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Trans-generational parasite protection associated with paternal diet

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Summary

1. Multiple generations of hosts are often exposed to the same pathogens, favouring the evolution of trans-generational defences. Because females have more opportunities to transfer protective molecules to offspring, many studies have focused on maternally derived protection. However, males of many species can transfer compounds along with sperm, including chemicals that could provide protection.

2. Here, we assess maternally and paternally derived protection in a monarch butterfly–protozoan parasite system where parasite resistance is heavily influenced by secondary plant chemicals, known as cardenolides, present in the larval diet of milkweed plants.

3. We reared monarch butterflies on medicinal and non-medicinal milkweed species and then measured resistance of their offspring to infection. We also measured cardenolide content in adult monarchs reared on the two species, and in the eggs that they produced.

4. We found that offspring were more resistant to infection when their fathers were reared on medicinal milkweed, while maternal diet had less of an effect. We also found that eggs contained the highest levels of cardenolides when both parents were reared on the medicinal species. Moreover, females reared on non-medicinal milkweed produced eggs with significantly higher levels of cardenolides if they mated with males reared on the medicinal milkweed species. However, we found an equivocal relationship between the cardenolides present in eggs and parasite resistance in the offspring.

5. Our results demonstrate that males reared on medicinal plants can transfer protection to their offspring, but the exact mechanism remains unresolved. This suggests that paternal protection from parasitism might be important, particularly when there are environmental sources of parasite resistance and when males transfer spermatophores during mating.

Key-words: *Asclepias*, *Danaus plexippus*, ecological immunology, *Ophryocystis elektroscirrha*, trans-generational immunity

Introduction

Parasites impose strong fitness costs on their hosts, and as a result, hosts have evolved a wide range of defences against parasitism. Such defences can protect the individual itself but also its genetic kin (Chapuisat *et al.* 2007; De Roode & Lefèvre 2012; Lefèvre *et al.* 2012; De Roode, Lefèvre & Hunter 2013). Because parents and offspring are often exposed to the same parasites, selection is expected to favour trans-generational defences (Little & Kraaijeveld 2004). Indeed, the transfer of maternal antibodies in vertebrates is well-established (Grindstaff, Brodie & Ketterson 2003; Hasselquist & Nilsson 2009), and recent studies have shown that trans-generational immunity also occurs in invertebrates where this phenomenon is referred to as trans-generational immune priming (Little *et al.* 2003; Little & Kraaijeveld 2004; Sadd *et al.* 2005; Moret 2006; Sadd & Schmid-Hempel 2007; Tidbury, Pedersen & Boots 2012). Because females can provide resources to their offspring via eggs, in utero environment, and lactation, many studies of trans-generational immunity have focused specifically on the maternal transfer of immune molecules, both passively and in response to an immune challenge. As a result, paternal transfer of

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immunity and the importance of non-immunological molecules – such as chemicals obtained from food – remain under-explored.

However, recent studies of flour beetles and pipefish have documented paternal immune priming (Roth *et al*. 2010, 2012). In addition, males are known to provide females with nutritive and defensive compounds during sperm transfer (Thornhill 1976; Bezzerides *et al*. 2004; Eisner *et al*. 2008), and paternal environment has been found to alter sperm phenotype resulting in an effect on offspring life history and survival (Crean, Dwyer & Marshall 2013). It is also increasingly clear that parasite resistance can be provided by secondary plant chemicals instead of canonical immune molecules (Keating, Hunter & Schultz 1990; De Roode *et al*. 2008; Parker *et al*. 2011; Sternberg *et al*. 2012), which could be transferred by males during mating. Thus, it is likely that males can play a major role in providing parasite resistance to offspring, especially through the transfer of secondary chemicals. Like maternally derived immunity, paternal transfer of secondary chemicals might occur passively or be increased in response to an immune challenge.

We investigated the relative importance of maternal and paternal trans-generational parasite protection in a system where antiparasitic resistance is strongly affected by host diet. In particular, we studied monarch butterflies (*Danaus plexippus*), their protozoan parasite *Ophryocystis elektroscirrha* and the milkweeds (*Asclepias* spp.) that monarchs use as their larval food. Monarchs are commonly infected with *O. elektroscirrha*, which reduces preadult survival as well as adult mating ability, life span, fecundity and flight ability (Bradley & Altizer 2005; De Roode, Yates & Altizer 2008; De Roode *et al*. 2009). Monarch larvae are specialist feeders on milkweed plants, which contain secondary chemicals called cardenolides. Milkweed species vary widely in the concentrations, diversity and polarity of their cardenolides, and previous studies have shown that the consumption of milkweeds with greater cardenolide concentrations provides greater protection against parasites (De Roode *et al*. 2008, 2011a; Sternberg *et al*. 2012). Moreover, monarchs are well known to sequester milkweed cardenolides into their own tissues (Malcolm & Brower 1989; Malcolm, Cockrell & Brower 1989), and it is reasonable that they might also provision their eggs with these chemicals. Cardenolide provisioning of eggs might occur not only by females, who produce the eggs, but also by males, who transfer large spermatophores during mating (Oberhauser 1988). It is therefore possible that monarchs of both sexes can provide trans-generational parasite protection by provisioning the eggs with diet-derived antiparasitic chemicals.

