Behavioural resistance against a protozoan parasite in the monarch butterfly

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Summary

1. As parasites can dramatically reduce the fitness of their hosts, there should be strong selection for hosts to evolve and maintain defence mechanisms against their parasites. One way in which hosts may protect themselves against parasitism is through altered behaviours, but such defences have been much less studied than other forms of parasite resistance.

2. We studied whether monarch butterflies (*Danaus plexippus* L.) use altered behaviours to protect themselves and their offspring against the protozoan parasite *Ophryocystis elektroscirrha* (McLaughlin & Myers (1970), *Journal of Protozoology*, **17**, p. 300). In particular, we studied whether (i) monarch larvae can avoid contact with infectious parasite spores; (ii) infected larvae preferentially consume therapeutic food plants when given a choice or increase the intake of such plants in the absence of choice; and (iii) infected female butterflies preferentially lay their eggs on medicinal plants that make their offspring less sick.

3. We found that monarch larvae were unable to avoid infectious parasite spores. Larvae were also not able to preferentially feed on therapeutic food plants or increase the ingestion of such plants. However, infected female butterflies preferentially laid their eggs on food plants that reduce parasite growth in their offspring.

4. Our results suggest that animals may use altered behaviours as a protection against parasites and that such behaviours may be limited to a single stage in the host–parasite life cycle. Our results also suggest that animals may use altered behaviours to protect their offspring instead of themselves. Thus, our study indicates that an inclusive fitness approach should be adopted to study behavioural defences against parasites.

Key-words: *Asclepias*, avoidance, *Danaus plexippus*, disease ecology, milkweed, monarch butterfly, *Ophryocystis elektroscirrha*, protozoan parasite, self-medication

Introduction

Free-living organisms are constantly confronted with a wide range of parasite species that can impose serious fitness costs, including reduced growth, reproduction and survival. Natural selection should therefore strongly favour host protective strategies to prevent and/or limit parasite infection (Combes 2001). The physiological immune system is perhaps the best-known mechanism that hosts use against parasitism and functions to limit parasite infection, growth and virulence when parasites have already made contact with or invaded the host body. Although the physiological immune system provides a major line of defence, it is not the only way in which hosts protect themselves against parasites. In particular, hosts may gain protection and/or minimize parasite-induced fitness loss by harbouring symbiotic bacteria, by sequestering protective chemicals from their diet and by changing their timing of reproduction (Parker *et al.* 2011). Moreover, it is often suggested that animals use behavioural mechanisms to avoid parasites, reduce infection risk and slow down parasite growth (Hart 1990). However, evidence for such behavioural defences remains relatively rare (Moore 2002).

To date, most studies on host behavioural defences have focused on disease avoidance, and the ability of hosts to detect and avoid contact with infective parasite stages has

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been documented across a broad range of taxa (Hart 1994; Moore 2002). For instance, healthy spiny lobsters are able to avoid conspecifics infected with a directly transmitted virus (Behringer, Butler & Shields 2006); herbivorous mammals display selective grazing to avoid faecal patches containing worm larvae (Hutchings *et al.* 2001; Ezenwa 2004); and gypsy moth caterpillars can avoid virus-contaminated foliage (Parker, Elderd & Dwyer 2010).

Hosts will not always manage to avoid contact with parasites, and another potential defence mechanism is self-medication: a series of behaviours by which hosts exploit additional species or compounds that prevent or reduce infection, whether mediated through defensive or nutritional properties (Clayton & Wolfe 1993; Lozano 1998; Hart 2005; Raubenheimer & Simpson 2009). Self-medication can be prophylactic when displayed by both uninfected and infected individuals to prevent infection. Wood ants, for example, incorporate pieces of conifer resin into their nests, and this resin inhibits the growth of bacteria and fungi (Christe *et al.* 2003; Chapuisat *et al.* 2007; Castella *et al.* 2008). Self-medication can also be therapeutic when used by already infected individuals. Although several correlative studies have suggested that great apes use therapeutic self-medication (Wrangham & Nishida 1983; Phillips-Conroy 1986; Huffman & Seifu 1989), the strongest experimental evidence for such behaviour comes from studies of phytophagous Lepidoptera. Some lepidopteran larvae actively change the composition of their diet, ingesting anti-parasitic plant toxins or altering their nutritional intake to fight their parasites (Lee *et al.* 2006; Povey *et al.* 2009; Singer, Mace & Bernays 2009).

Recently, we have shown that western North American monarch butterflies (*Danaus plexippus*) preferentially lay their eggs on anti-parasitic milkweed when infected with the protozoan parasite *Ophryocystis elektroscirrha*, thereby reducing parasite infection and virulence in their offspring (Lefèvre *et al.* 2010). Here, we follow up from that work to determine whether such trans-generational medication is common across multiple monarch populations and whether monarchs are also able to defend themselves against parasites using additional behaviours.

Ophryocystis elektroscirrha (McLaughlin & Myers 1970) occurs throughout the distribution of monarch butterflies, including in the geographically separated populations in western and eastern North America (Leong *et al.* 1997; Altizer, Oberhauser & Brower 2000; Altizer 2001). Parasite infection occurs when larvae ingest infective spores deposited on the egg shells and milkweed foliage by infected females during oviposition. Newly hatched monarch caterpillars, like many other larvae of plant-feeding Lepidoptera, often feed initially on their chorion (Nielsen & Common 1991) and can become infected this way (De Roode, Gold & Altizer 2007). Additionally, monarch caterpillars can become infected when ingesting parasite spores with milkweed foliage (De Roode, Gold & Altizer 2007). Upon ingestion, spores lyse in the larval gut to release sporozoites that traverse the midgut wall and invade the host's hypoderm (McLaughlin & Myers 1970). Here, the parasite undergoes vegetative asexual replication before completing sexual reproduction during monarch pupation. Upon eclosion, adult butterflies emerge from the chrysalis covered with infective parasite spores on the outside of their bodies (Leong *et al.* 1992). These spores undergo no further replication and must be ingested by larvae to cause new infections. *Ophryocystis elektroscirrha* has strong detrimental effects on monarch fitness by reducing adult life span, mating ability, fecundity and flight ability (Bradley & Altizer 2005; De Roode, Gold & Altizer 2007; De Roode, Yates & Altizer 2008; De Roode *et al.* 2009).

