemed comparable or even better than monarchs for the performance of *P. cassotis*, based on their rates of success and brood characteristics. Unfortunately, due to their foreign status, we were unable to test *E. core* in the same laboratory space as the monarch research, obviating any statistical comparisons between hosts. *Pteromalus cassotis* has never been found outside of continental North America, nor as a parasitoid of milkweed butterflies other than the monarch. However, given its performance on this novel danaid, it is plausible that parasitism of other North American danaids (such as *Danaus gilippus* or *Danaus eresimus*) occurs, but has yet to be been documented. Accidental introductions of *P. cassotis* to regions such as the Caribbean, Africa, or tropical Asia could harm native milkweed butterfly populations.

Our comparison of monarch and *E. core* hosts aimed to clarify the role of cardenolides in milkweed butterflies' interactions with potential parasitoids. We tested three cardenolide concentrations but recognize that Asclepiads and their specialized herbivores also vary in the types of cardenolides present. Cardenolides differ in their sidechains

and polarity, and these characteristics might influence sequestration and the outcomes of host–parasitoid interactions (Agrawal et al. 2012). Intraspecific manipulations of nutrient, light, or water availability to a single species of host plant could be used to modify total foliar cardenolides, though they should be done with care because manipulations such as these can also generate considerable variation in the types of cardenolides present, carbon:nitrogen ratio, and water content (Couture et al. 2010; Agrawal et al. 2012). Alternatively, greater concentrations of foliar cardenolides might be further tested by rearing monarchs on even more toxic milkweeds such as *A. masonii* or *A. albicans* (Malcolm 1991). Finally, future research should include a broader representation of milkweed butterflies that vary in cardenolide sequestration, such as *Danaus petilia* (Stoll), a congener of monarchs whose adults have been found to contain little to no sequestered cardenolides (Nelson 1993). Studies such as these would clarify the extent to which cardenolides influence the broader ecology of milkweeds, milkweed butterflies, and their natural enemies.

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