Due to its life cycle, *O. elektroscirrha* is particularly likely to exert selection for trans-generational monarch defences. *Ophryocystis elektroscirrha* forms dormant spores on the abdomen of infected monarch butterflies and during oviposition; some of these spores are passively transferred to monarch eggs and the milkweed leaves on which the eggs are laid. Hatching larvae consume their eggshells and the surrounding milkweed, thereby ingesting the parasite spores. *Ophryocystis elektroscirrha* then undergoes asexual and sexual replication in larvae and pupae to form a new generation of dormant spores on emerging adult butterflies. Although horizontal parasite transmission between adults and unrelated monarch larvae can occur (Altizer, Oberhauser & Geurts 2004), most transmission occurs vertically, either from infected mothers to their offspring or through the transfer of spores from males to females during mating and subsequently to offspring (De Roode *et al*. 2009).

To investigate the potential transfer of cardenolides across generations and the effects of parental diet on offspring resistance, we carried out three experiments in which monarchs were reared on *Asclepias incarnata* (swamp milkweed, low-cardenolide concentration) or *Asclepias curassavica* (tropical milkweed, high-cardenolide concentration), the latter of which reduces parasite infection, growth and virulence (De Roode *et al*. 2008; Lefèvre *et al*. 2010; Sternberg *et al*. 2012). We then mated these monarchs using fully factorial designs in which parents were fed on either the same species or different species of milkweed. In experiments 1 and 2, we collected the eggs from each mating and deposited parasite spores directly on each egg. We then measured parasite infection in the offspring. In experiment 3, we analysed the chemical content of the plants eaten by the parents during their larval development, the wings of the parents and the eggs produced from each mated pair. Our results show that offspring experience greater resistance to parasite infection when their parents – and especially their fathers – are reared on the medicinal *A. curassavica*. Moreover, our chemical analyses show that eggs contain greater concentrations of cardenolides when parents are reared on *A. curassavica*. However, when analysing the chemistry of eggs from the first and second experiments, we did not find a significant relationship between the cardenolide concentrations of individual females' eggs and infection in their offspring. These results suggest that additional factors might explain the transfer of parasite resistance from parents to offspring.

Materials and methods

EFFECTS OF PARENTAL DIET ON PARASITE RESISTANCE

Monarch and parasite sources

Adult monarchs were collected in Atlanta, GA, USA (experiment 1) and St. Marks, FL, USA (experiment 2). Both of these sampling locations are within the range of a single, panmictic North American monarch population (Lyons *et al*. 2012). The offspring of these monarchs were used to create unrelated family lineages (two lineages from Atlanta, GA, USA, and seven lineages from St. Marks, FL, USA). Twenty individuals from each lineage were reared individually in 1-L plastic containers in the laboratory on

the low-cardenolide milkweed species *A. incarnata* (swamp milkweed) and twenty were reared on the high-cardenolide, antiparasitic *A. curassavica* (tropical milkweed). This resulted in approximately 10 males and 10 females per lineage reared on each food plant species, which served as the parental generation used for our experiments. In experiment 1, females in the parental generation were only permitted to mate with males from the other lineage. In experiment 2, larval food plant treatment was represented in similar numbers across lineages such that there was no treatment represented in only one lineage combination. All of the milkweed plants were grown from seeds (Butterfly Encounters, Inc., San Ramon, CA, USA) under uniform greenhouse conditions. Prior to mating, all monarchs in the parental generation were confirmed to be uninfected using a non-destructive sampling method. This was performed by applying clear tape to the abdomen of the monarch, which was then transferred to a white index card and examined under a microscope for the presence of parasite spores (Altizer, Oberhauser & Brower 2000). One clonal parasite line was used per experiment, with each line originating from an infected adult monarch collected at the same time as the family lineages for the respective experiments. To create these lines, monarch larvae were inoculated with one haploid spore, resulting in single-genotype infections as described by De Roode, Gold & Altizer (2007). The infected larvae were reared to adulthood and used as a source of clonal parasites for our experiments (denoted as C1E23-P1-1 for experiment 1 and C1E25-P1-1 for experiment 2).

