Journal of Animal Ecology 2008, **77**, 120–126 doi: 10.1111/j.1365-2656.2007.01305.x

Host plant species affects virulence in monarch butterfly parasites

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Summary

1. Studies have considered how intrinsic host and parasite properties determine parasite virulence, but have largely ignored the role of extrinsic ecological factors in its expression.

2. We studied how parasite genotype and host plant species interact to determine virulence of the protozoan parasite *Ophryocystis elektroscirrha* (McLaughlin & Myers 1970) in the monarch butterfly *Danaus plexippus* L. We infected monarch larvae with one of four parasite genotypes and reared them on two milkweed species that differed in their levels of cardenolides: toxic chemicals involved in predator defence.

3. Parasite infection, replication and virulence were affected strongly by host plant species. While uninfected monarchs lived equally long on both plant species, infected monarchs suffered a greater reduction in their life spans (55% vs. 30%) on the low-cardenolide vs. the high-cardenolide host plant. These life span differences resulted from different levels of parasite replication in monarchs reared on the two plant species.

4. The virulence rank order of parasite genotypes was unaffected by host plant species, suggesting that host plant species affected parasite genotypes similarly, rather than through complex plant species–parasite genotype interactions.

5. Our results demonstrate that host ecology importantly affects parasite virulence, with implications for host–parasite dynamics in natural populations.

Key-words: Apicomplexa, *Asclepias*, evolution of virulence, pathogenicity, tritrophic interaction.

Introduction

Many parasites cause harm to their hosts (i.e. virulence), and hence play an important role in host ecology and evolution (e.g. Dobson & Hudson 1986). Many studies have shown that virulence is a genetically determined parasite trait (e.g. Diffley *et al*. 1987; Mackinnon & Read 1999), and that its expression depends on specific interactions between host and parasite genotypes (e.g. Carius, Little & Ebert 2001; Grech, Watt & Read 2006; Lambrechts, Fellous & Koella 2006). How host ecology determines virulence, however, is less well understood (but see Brown, Loosli & Schmid-Hempel 2000; Thomas & Blanford 2003; Mitchell *et al.* 2005).

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Recent studies on tritrophic interactions between pathogens, phytophagous insects and their larval food plants have highlighted the role of environmental factors on parasite infection and virulence (reviewed in Cory & Hoover 2006). For example, studies on baculoviruses have shown that both host mortality rate (e.g. Keating & Yendol 1987; Forschler, Young & Felton 1992; Farrar & Ridgway 2000) and viral replication (Ali *et al*. 2002; Raymond *et al*. 2002) can vary depending on the host plant on which infection occurs. Most of these studies, however, have focused on single isolates of viruses, and hence provided limited insights into how host plant species and distinct parasite genotypes can interact to determine virulence phenotypes in the host.

Here, we ask how parasite genotype and host plant species affect virulence caused by the protozoan parasite *Ophryocystis elektroscirrha* in monarch butterflies (*Danaus plexippus*). Monarchs are commonly exposed to parasite genotypes differing in virulence (De Roode, Gold & Altizer 2007), and feed on milkweed species that differ in levels of cardenolides: secondary compounds that monarchs can sequester and use

in predator defence. We compare four parasite genotypes on two common host plant species that differ dramatically in their cardenolide levels, and provide evidence that host plant properties play an important role in determining parasite virulence.

Materials and methods

THE HOST–PARASITE SYSTEM

In North America, monarch butterflies occur in several populations. The best known is the population in eastern North America that migrates annually to overwintering sites in central Mexico (Brower 1995). A second, non-migratory population inhabits South Florida (Ackery & Vane-Wright 1984; Brower 1995; Knight 1998). Monarchs can use at least 27 milkweed species as their larval host plants (Ackery & Vane-Wright 1984; Malcolm & Brower 1989; Vickerman & de Boer 2002) and encounter different species in different populations. For example, monarchs in the eastern North American population frequently encounter *Asclepias incarnata* L. (swamp milkweed), whereas a common host plant in South Florida is *A. curassavica* L. (tropical or red butterfly milkweed) (Woodson 1954).