Given the fitness costs of *O. elektroscirrha* infection, we hypothesized that monarch butterflies would benefit greatly from behavioural defensive mechanisms against this parasite and identified several distinct ways in which such defences may be achieved. First, monarch larvae may actively avoid infection by preventing the consumption of contaminated egg shells or contaminated milkweed foliage (avoidance behaviours 1 and 2 in Fig. 1). Second, monarch larvae may actively medicate themselves by ingesting therapeutic food plants. Monarchs form a tight association with their milkweed larval food plants (Ackery & Vane-Wright 1984), and previous studies have shown that some milkweed species can strongly reduce the probability of parasite infection and subsequent parasite reproduction (De Roode *et al.* 2008, 2011a,b; Lefèvre *et al.* 2010). Although the precise mechanism of parasite inhibition by milkweed plants is not yet clear, previous work (De Roode *et al.* 2008, 2011a,b) has implicated cardenolides, toxic steroids produced by many milkweed species (Agrawal & Fishbein 2006), as potential anti-parasitic agents. In particular, parasites produced lower spore loads and caused lower virulence on monarchs reared on a milkweed species with high cardenolide concentrations (the tropical milkweed *Asclepias curassavica*) than on a species with low concentrations (the swamp milkweed *Asclepias incarnata*) (De Roode *et al.* 2008, 2011a,b; Lefèvre *et al.* 2010). Hence, it is possible that infected monarch larvae preferentially consume high-cardenolide anti-parasitic milkweeds or, in the absence of choice, ingest a larger quantity of anti-parasitic milkweed foliage than uninfected counterparts (self-medication behaviours 1 and 2 in Fig. 1). Third, infected female butterflies may preferentially lay their eggs on anti-parasitic milkweed (trans-generational medication behaviour in Fig. 1). Adult butterflies cannot cure themselves of their parasites, nor can they avoid parasite transmission to their offspring. However, by preferentially laying their eggs on anti-parasitic milkweeds, females may reduce the infection probability of their offspring as well as the parasite growth in infected offspring.

We have previously found that caterpillars are unable to self-medicate when offered a limited choice between two milkweed species that differ in their anti-parasitic properties but that female butterflies from a population that inhabits western North America engage in trans-generational medication (Lefèvre *et al.* 2010). Here, we test whether monarch caterpillars can avoid infective parasite spores, whether caterpillars can self-medicate when provided with a wider range of milkweed species and whether caterpillars can increase their
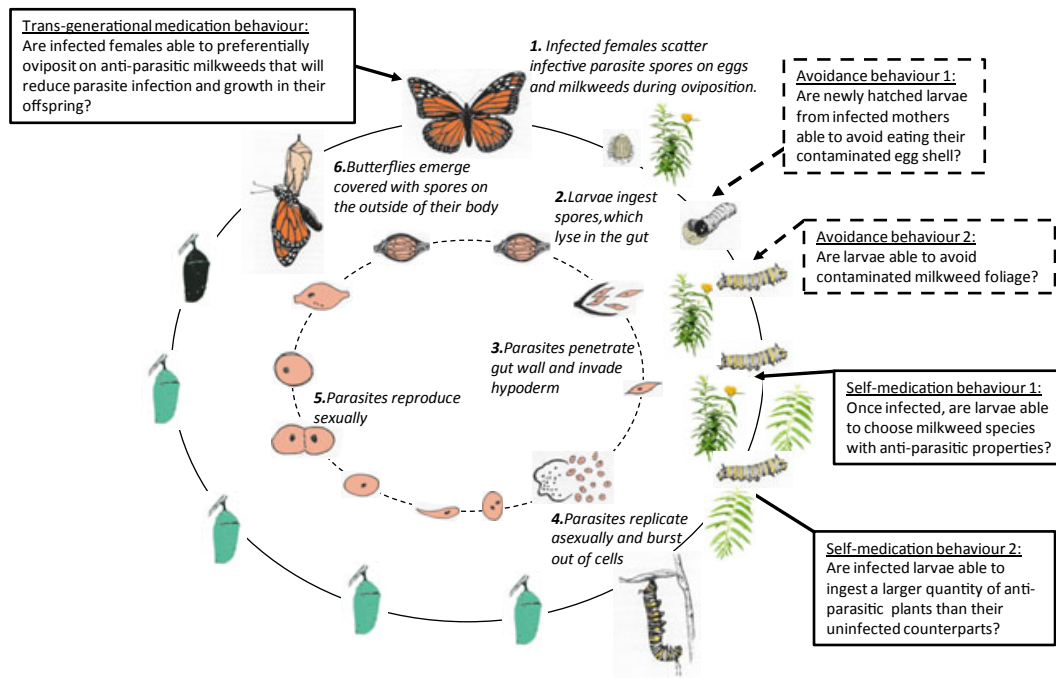
Fig. 1. Life cycle of the monarch butterfly, *Danaus plexippus* (outer circle) and its protozoan parasite, *Ophryocystis elektroscirrha* (inner circle). Text boxes represent expected monarch behavioural defences: dashed boxes indicate possible avoidance behaviours, while solid boxes indicate possible medication behaviours.