Experiment 1

Female monarchs from the parental generation were allowed to mate once with a single, unrelated male in all four possible combinations of parental food plant (i.e. both parents reared on *A. curassavica*, both parents reared on *A. incarnata*, female reared on *A. curassavica* and male reared on *A. incarnata*, and vice versa). Mated females were provided with the low-cardenolide milkweed species *Asclepias tuberosa* (butterfly weed) for oviposition. Three days after oviposition, 50 eggs per female were collected and stored in 1 mL of methanol at −80 °C for later chemical analysis. Fifteen eggs per female were placed singly on leaf discs in individual Petri dishes lined with wet filter paper. For ten of these eggs, a drawn-out capillary tube was used to manually place 10 parasite spores on each egg, while five eggs were not inoculated, to serve as controls. The eggs were checked daily until the larvae hatched and consumed their eggshells. The larvae were then transferred into individual 1-L plastic containers with *A. tuberosa* cuttings in florist tubes. These containers were kept in a climate-controlled room at 26 °C on a 16 L : 8 D light cycle until pupation. Fresh *A. tuberosa* cuttings were added as needed throughout this period.

Six days after pupation, monarchs were transferred to a new room held under the same temperature and light conditions. This was carried out to prevent parasite transmission from the emerging adult butterflies. At this point, the pupae were checked daily and scored on a 0–5 scale for discoloration associated with parasite infection. On this scale (referred to as pupal score), 0 corresponds to the absence of parasite-associated discoloration, indicating few to no parasites present, and 5 corresponds to large patches of discoloration, indicating high numbers of parasites (De Roode *et al.* 2009). For our analysis, we used the score recorded the day prior to eclosion.

Experiment 2

The design for experiment 2 was similar to that of experiment 1, but the experimental protocol was refined, and additional measurements were taken from adult monarchs. Specifically, we increased the inoculation dose from 10 to 20 spores to increase the infection probability in the monarchs, which was lower than expected in experiment 1. By increasing the inoculation dose in experiment 2, we increased the rate of infection and consequently increased our sample size of infected offspring. Additionally, eggs were checked twice daily, instead of once daily, to better monitor hatching and consumption of eggshells. Post-hatching larvae were provided with fresh *A. incarnata* instead of *A. tuberosa*. On the day that they emerged, adult monarchs were placed in glassine envelopes, weighed and then checked daily to determine longevity. Prior work has shown that the effects of infection and parasite burden on monarch longevity under starvation conditions are similar to the effects under more natural conditions (De Roode, Yates & Altizer 2008; De Roode *et al.* 2009). After adult monarchs died, their bodies were placed in 5 mL of water and vortexed for 5 min to shake off the parasite spores. These spores were then counted using a haemocytometer to obtain a second measure of parasite burden (referred to as spore load). Because *O. elektroscirrha* only replicates in the larval and pupal stages of the monarch, this measure represents the total replication of parasites within the host.

SECONDARY CHEMICALS IN PLANTS, MONARCHS AND EGGS

Monarchs used in experiment 3 were the non-inbred descendants of butterflies collected in St. Marks, FL, USA. The offspring of these monarchs were mated to create four unrelated family lineages, and a minimum of 40 newly hatched larvae per family lineage were divided evenly between *A. curassavica and A. incarnata*, with the goal of rearing 12 female and 12 males per family line per plant for subsequent mating (described below). Larvae were reared individually in plastic containers at 25 °C, 16 : 8 L : D, and fed their assigned plant diets *ad libitum* until pupation (about 16 days). The plants used to rear monarchs were grown from seed (Butterfly Encounters, Inc.) at 25 °C, 16 : 8 L : D. Larvae were provided with cut leaves that were renewed every 2 days during the first 8 days of larval growth and daily thereafter. Monarchs grew too large to receive all of their food from single milkweed plants, and each larva received food from three to five individual plants during the larval period. Therefore, we cannot match the cardenolide chemistry of individual plants to the chemistry of specific adults and their eggs. Rather, we pooled foliar samples, separately for each milkweed species, from all plants that were used to feed larvae on a given feeding day. This resulted in 24 separate plant samples for each milkweed species for subsequent chemical analysis (methods below). Foliar cardenolide concentrations in the plants used to feed monarchs did not change during the course of the experiment ($F_{1,44} = 1{\cdot}88$, $P = 0{\cdot}1777$).