Ophryocystis elektroscirrha (McLaughlin & Myers (1970) is an apicomplexan protozoan that infects monarchs throughout their range (Leong, Yoshimura & Kaya 1997; Altizer, Oberhauser & Brower 2000). Parasite prevalence differs between monarch populations, with less than 5% of monarchs from the eastern North American population infected, and up to 80% of monarchs from South Florida (Leong *et al*. 1997; Altizer *et al*. 2000). Infections occur when caterpillars ingest spores from contaminated eggs or leaves of the host plant; spores lyse in the larval gut and parasites penetrate the intestinal wall to undergo asexual replication in the larval hypoderm. After host pupation, the parasite undergoes a sexual phase and forms spores around the scales of the developing host, such that adult butterflies emerge covered with spores on the outsides of their bodies. Parasites do not continue to replicate on adults, and spores must be ingested by larvae to cause new infections.

HOST, PARASITE AND HOST PLANT SOURCES

Monarchs used in this study were the F_1 offspring of four genetic crosses between monarch lineages from the eastern North American population. Parental lineages consisted of wild-caught monarchs collected in Georgia and Delaware (USA) in August 2006 and laboratory-reared great-grand progeny of wild-caught monarchs obtained from Georgia in April 2006. They were mated in 0·6 m^3 mesh cages, held at 26·3 °C (min 23·9–max 29·0) and 47% relative humidity (RH) (41–61) and fed *ad libitum* with a 10% honey solution. Mated females within a genetic cross were pooled into a single cage and provided with greenhouse-grown potted *A. incarnata* plants for oviposition.

We used four parasite genotypes, which were the progeny of parasite spores harvested either directly from wild-caught butterflies or from laboratory-reared butterflies infected with wild-collected parasites. Parasites were cloned to avoid the use of mixed-genotype parasite inocula in the experiment. They were denoted E1 (eastern North America population: Ithaca, New York, USA) and E11 (eastern North America population: Mt Lake, Virginia, USA), and F3 and F13 (South Florida population: Miami, USA).

Monarch larvae were reared from hatching to pupation on either *A. incarnata* or *A. curassavica*. Plant seeds were obtained from Butterfly Encounters (Dublin, California, USA), and over 300 plants of each species were grown in individual pots under identical conditions in the same room of a greenhouse. Plant species were chosen for their marked difference in cardenolide levels, with *A. curassavica* reported to have much higher concentrations than *A. incarnata* (Malcolm & Brower 1986; Zalucki, Brower & Malcolm 1990). Apart from this difference in cardenolides, the two plant species are very similar in terms of height and leaf morphology (Woodson 1954).

EXPERIMENTAL DESIGN

The experiment was fully factorial, with infection treatment (E1, E11, F3, F13 or control) and host plant species (*A. incarnata* or *A. curassavica*) as experimental factors. Initially, each parasite clone by host plant species treatment included 30 replicate larvae, resulting in a total of 300 larvae. Larvae were assigned randomly to treatment groups. Host sex was determined upon emergence of adult butterflies and was also included as a factor in the analyses.

Experimental larvae were obtained from eggs laid by mated females on *A. incarnata*. To ensure that larvae encountered their assigned host plant species immediately upon hatching, we transferred eggs or hatching larvae manually to leaves of either host plant species. Two days after hatching, when larvae had reached the second instar, they were transferred to individual 10-cm Petri dishes for parasite inoculation. These dishes contained moist filter papers and 1 cm^2 pieces of milkweed onto which we had deposited 10 parasite spores manually.