absolute consumption of anti-parasitic milkweed foliage in the absence of diet choice. Moreover, we investigate the generality of trans-generational medication by testing whether monarchs from the population that inhabits eastern North America also preferentially lay their eggs on anti-parasitic milkweed when infected. Overall, we carried out a series of experiments to: (i) determine the anti-parasitic effects of five milkweed species; (ii) test whether monarch larvae can avoid infection by avoiding parasite-contaminated egg chorion and milkweed foliage; (iii) test whether monarch larvae can self-medicate by preferentially consuming anti-parasitic milkweeds or, in the absence of choice, by ingesting greater amounts of anti-parasitic milkweed foliage; and (iv) assess whether infected monarch butterflies from eastern North America preferentially lay their eggs on anti-parasitic milkweed as do those from western North America (Lefèvre *et al.* 2010). Our results suggest that trans-generational medication is the only behavioural mechanism by which monarchs protect themselves against parasitism.

Materials and methods

HOST, PARASITE AND MILKWEED SOURCES

Monarchs used in all experiments were the laboratory-reared grand-progeny of monarchs collected from North America. Parasites used were cloned isolates derived from wild-collected parasite strains. Each experiment used sympatric host and parasite combinations. Milkweed seeds were obtained from Butterfly Encounters, CA, USA and were germinated and reared to adulthood in a climate-controlled greenhouse.

DETERMINING THE ANTI-PARASITIC PROPERTIES OF FIVE MILKWEED SPECIES

We investigated the anti-parasitic properties of five milkweed species: *Asclepias erosa* (desert milkweed), *A. curassavica* (tropical milkweed), *Asclepias syriaca* (common milkweed), *A. incarnata* (swamp milkweed) and *Asclepias tuberosa* (butterfly weed). We randomly assigned 50 monarch larvae obtained from 12 genetic lineages (obtained from Pismo Beach, CA, USA) to each milkweed species and infected 40 larvae with 10 parasite spores and left 10 larvae uninfected. Infections were performed by feeding 2-day-old larvae a milkweed leaf disc on which we had deposited 10 parasite spores (from a single parasite clone); uninfected control larvae received leaf discs without parasites. Upon consumption of their leaf disc, larvae were transferred to individual plastic containers kept at 26 °C and a 16L : 8D light cycle. Containers contained a florist tube with milkweed cuttings. Individual plants were assigned to individual larvae, such that each larva completed its development on one plant. In some cases, larvae finished their assigned plant before reaching the pupal stage, and in such cases, they received additional milkweed foliage from a different plant individual from the same species. Upon eclosion, adult monarchs were transferred into individual glassine envelopes and kept at 12 °C. We then checked daily for mortality to calculate their longevity. This measure of longevity provides a combined measure of life span and starvation index and responds to parasite infection and increasing parasite spore loads in a similar manner as life span measured under more natural conditions (De Roode *et al.* 2009). After monarchs died, their bodies were vortexed at high speed in 5 mL H_2O to shake off parasite spores; we then determined the number of parasites that were washed off – referred to as spore load – using a haemocytometer (De Roode, Gold & Altizer 2007). We used analysis of variance to test for the effects of milkweed species on parasite spore load and monarch adult life span.

We also used analysis of covariance to test for the effects of milkweed species and parasite spore load on the longevity of infected monarchs. Analyses were carried out in R version 2.10.1 (R Development Core Team 2010). Models were checked for homogeneity of variance by using the Fligner–Killeen test (Crawley 2007).

AVOIDANCE EXPERIMENTS

Avoidance experiment 1: do newly hatched caterpillars from infected mothers avoid their contaminated egg shell?

This experiment tested whether monarch offspring from infected mothers can prevent infection by avoiding the consumption of their contaminated egg shell. To test this, we obtained eggs from infected and uninfected females and then compared the proportion of larvae that did not eat their egg shell. We obtained eggs from infected and uninfected females as follows: larvae from three non-inbred family lines (obtained from Marietta, GA, USA) were randomly assigned to an infected group or an uninfected control group. Larvae were infected as described earlier and reared to adulthood on greenhouse-grown *A. incarnata* cuttings. Seven days after pupation, pupae were scored for parasite infection using discoloration of the pupal case (De Roode, Gold & Altizer 2007). After eclosion, clear tape discs (2·54 cm diameter) were pressed onto the abdomens of uninoculated male and female butterflies and examined under a dissecting microscope at 60× to verify the absence of parasite spores: all individuals in the uninfected group were parasite free. Infected females were transferred to one of three mating cages and control female monarchs to one of three other mating cages. Uninfected and unrelated males were added to these cages to serve as mating partners. Mated infected (n = 30) and uninfected (n = 50) females were kept in two separate holding cages and supplied with *ad libitum* 10% honey water solution. Four potted *A. incarnata* plants were introduced in each of the holding cages until one egg was laid on each of 10 different leaves of each individual plant. One day before hatching, the leaves carrying an egg were detached from the plants and transferred to individual 10-cm Petri dishes containing moist filter paper. Upon hatching, the proportion of caterpillars from infected (n = 40) and uninfected female butterflies (n = 40) that ate their egg shell was recorded. Fisher exact tests carried out in R version 2.10.1 were used to compare these two proportions.

Avoidance experiment 2: do foraging caterpillars avoid contaminated milkweed foliage?