When butterflies emerged from their pupae, they were paired with unrelated partners to generate eggs for chemical analysis. Pairings were established to generate all possible factorial combinations of male and female diet: *A. curassavica* male with *A. curassavica* female ($n = 11$), *A. curassavica* male with *A. incarnata* female ($n = 10$), *A. incarnata* male with *A. curassavica*

female ($n = 12$) and *A. incarnata* male with *A. incarnata* female ($n = 12$); sample sizes vary slightly because not all pairings produced eggs, but there was no bias in egg production among family lines. Mating cages were examined daily, and new eggs were removed, counted and frozen at −80 °C. When the number of eggs removed from a mating reached 50, or three consecutive days passed without new eggs, the mating was terminated. Final egg numbers per mating used for chemical analysis varied from 20 to 50 (mean = 48·13). The accumulated eggs from each mating were weighed on a microbalance and placed in methanol for chemical analysis (described below). Butterflies were dried, and wing samples were collected in methanol for chemical analysis.

We measured cardenolides in plants, butterflies and eggs using methods described by Zehnder & Hunter (2007). For plants, six leaf discs from the fourth pair of leaves of each plant were ground in methanol using a ball mill and sonicated at 60 °C for 1 h. Another six leaf discs were taken and oven-dried to provide estimates of sample dry mass. Samples were then pooled into a single sample for each milkweed species for each day of the experiment, for a total of 24 samples per milkweed species. For butterfly chemistry, we weighed and ground the right forewing and then extracted it in methanol. Eggs were also ground in methanol for cardenolide analysis. The supernatant from samples in methanol was evaporated at 45 °C until dry. Samples were then resuspended in 150 μL of methanol containing 0·15 mg mL^{-1} digitoxin as an internal standard and analysed using reverse-phase high-performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA). Running time for each sample was 9 min. Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300 nm. Peaks with symmetrical absorption maxima between 216 and 222 nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of the internal standard (digitoxin) and the estimated sample mass.

STATISTICAL ANALYSIS

Effects of parental diet on parasite resistance

Data from experiments 1 and 2 were first analysed separately and then combined for a second set of analyses that included experiment as a factor (see Tables 1 and 2 for the factors included in the two sets of analyses). The combined analyses served to test whether the effect of parental diet was consistent across both experiments, despite the changes in experimental protocol (e.g. higher dose in experiment 2), or whether the rank order of parasite resistance in offspring differed between experiments, which would be indicated by a significant interaction between experiment and parental diet.

In both sets of analyses, mixed effects models were used to assess the effect of parental food plant on pupal score (a combined measure of resistance to becoming infected and resistance to the subsequent reproduction of the parasites in those monarchs that become infected) and infection probability. In the analyses of experiment 2, additional mixed effects models were used to analyse the effect of parental food plant on the $\log_{10}$-transformed spore load of offspring (data only collected during experiment 2). Maximal models included paternal and maternal food plant species as fixed effects, as well as the interaction between these fixed effects. Likewise, mixed effects models were used to analyse the effect of parental food plant and infection status on three measures of general vigour in the offspring produced in experiment 2, namely larval development time, adult mass and adult longevity. In addition to paternal and maternal

Table 1. Analysis of variance in three measures of disease resistance using mixed effects models, separated by experiment. Listed are all the fixed effects and interaction terms included in the maximal models prior to stepwise model simplification. Maternal identity was included in all models as a random effect

	χ^2	d.f.	*P*
Experiment 1			
Pupal score			
Paternal food plant	0·445	1	0·505
Maternal food plant	0·814	1	0·367
Pat. × Mat. food plant	0·031	1	0·861
Infection probability			
Paternal food plant	0·601	1	0·438
Maternal food plant	0·182	1	0·669
Pat. × Mat. food plant	0·648	1	0·421
Experiment 2			
Pupal score			
Paternal food plant	4·72	1	0·030*
Maternal food plant	2·15	1	0·143
Pat. × Mat. food plant	0·055	1	0·814
Infection probability			
Paternal food plant	3·70	1	0·054
Maternal food plant	1·20	1	0·274
Pat. × Mat. food plant	0·264	1	0·607

Significance ($P < 0·05$) is indicated by asterisks; these terms were retained in the minimal adequate model.