Once larvae had completely eaten their milkweed pieces – and hence ingested the full inoculum of parasites – they were transferred to individual 0·47-litre plastic containers with meshed lids. Containers were lined with moist paper towels and caterpillars provided with fresh milkweed cuttings. Containers were checked daily and fresh milkweed cuttings and towels provided as needed; over the course of the experiment, each caterpillar received milkweed shoots from several individual plants. Once monarchs had been in the pupal stage for 7 days (an average of 2 days before adult emergence), they were transferred to a different laboratory, maintained at 23·8 °C (min 22–max 24·8) and 53% RH (min 40–max 70). This was performed to avoid risk of parasite contamination of the larval rearing laboratory by emerging infected adults. Upon emergence, adult monarchs were transferred to individual glassine envelopes and held in a 14 °C controlled environment chamber.

INFECTION AND VIRULENCE MEASUREMENTS

We quantified the proportion of animals that became infected to determine the infection success of the different parasite genotypes on larvae reared on the two host plant species. As a measure of parasite replication, we quantified the parasite spore loads of adult butterflies (as described in De Roode *et al*. 2007).

To quantify virulence, we calculated adult life spans as the difference (in days) between the day of emergence and the day of death (see De Roode *et al*. 2007). We also compared monarch mortality, adult weight and development times among parasite genotypes and host plants. Adult weight was measured to the nearest mg 1 day after emergence, and development time was estimated both as the number of days between hatching and pupation and the number of days between hatching and adult emergence.

CARDENOLIDE MEASUREMENTS

During larval development, each caterpillar received shoots and leaves from multiple plant individuals within a single milkweed species. To assess the average cardenolide chemistry of these species,

leaves were collected from 32 plants of each species during the rearing process (64 samples total), freeze-dried and ground (after being held at –80 °C for 6 weeks). Four mL of 100% methanol was added to 200 mg of ground leaf material, and samples were vortexed, sonicated for 30 min and left overnight at 55 °C. Samples were then vortexed again and centrifuged. Supernatants were decanted and held to one side. An additional 2 mL of 100% methanol was then added to the remaining precipitate, which was vortexed, centrifuged and decanted to give a final extracted volume of 6 mL. Samples were dried at 70 °C and resuspended in 1 mL of 100% methanol containing 0·15 mg/mL of the cardenolide digitoxin (Sigma Chemical Company, St. Louis, Missouri, USA) as an internal standard for quantification of foliar cardenolides. Two samples from *A. currassavica* were lost during sample preparation, leaving 30 samples of this species for final analysis.

Cardenolide concentrations were estimated for each plant sample by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC with PDA detector (Waters Corporation, Milford, MA, USA). The UPLC is a capillary HPLC system that works under high pressures (up to 15 000 psi) and reduces run times for cardenolides from over 60 min to under 10 min per sample. Methods followed the solvents and gradients in Malcolm & Zalucki (1996), calibrated for shorter run times.

STATISTICAL ANALYSIS

All analyses were carried out in R version 2·4·0 (R Development Core Team 2006) using generalized linear models (GLM). Proportions of animals that died or became infected were analysed using GLM with binomial error distributions, while numbers of chemically distinct cardenolides in host plants were analysed using GLM with Poisson error distributions; cardenolide concentrations, monarch development time, parasite spore load and monarch adult longevity were analysed using GLM with normal error distributions. Models were checked for normality of error distributions and homogeneity of variance. Cardenolide concentrations and parasite spore loads were log-transformed prior to analysis. Full models included infection treatment, host plant species and monarch sex as explanatory variables, and all relevant interactions between them. Minimal models were derived by removing model terms followed by model comparison. Only terms for which removal significantly ($P < 0·05$) reduced the explanatory power of the model were retained in the minimal model (Crawley 2002).

Results

CARDENOLIDES

Total cardenolide concentrations were on average 13-fold higher in *A. curassavica* than in *A. incarnata* (Fig. 1a; $F_{1,60} = 293$, $P < 0·001$). Additionally, *A. curassavica* foliage contained a much larger number of chemically distinct cardenolides than did the foliage of *A. incarnata* ($\chi^2_{1,60} = 236$, $P < 0·001$). Some *A. curassavica* plants harboured up to 22 distinct cardenolides, whereas most *A. incarnata* plants harboured only a single cardenolide (Fig. 1b).