This experiment determined whether uninfected caterpillars preferentially consume uncontaminated over parasite-contaminated milkweed. Seventy-two 2-day-old larvae from three out-bred monarch families (obtained from Miami, FL, USA, and Pismo Beach, CA, USA) were individually transferred to 10-cm Petri dishes lined with moist filter paper and were given a choice between two 0·8-cm-diameter *A. incarnata* leaf discs that differed only in the presence or absence of parasite spores. Within a dual-choice test, the two leaf discs originated from the same leaf to account for potential choice biases resulting from reasons other than the presence/absence of parasite spores, such as nutritional differences. To rule out any positional effect, the relative left/right position of the contaminated leaf disc was randomized. Contaminated discs received either 10 or 100 infective spores to measure a potential effect of parasite concentration on larval avoidance behaviour. The first disc chosen was recorded, and GLM with a binomial error structure and logit link function in R version 2.10.1 was used to examine the effect of foliage contamination, monarch family and parasite concentration on larval choice.

SELF-MEDICATION EXPERIMENTS

Medication experiment 1: do infected caterpillars preferentially consume anti-parasitic milkweeds?

To test for the existence of self-medication behaviour, we conducted a cafeteria assay that measured the preference of infected and uninfected caterpillars among the same five milkweed species used for the milkweed experiment. These species were chosen because they vary widely in the extent to which they reduce parasite growth (Fig. 2a). Hatching larvae (n = 240) from two out-bred monarch families (one obtained from Miami, FL, USA and one from the eastern monarch overwintering site in Cerro Pelon, Mexico) were either infected (n = 120) by adding 100 spores of one parasite clone to their egg chorion (De Roode, Gold & Altizer 2007) or left uninfected (n = 120). Infected and uninfected larvae were then assigned to one of three groups: multiple choices at either (i) day 1 (first-instar caterpillars); (ii) day 5 (third-instar caterpillars); or (iii) day 11 (fifth-instar caterpillars). All larvae were reared on *Asclepias fascicularis*, a species not used for the multiple-choice tests. First- and third-instar larvae were placed in the centre of 10-cm Petri dishes containing moist filter paper, with leaf discs of the five milkweed species arranged in a circle around them in random order. Fifth-instar caterpillars were placed in individual cages with a bundle of milkweed shoots of the five species kept in a water bottle. Prior to adult emergence, pupae were scored for parasite infection using discoloration of the pupal case on a scale of 0–5, with 0 being uninfected (no dark patches under the pupal integument) and 5 heavily infected (dark patches forming under the majority of pupal integuments). We recorded the first milkweed species chosen by foraging caterpillars and used chi-square tests in R version 2.10.1 to compare the proportions of caterpillars that preferred a given plant species.
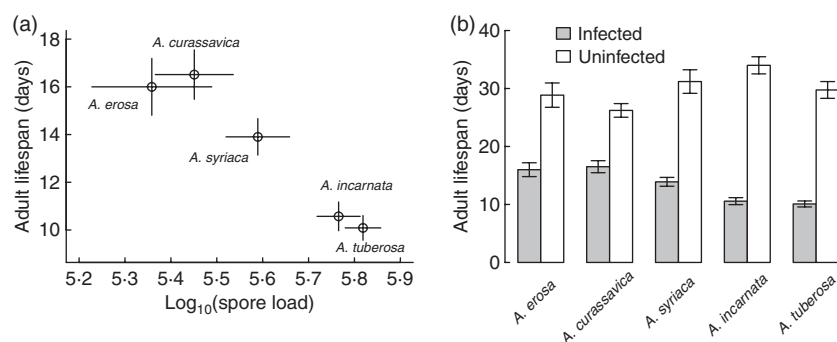

Fig. 2. Effect of five milkweed species on (a) parasite spore loads and adult lifespan of infected monarchs and (b) adult life span of infected and uninfected monarchs. Bars show mean ± SEM.

Medication experiment 2: do infected caterpillars ingest a larger quantity of anti-parasitic milkweed?

To compare the amount of anti-parasitic and non-anti-parasitic milkweeds eaten by infected and uninfected caterpillars, we conducted a no-choice experiment. Initially, 64 larvae from four out-bred monarch families (obtained from Pismo Beach, CA, USA) were assigned to an infected group ($n = 32$; eight larvae from each family infected with 10 spores from one parasite clone following the same method as described earlier) or an uninfected control group ($n = 32$). Larvae were kept in individual 0·94-L containers with meshed lids and were provided with milkweed foliage from either *A. incarnata* (16 infected and 16 uninfected larvae) or *A. curassavica* (16 infected and 16 uninfected larvae). These species were chosen for their marked differences in anti-parasitic effects, with *A. curassavica* being much more medicinally active than *A. incarnata* (De Roode *et al.* 2008, 2011a,b; Lefèvre *et al.* 2010). The amount of milkweed consumed over the entire caterpillar developmental period was measured as described previously (Lefèvre *et al.* 2010). Prior to adult emergence, pupae were scored for parasite infection using discoloration of the pupal case. The overall *A. incarnata* or *A. curassavica* intake was analysed using multi-way ANOVA. Models were checked for homogeneity of variance by using the Fligner–Killeen test (Crawley 2007) in R version 2.10.1 and full models included infection treatment and monarch family as explanatory variables, and interactions between them.

TRANS-GENERATIONAL MEDICATION EXPERIMENT: DO INFECTED ADULT FEMALES PREFERENTIALLY OVIPOSIT ON ANTI-PARASITIC MILKWEEDS?

In this experiment, we determined the oviposition preferences of infected and uninfected female butterflies for *A. curassavica* and *A. incarnata*, again because these species vary greatly in their anti-parasitic properties. Larvae from six out-bred family lines (obtained from San Antonio, TX, USA) were randomly assigned to an infected group or an uninfected control group, and 2-day-old larvae were infected with 10 spores from one parasite clone or left uninfected as described earlier. Infected and uninfected larvae were reared on *A. tuberosa* over their entire development, and infection status of butterflies was determined based on the discoloration of the pupal case (as described above). After eclosion, uninoculated male and female butterflies were checked for the absence of parasite spores as described earlier (avoidance experiment 1). Males from the infected group were not used for the experiment. Infected females were transferred to one of six mating cages and control female monarchs to one of six other mating cages. Uninfected and unrelated males were added to these cages to serve as mating partners. Females were kept in the mating cages between 2 and 5 days. Mated infected ($n = 10$) and uninfected ($n = 10$) females were then kept in two separate holding cages and supplied with *ad libitum* 10% honey water solution. Three days after mating (i.e. average time required for egg maturation), infected and uninfected females were released individually in one of five 7·1 m³ flight cages (3·1 m length, 1·8 m height and 1·27 m width) with a choice between one potted *A. curassavica* and one potted *A. incarnata* plant. Females were allowed to oviposit for a period of 1 h, after which they were returned to their original holding cage, and the number of eggs laid on each of the two plant species was recorded. To account for differences in oviposition caused by variations in the biomass of milkweeds, the dry weight of plant foliage was measured. Two days after their first oviposition test, females were tested again on a second trial. Logistic regression by generalized linear mixed model (GLMM, binomial errors, logit link) in R version 2.10.1 was used to investigate the effect of infection on the proportion of eggs laid on the anti-parasitic *A. curassavica*. The full model included infection, plant dry mass, monarch family and position of the plants in the cage. As ovipositing females were tested twice, the model was fitted by specifying female identity as a random effect (Crawley 2007). Finally, Poisson regression was used to investigate the effect of infection, monarch family, position of the plants in the cage and plant biomass on the total number of eggs laid by ovipositing females.

Results

DETERMINING THE ANTI-PARASITIC PROPERTIES OF FIVE MILKWEED SPECIES

The milkweed species on which infected larvae were reared strongly affected parasite spore load (ANOVA, $F_{4,128} = 5·7$, $P = 0·0003$). Parasite spore loads were lowest on *A. erosa* followed by *A. curassavica*, *A. syriaca*, *A. incarnata* and *A. tuberosa* (Fig. 2a).

As expected, infected monarchs had much shorter lives than did uninfected individuals (Fig. 2b; $F_{1,191} = 455·9$, $P < 0·001$). We also found a significant effect of milkweed species on host life span (Fig. 2a,b; $F_{4,191} = 8·56$, $P < 0·001$), and as expected, infected monarchs lived longer on milkweed species on which they experienced lower parasite spore loads (Fig. 2a, $F_{1,123} = 55·7$, $P < 0·001$). There was also a strong infection by milkweed species interaction on monarch adult life span (Fig. 2b, $F_{4,191} = 9·84$, $P < 0·001$). This interaction indicates that anti-parasitic milkweed species are not anti-parasitic by providing greater overall health to monarchs but that milkweed species affect infected and uninfected individuals differentially. Finally, there was no spore load by milkweed species interaction ($F_{1,123} = 0·52, P = 0·72$).

AVOIDANCE EXPERIMENTS

Avoidance experiment 1: do newly hatched caterpillars from infected mothers avoid eating their egg shell?

Overall, 37 of 40 eggs from infected females and 39 of 40 eggs from uninfected females hatched (Fisher's exact test, $P = 0·6$). There was no difference in the proportion of caterpillars that ate their egg shell between larvae from infected females and those from uninfected females (Fig. 3a; uninfected: 79%, infected: 81%, $P = 0·9$), suggesting that newly hatched larvae from infected female butterflies do not avoid eating their contaminated egg shell.

Avoidance experiment 2: do foraging caterpillars avoid contaminated foliage?

Even if hatching larvae from infected females are unable to avoid consumption of contaminated egg shells, uninfected caterpillars may still be able to prevent potential infection by