Under reverse-phase HPLC, polar compounds elute earlier than do non-polar compounds. Using retention time as an estimate of polarity, the dominant cardenolide in *A. incarnata* was highly polar, whereas *A. curassavica* contained a broad range of cardenolides ranging from highly polar to highly non-polar (Fig. 1b).

PRE-ADULT MORTALITY AND DEVELOPMENT

A total of 65 monarchs died before reaching the adult stage; this mortality did not differ by host plant species ($\chi^2_{1,8} = –0·92$, $P = 0·34$) or parasite infection, there being no difference in the proportion of monarchs that died between infection treatment groups ($\chi^2_{4,5} = 2·6$, $P = 0·63$). The remaining analyses are restricted to the 235 individuals that survived to the adult stage.

There were no differences in development time for monarchs reared on the two host plant species, whether development time was measured as the time between hatching and pupation ($F_{1,229} = 0·29$, $P = 0·59$) or between hatching and adult emergence ($F_{1,228} = 3·2$, $P = 0·074$). However, adult monarchs weighed more when reared on *A. curassavica* than on *A.*

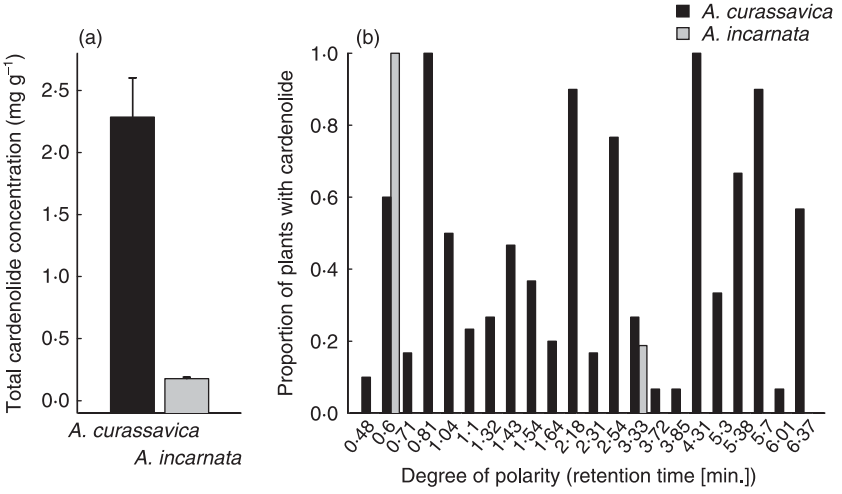

Fig. 1. (a) Total concentration of foliar cardenolides of *A. curassavica* and *A. incarnata*; bars show mean + 1 SEM. (b) Relative polarity, estimated by retention time under reverse-phase HPLC, of foliar cardenolides from *A. curassavica* and *A. incarnata*. Increasing retention times represent decreasing polarity. Bars represent the proportion of plants that produced a cardenolide of a given polarity. Sample sizes are 30 and 32 plants for *A. curassavica* and *A. incarnata*, respectively.

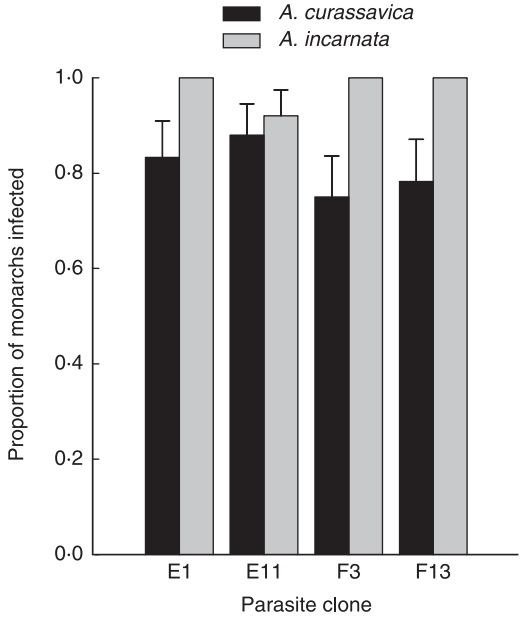

Fig. 2. Proportions of monarchs that became infected (± 1 SEM) when inoculated with parasite clones E1, E11, F3 or F13. Monarch larvae were reared on either *A. curassavica* or *A. incarnata*.