Fig. 3. Monarch avoidance behaviour experiments. (a) Proportions of newly hatched caterpillars from infected and uninfected adult females that ate their egg shell. (b) Proportion of foraging monarch larvae that chose a contaminated leaf disc over an uncontaminated disc as a function of parasite concentration. Bars show mean ± 95% CI.
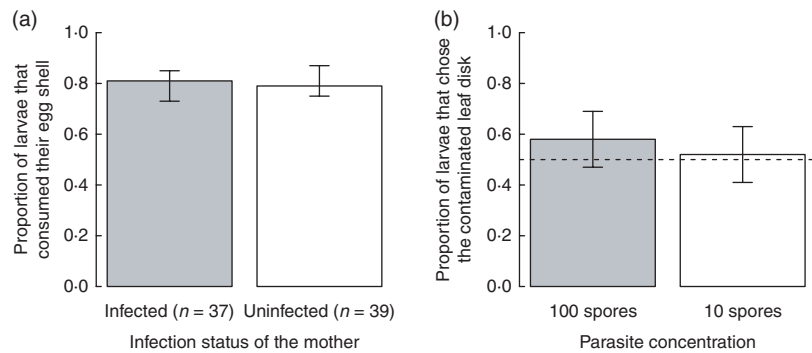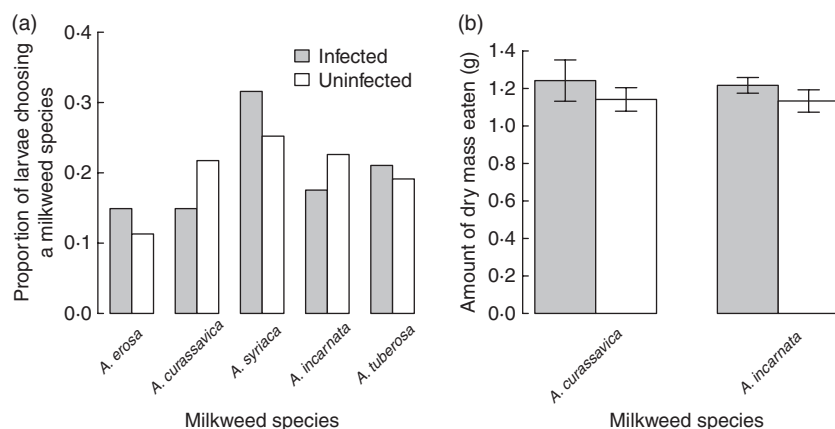
avoiding contaminated milkweed foliage. However, caterpillars presented with a choice between a parasite-free *A. incarnata* leaf disc and a parasite-contaminated disc from the same leaf did not avoid contact with the infective spores. The proportion of larvae that chose the parasite-contaminated disc was consistent with a random choice [Fig. 3b; logistic regression: odds ratio (OR) = 1·02, 95% confidence interval (CI) = 0·14, 1·9, P = 0·7]. There were also no significant effects of monarch family (OR = 1, CI = 0·99, 1·01, P = 0·6) or parasite concentration (Fig. 3b; OR = 1·2, CI = 0·25, 2·1, P = 0·6). Together, avoidance experiments 1 and 2 indicate that monarch caterpillars appear unable to avoid the infective spores of *O. elektroscirrha*.

SELF-MEDICATION EXPERIMENTS

Medication experiment 1: do infected caterpillars choose anti-parasitic milkweeds?

Overall, monarch caterpillars did not exhibit any preferences among the five milkweed species (chi-square test, χ^2 = 6·8, d.f. = 4, P = 0·15). There was no significant difference in the proportion of caterpillars choosing a given milkweed species between infected and uninfected individuals (Fig. 4a; χ^2 = 3·7, d.f. = 4, P = 0·45). The milkweed preferences of first, third and fifth instars were also consistent with a random choice (χ^2 = 10·4, d.f. = 8, P = 0·24). Finally, there was no effect of monarch family (χ^2 = 5·2, d.f. = 4, P = 0·27).

Medication experiment 2: do infected caterpillars ingest larger quantities of anti-parasitic milkweed?

When reared on the anti-parasitic larval host plant, *A. curassavica*, infected and uninfected monarch caterpillars ingested a similar amount of milkweed over their larval development (Fig. 4b; mean ± SE, 1242 mg of dried mass ± 110 and 1141·46 mg ± 62·7, ANOVA, $F_{1,15}$ = 0·136, P = 0·72). We found a significant effect of monarch family with one lineage eating more than the others ($F_{1,15}$ = 10, P < 0·001). There was no significant lineage by infection interaction ($F_{2,15}$ = 0·49, P = 0·62). Infected and uninfected larvae also ate a similar amount of *A. incarnata* over their larval development (Fig. 4b; 1216·66 ± 41·8 and 1133·19 mg ± 59·7, $F_{1,14}$ = 0·16, P = 0·70). There was a significant lineage effect, with again the same lineage ingesting a larger quantity of *A. incarnata* than the other monarch families ($F_{3,14}$ = 12·48, P < 0·001). Finally, we found a significant lineage by infection interaction ($F_{3,14}$ = 4·74, P = 0·02). Individuals from two lineages ingested a higher quantity of *A. incarnata* when infected than when uninfected, whereas individuals from another lineage exhibited a reverse tendency.

TRANS-GENERATIONAL MEDICATION EXPERIMENT: DO INFECTED ADULT FEMALES PREFERENTIALLY OVIPOSIT ON ANTI-PARASITIC MILKWEEDS?

Infection significantly affected the proportion of eggs laid on the anti-parasitic *A. curassavica* (Fig. 5; GLMM, OR = 1·51; 95% CI = 1·1, 2; P = 0·001) with parasitized females
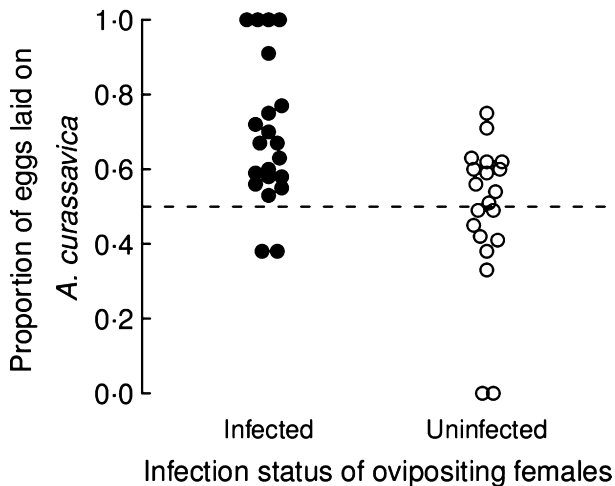

Fig. 4. Monarch self-medication behaviour experiments. (a) Preferences of infected and uninfected caterpillars (first, second and fifth instars combined) among five milkweed species in cafeteria choice tests. (b) Amount of the medicinal *Asclepias curassavica* and the non-medicinal *A. incarnata* eaten by infected and uninfected caterpillars in no-choice tests during their larval development. Bars show mean ± SEM.