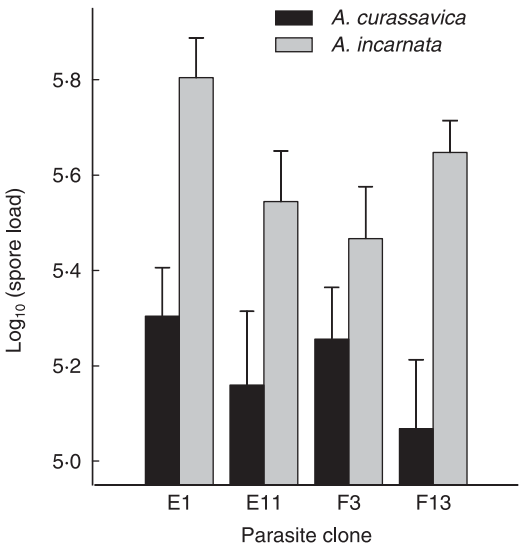

Fig. 3. Parasite spore loads on monarchs infected with parasite clones E1, E11, F3 or F13. Monarch larvae were reared on either *A. curassavica* or *A. incarnata*. Bars show mean + 1 SEM.

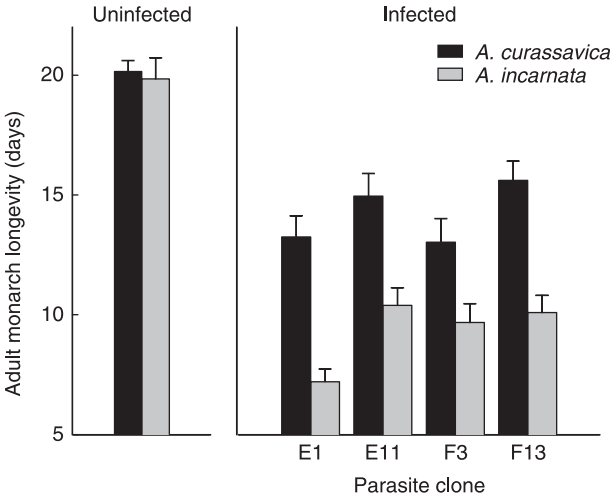

Fig. 4. Adult life span of monarchs that were uninfected or infected with parasite clones E1, E11, F3 or F13, and reared on either *A. curassavica* or *A. incarnata*. Uninfected monarchs consist of control animals – which were not inoculated with parasites – and animals that were inoculated but did not become infected. Bars show mean + 1 SEM.

incarnata ($F_{1,231}$ = 34·8, P < 0·001), and this was true for all infection treatments (infection treatment × host plant species not significant).

PROPORTION INFECTED AND PARASITE INFECTION LOADS

None of the control monarchs became infected, whereas between 75 and 100% of inoculated individuals did. For all parasite clones and both sexes, more monarchs became infected when inoculated and reared on *A. incarnata* than on *A. curassavica* (Fig. 2; $\chi^2_{1,6}$ = 14·9, P < 0·001).

Monarchs also became more heavily infected when reared on *A. incarnata* (Fig. 3): among infected individuals, monarchs reared on *A. incarnata* had on average 2·6 times as many parasites as those reared on *A. curassavica* ($F_{1,162}$ = 28·7, P < 0·001), and this was true for all parasite genotypes and both host sexes (two- and three-way interactions with infection treatment and host sex non-significant). Parasite clones did not differ in the spore loads they produced ($F_{3,159}$ = 1·97, P = 0·12), but female monarchs had slightly higher spore loads than did males ($F_{1,162}$ = 4·6, P = 0·034).