Fig. 5. Monarch trans-generational medication. Proportion of eggs laid on the anti-parasitic *Asclepias curassavica* by infected and uninfected females in dual-choice tests. Infected females laid 69.4% of their eggs on *A. curassavica* while uninfected females laid only 48.5% of their eggs on *A. curassavica*.

exhibiting a strong oviposition preference for *A. curassavica*, while uninfected females exhibited no preference between the two plant species. The dry weight of *A. curassavica* foliage was significantly greater than that of *A. incarnata* (mean ± SE 1528 ± 60 and 1137 ± 37 mg, *t*-test, t = 5·4, d.f. = 74, P < 0·001). However, the effect of plant biomass on oviposition choice was not statistically significant (OR = 1·4, CI = 0·95, 2·1, P = 0·08) and did not remove the preference for *A. curassavica* when included as a covariate in the analysis. Female choice was influenced neither by the position of the plants in the cage (OR = 1·1, CI = 0·82, 1·48, P = 0·6) nor by monarch families (lineage B: OR = 1·6, CI = 0·9, 2·9, P = 0·9; lineage C: OR = 2, CI = 1·1, 3·5, P = 0·01; lineage D: OR = 1·19, CI = 0·57, 2·44, P = 0·64; lineage J: OR = 1·35, CI = 0·8, 2·24, P = 0·23; and lineage K: OR = 1·2, CI = 0·7, 2, P = 0·5).

The total number of eggs laid on both milkweed species over the 1-h period of choice test did not differ between infected and uninfected females (mean ± SE, 33·85 ± 5·3 and 35·1 ± 4·7, respectively; GLM poisson, χ^2 = 0·005, P = 0·94), but monarch families varied in the number of eggs they laid (χ^2 = 79, P < 0·001). The relative position of milkweed species and plant biomass did not influence the total number of eggs laid (χ^2 = 0·05, P = 0·81, and χ^2 = 0·96, P = 0·32).

Discussion

Our study confirms that animals may use behavioural defences to protect themselves against parasites and suggests that such behaviours may be limited to a single stage in the host–parasite life cycle. In particular, we showed that monarch caterpillars could not avoid contact with infective spores of a protozoan parasite. Caterpillars could also not cure themselves of disease by preferentially consuming anti-para-

sitic host plants or by increasing their intake of anti-parasitic food. In contrast, we found that infected female monarch butterflies preferred to lay their eggs on anti-parasitic milkweeds, on which their offspring experience reduced infection risk, lower parasite burdens and smaller reductions of fitness.

Monarch butterflies become infected with the protozoan *O. elektroscirrha* when larvae ingest infective parasite spores on egg shells and milkweed foliage. We therefore hypothesized that monarch larvae may be able to avoid infection by avoiding the consumption of parasite-contaminated egg shells and milkweed foliage, but our experiments found no support for this hypothesis. On average, 80% of hatching caterpillars from uninfected butterflies consumed their egg shell upon hatching, and offspring caterpillars from infected butterflies were no different. In many Lepidoptera species, the egg chorion provides beneficial nutrients to first-instar caterpillars, and its ingestion can positively affect adult performance (Barros-Bellanda & Zucoloto 2001). Given these nutritional benefits, it could be argued that the benefits of chorion consumption outweigh the costs of becoming infected. However, the fitness consequences of *O. elektroscirrha* infection are severe, and we favour the hypothesis that caterpillars are unable to detect the infective spores of *O. elektroscirrha* on their chorion. This hypothesis is also supported by the finding that second-instar larvae did not avoid the consumption of parasite-contaminated milkweed foliage. In this case, the cost-benefit analysis is straightforward: larvae had the choice between two equally nutritious leaf discs – one of which contained disease-causing parasites – but they did not avoid the contaminated foliage. Overall, the two avoidance experiments indicate that, when foraging, monarch caterpillars are unable to detect the presence of infective parasite spores and avoid their ingestion accordingly. In a way, this is not surprising because these parasites are very small and may be hard to detect. Indeed, in cases where avoidance behaviour has been demonstrated conclusively, parasite detection is much easier than it would be in the monarch-protozoan system. For instance, gypsy moth larvae avoid nucleopolyhedrosis viruses by avoiding the consumption of virus-killed larval corpses, which ooze into black patches on food plants (Parker, Elderd & Dwyer 2010).

We also did not find support for the existence of prophylactic or therapeutic self-medication in monarch butterfly caterpillars. Infected and uninfected caterpillars displayed no significant preference among the five milkweed species, even though these species differed greatly in their therapeutic effects. Furthermore, in the no-choice feeding experiment, parasitized and unparasitized caterpillars consumed a similar amount of the anti-parasitic *A. curassavica*. These results support a previous study, in which we allowed monarch larvae a choice between *A. incarnata* and *A. curassavica* and found that infected larvae did not preferentially consume the anti-parasitic *A. curassavica* (Lefèvre *et al.* 2010). Our results contrast with recent studies on different species of Lepidoptera. *Spodoptera* caterpillars, in response to viral or bacterial infection, are able to offset protein costs of pathogen resistance by self-regulating their nutritional intake (Lee *et al.*

2006; Povey *et al.* 2009); and parasitoid-infected arctiid caterpillars increase the uptake of anti-parasitic chemicals from their diet (Singer, Mace & Bernays 2009). Our results may differ from those of other studies for two reasons. First, arctiid and *Spodoptera* larvae are known to feed on multiple species of plant during their development (Brown & Dewhurst 1975; Haggis 1986; Singer, Bernays & Carriere 2002; Bernays & Chapman 2004; Singer *et al.* 2004a,b). In contrast, monarch butterfly larvae usually complete their larval development on a single milkweed plant. Female monarchs lay a single or a few eggs on individual plants that have a biomass sufficient to support the development of the offspring, and larval movements between plants are risky (Borkin 1982; Malcolm & Brower 1986; Zalucki, Brower & Malcolm 1990). Second, a recent study has suggested that medicinal milkweeds mediate their anti-parasitic effect before and during infection, but have no further effects once caterpillars are infected (De Roode *et al.* 2011a). Milkweeds may therefore be therapeutic by reducing the effective number of parasites initiating an infection, but may not reduce parasite growth after infection has occurred. These results imply that caterpillars would benefit little from consuming anti-parasitic milkweed once they are already infected.