VIRULENCE

Infected monarchs had shorter life spans than uninfected monarchs (Fig. 4). While there was no difference in adult life spans between uninfected monarchs reared on the two host plant species, infected monarchs lived for a shorter time when reared on *A. incarnata* than when reared on *A. curassavica* (host plant–infection treatment interaction: $F_{4,223}$ = 5·4, P < 0·001). This host plant difference occurred for both host sexes (two- and three-way interactions with host sex non-significant), although males lived an average of 1·8 days longer than females ($F_{1,223}$ = 14·0, P < 0·001). Restricting the analysis to infected animals only showed that the host plant difference was consistent across parasite genotypes: monarchs infected with different parasite genotypes had different life spans (Fig. 4: $F_{3,162}$ = 5·1, P = 0·002), but differences in life span between individuals reared on different host plant species were similar for all parasite genotypes and the virulence ranking of the parasite clones was unaltered by host plant (host

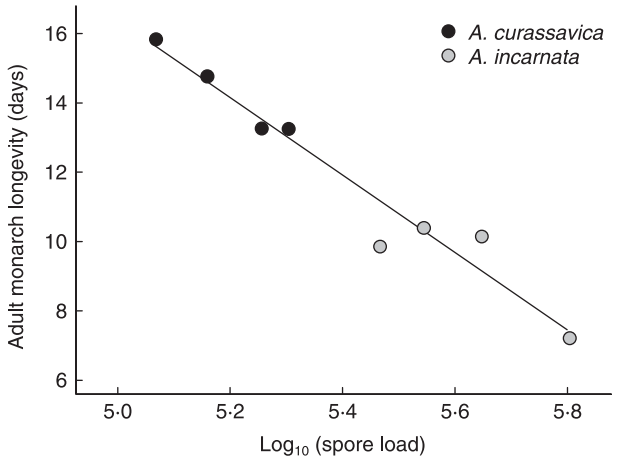

Fig. 5. Relationship between adult monarch life span and parasite spore load. Each point is the mean of a parasite clone; the line is the least-squares regression line ($R^2 = 0{\cdot}94$).

plant–infection treatment interaction: $F_{3,159} = 1{\cdot}1$, $P = 0{\cdot}36$). Comparison of the adjusted sums of squares of the minimal model showed that host plant species was a more important determinant of longevity than parasite clone (SS: 958 and 59, respectively).

To determine whether the virulence differences associated with host plant species could be attributed directly to the observed differences in parasite spore loads, we analysed the relationship between average spore load and monarch longevity for each clone on each host plant species (Fig. 5). In this analysis, mean longevity decreased strongly with increasing mean spore load ($F_{1,6} = 113$, $P < 0{\cdot}001$), while host plant and parasite clone were not significant. Thus the different spore loads of monarchs reared on the two host plant species explained the observed differences in adult life spans.

Discussion

Our results show that host ecology affects parasite infection, replication and virulence. Infected adult monarch butterflies had higher parasite loads and lived for a shorter time when reared on *A. incarnata* than when reared on *A. curassavica*. Although parasite clones varied in the levels of virulence they caused, host plant species was a more powerful determinant of virulence than parasite genotype. Importantly, the relative differences in virulence associated with host plant species were similar for all parasite genotypes. Thus, host plant species affected the expressed virulence of parasites irrespective of their genotype, rather than through a complex interaction between specific genotypes and host plants (cf. Hodgson *et al*. 2002).

Virulence modulation by host plant species could affect many host–parasite processes, including parasite virulence evolution. For example, if host longevity is an important determinant of parasite transmission, it is possible that plants on which monarchs suffer reduced life spans could select against highly virulent parasite genotypes. This is because a combination of highly virulent pathogens and host plants that lead to expression of high virulence could result in very short host life spans, which would reduce parasite fitness through a reduction in transmission opportunities. If, on the other hand, high parasite spore load is more important for parasite transmission than host longevity, such plants would not select against highly virulent parasites. How host plant species affect virulence evolution depends therefore on the degree to which host longevity and parasite spore load ultimately determine parasite fitness.

Our results also have implications for parasite local adaptation, as they suggest that environmental conditions may strongly determine parasite infection rates and virulence and, with that, parasite fitness. Local adaptation studies often use cross-infection experimental designs to test whether parasite infection rates are higher on sympatric than allopatric host–parasite combinations, and many have found that this is indeed the case (e.g. Lively 1989; Ebert 1994; Thrall, Burdon & Bever 2002). However, other studies have not found such patterns. Although there are many potential reasons for this (Kaltz & Shykoff 1998; Lively 1999; Greischar & Koskella 2007), the lack of such patterns could be caused by the environmental conditions under which testing occurs. Thus, if parasites are adapted to local host genotypes in particular local environments, then cross-infection studies that are carried out under environmental conditions different from local environments could obscure parasite local adaptation.

In contrast with previous experiments (Erickson 1973; Zalucki, Brower & Alonso 2001; Ladner & Altizer 2005), we found no host plant species effects on monarch survival in the absence of infection. However, monarchs weighed more when reared on *A. curassavica* than when reared on *A. incarnata*. If plant species also affected general host condition, this may have resulted in stronger antiparasite defence and immunity and hence lower parasite burdens. Alternatively, the reduced infection rates and parasite burdens on *A. curassavica* could have resulted from plant chemicals interfering with initial parasite infection or later stages of replication and spore production. Plant chemicals – such as oxidizing agents and tannins – can reduce infection rates of insect baculoviruses (Felton & Duffey 1990; Keating, Hunter & Schultz 1990), and it is possible that *A. curassavica* contains higher levels of parasite-inhibiting chemicals than *A. incarnata*.

One obvious candidate group of such chemicals is the cardenolides, which occurred in greater variety and higher concentrations in *A. curassavica* than in *A. incarnata*. Monarchs reared on *A. curassavica* should have encountered and sequestered higher levels of these chemicals than those reared on *A. incarnata*, as past work has demonstrated a positive relationship between cardenolide concentrations in plants and monarch tissues (Malcolm & Brower 1989). Notably, *A. curassavica* contained several highly non-polar cardenolides whereas the dominant cardenolide in *A. incarnata* was highly polar. Because non-polar cardenolides are more likely to move across membranes (Fordyce & Malcolm 2000), it is possible that the non-polar cardenolides in *A. curassavica* played a role in inhibiting parasite growth. A comparison of two plant species is insufficient to correlate virulence differences with

cardenolides directly, but future work will pursue the role that these chemicals play in modulating parasite virulence. Cardenolides have often been implicated as predator-deterring chemicals (Brower *et al*. 1968; Malcolm & Brower 1989; Malcolm 1991), and it is an interesting possibility that these chemicals may also confer protection against disease: the ability of monarchs to sequester these chemicals may then serve more than one purpose.

To our knowledge, our study is the first to demonstrate that host plant species can affect the virulence of protozoan parasites. Together with studies on viruses (Cory & Hoover 2006), bacteria (Kouassi *et al*. 2001; Ali, Young & McNew 2004) and nematodes (Barbercheck, Wang & Hirsh 1995), these results provide evidence that host diet is an important determinant of parasite fitness. In the current study, host plant effects were greater than those caused by parasite genotypes, suggesting that host plant species may not just *affect*, but indeed *drive* the ecology and evolution of this monarch–parasite system. More generally, our results suggest that host ecology is an important determinant of parasite fitness and host–parasite interactions.

Acknowledgements

We thank Jean Chi, Natalie Kolleda, Byron Ledbetter, Rachel Rarick, Julie Rushmore and Star Scott for help with the experiment, Melanie Smith, Michael Boyd and Andy Tull for help with growing and maintaining plants, and Catherine Bradley, Kristin Harper, Daniel Streicker, Helen Wearing, Jamie Winternitz and two anonymous reviewers for constructive comments on the manuscript. The work was supported by the University of Georgia and a Marie Curie Outgoing International Fellowship (FP6-2004-Mobility-6, Proposal no. 021353) to J.C.dR.

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Received 20 March 2007; accepted 31 July 2007
Handling Editor: Mike Boots