We found evidence in our study for one behavioural defence against parasites, the preferential oviposition by infected monarch butterflies on anti-parasitic milkweed. Specifically, we observed that infected females preferentially laid their eggs on the anti-parasitic *A. curassavica* in dual-choice tests with *A. incarnata*. Our results suggest that monarch caterpillars cannot avoid infection or use plants to reduce parasite growth but that adults can provide their offspring with medication by laying their eggs preferentially on anti-parasitic larval host plants. These offspring will suffer lower rates of infection and lower parasite burdens, thereby mitigating the fitness reductions that are expressed on less anti-parasitic milkweeds (Fig. 2). However, therapeutic milkweeds such as *A. curassavica* can also come at a cost. For example, the longevity of uninfected individuals reared on *A. curassavica* is shorter than that of uninfected monarchs reared on *A. incarnata* (Fig. 2b). This cost may partly explain why uninfected individuals do not display an oviposition preference for *A. curassavica*.

This oviposition experiment was carried out with monarchs collected from eastern North America and confirms the finding of an oviposition preference for *A. curassavica* by infected monarchs collected from western North America (Lefèvre *et al.* 2010). Future population genetic studies, in combination with behavioural assays on monarchs from other geographic areas, will tell us whether the western and eastern North America populations are genetically distinct and whether this fascinating behaviour represents a case of convergent evolution or a legacy from a common ancestor.

We currently do not know the proximate mechanism by which infected butterflies alter their oviposition preference. One possibility is that infection induces changes in receptors involved in milkweed-seeking behaviour and preference, including the olfactory and contact chemoreceptor sensilla

on the legs and antennae of the butterfly (Haribal & Renwick 1998). Similar mechanisms have been proposed to explain changes in diet preference of infected arctiid larvae (Singer, Mace & Bernays 2009), which show increased responsiveness of taste receptors to protective plant toxins (Bernays & Singer 2005). In the case of milkweeds, cardenolides may provide cues about – and also mediate – anti-parasitic effects.

An important question raised by our results is why hosts do not evolve a complete arsenal of behavioural defences to cope with their parasites. There are at least two explanations for this. First, hosts and parasites continuously co-evolve: hosts are under strong selective pressures to avoid parasites, but parasites are under strong selection to avoid detection. Indeed, it has been suggested that parasites have more to lose (their whole fitness) than hosts (part of their fitness) such that selective pressures acting on parasite transmission may be stronger than those acting on host avoidance (life-dinner principle, Dawkins & Krebs 1979). Second, while each potential defence mechanism is effective in fighting off parasites, it may be too costly for hosts to evolve a full arsenal of defence mechanisms. Indeed, parasite defence mechanisms are often costly to maintain, and it is often expected that hosts will evolve or use only a subset (Sheldon & Verhulst 1996; Castella *et al.* 2008; Simone, Evans & Spivak 2009; Baucom & De Roode 2010). With regard to monarch butterflies and their parasites, our results show that monarchs may protect their offspring against parasites by preferentially laying their eggs on anti-parasitic milkweeds (see also (Lefèvre *et al.* 2010)). Moreover, recent studies have shown that monarchs possess physiological qualitative and quantitative resistance mechanisms against *O. elektroscirrha* (De Roode & Altizer 2010; Lefèvre, Williams & de Roode 2011).

Beyond demonstrating that animals may use behavioural mechanisms as a protection against parasites, our results also demonstrate that an inclusive fitness approach should be used to study behavioural defences against parasites in nature. As described earlier, we found that monarchs cannot protect themselves from disease but that they can protect their offspring, and it is possible that other species have also evolved the ability to protect their offspring from the detrimental effects of parasites. For example, when infected, mammals, birds and some insects can increase their offspring resistance via maternal trans-generational immune priming (e.g. Little *et al.* 2003; Hasselquist and Nilsson 2009; Tidbury, Pedersen & Boots 2010). Trans-generational anti-infection behaviours may also be constitutive. For instance, the tree frog *Hyla versicolor* can discriminate between oviposition sites and preferentially lays eggs in pools without trematode-infected snails (Kiesecker & Skelly 2000). Similarly, great tits (*Parus major*) prefer to roost in boxes free of haematophagous fleas (Christe, Oppliger & Richner 1994). Thus, it is possible that important behavioural defence mechanisms are missed when focusing on mechanisms that protect individuals directly rather than their offspring.

Finally, our study has major implications for the understanding of the evolutionary ecology of host–parasite interactions. In particular, it emphasizes the importance of host

behaviour in mitigating disease, and the crucial role of the environment in shaping the dynamics of antagonistic co-evolution. From an ecological perspective, this study highlights that parasites, like predators, can influence the distribution patterns of animals and that oviposition choices in response to infection can be a determinant of the structure of ecological communities.

Acknowledgements

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