Table 2. Analysis of variance in three measures of disease resistance using mixed effects models, experiments 1 and 2 combined with experiment included in the models as a fixed effect. Listed are all the fixed effects and interaction terms included in the maximal models prior to stepwise model simplification. Maternal identity was included in all models as a random effect

	χ^2	d.f.	*P*
Pupal score			
Paternal food plant	5·31	1	0·021*
Maternal food plant	2·72	1	0·099
Experiment	9·56	1	0·002*
Pat. × Mat. food plant	0·003	1	0·954
Pat. food plant × Experiment	0·238	1	0·626
Mat. food plant × Experiment	0·003	1	0·959
Pat. × Mat. food plant × Experiment	0·131	1	0·718
$\log_{10}$(spore load)			
Paternal food plant	5·25	1	0·022*
Maternal food plant	0·744	1	0·388
Mat. × Pat. food plant	0·073	1	0·787
Infection probability			
Paternal food plant	3·66	1	0·056
Maternal food plant	1·39	1	0·238
Experiment	2·08	1	0·149
Pat. × Mat. food plant	0·004	1	0·961
Pat. food plant × Experiment	0·183	1	0·669
Mat. food plant × Experiment	0·002	1	0·961
Pat. × Mat. food plant × Experiment	0·717	1	0·397

Significance ($P < 0·05$) is indicated by asterisks; these terms were retained in the minimal adequate model.

food plant species and the interaction term, infection status was included as a fixed effect in the full models for these three measures of vigour. Maternal identity, which corresponded to sibling groups, was included as a random effect in all models. In all but two cases in experiment 1 (where two males were mated with two females each), males were not permitted to mate multiple times, so maternal identity almost always corresponds to a unique paternal identity.

In the second set of analyses, where data from experiments 1 and 2 were combined, experiment was included in the full model as a fixed effect, as well as all possible two- and three-way interactions between experiment, maternal food plant and paternal food plant. All variables included in the maximal models are listed in Table 1 (experiments separated), Table 2 (experiments combined) and Table S1 (Supporting information) (measures of general vigour, data from experiment 2 only).

Analyses were performed in R v. 3.1.0 using the LME4 package v. 1.1-6 (Bates, Maechler & Bolker 2012) to allow for binomial error structures in the analysis of infection probability. Models with binomial error structures were fitted using Laplace approximation. Normal error structures were used in the analysis of pupal score and spore load, again in the LME4 package. Models were fitted using maximum likelihood, to allow for comparison of models with different fixed effect structures. Minimal adequate models were derived by the stepwise removal of terms followed by model comparison based on likelihood ratios, using the ANOVA function in R; terms were retained if their removal significantly ($P < 0{\cdot}05$) reduced the explanatory power of the model (Crawley 2007). Following model simplification, minimal adequate models were fitted using restricted maximum likelihood (REML) to produce less biased estimates of standard deviations (Bolker *et al.* 2009).

Secondary chemicals in plants, monarchs and eggs

Total cardenolide concentrations in plants, butterflies and eggs were log-transformed prior to analysis to meet assumptions of homogeneity of variance. To compare total cardenolide concentrations in adult butterflies, we used mixed model analysis of variance, with larval host plant and butterfly sex as fixed effects and family of origin as a random effect. To compare total cardenolide concentrations in monarch eggs, we used mixed model analysis of variance with paternal and maternal host plant as fixed effects and the combination of families in the mating as a random effect. To compare total cardenolide concentrations between milkweed species, and to assess any changes in plant chemistry over the course of the experiment, we used a general linear model, with milkweed species as a class variable and Julian date as a continuous variable (individual plants were never used on more than a single day, so repeated-measures analysis is not appropriate here). Models were run using SAS 9.2 for Windows (SAS Institute Inc., Cary, NC, USA).

To compare the composition of cardenolides (i.e. the presence of cardenolide peaks and their relative concentrations) in monarch eggs, we used permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) following Bray–Curtis ordination with maternal and paternal host plant as independent variables. Analysis was conducted using the Adonis procedure of the VEGAN package in R version 2.7.1 (Oksanen *et al.* 2013). Note that eggs from four mated pairs were dropped from this analysis because they contained no cardenolides at all and therefore could not be analysed by PERMANOVA. We used the same technique to compare the cardenolide composition of the eggs from experiments 1 and 2.

To visualize differences in the cardenolide composition of milkweed plants, monarch adults and eggs, we used metaMDS in Vegan for Nonmetric Multidimensional Scaling (NMDS) (McCune & Grace 2002), stepping down from a six-dimensional model to a one-dimensional model, with 999 permutations per model run and a maximum of 20 runs per dimension. Inspection of the scree plot illustrated that model stress declined rapidly from a one-dimensional to a two-dimensional model, declining only slightly thereafter. We therefore used a two-dimensional model for visualization [model stress = 0·1251, well within the range that is typical of ecological data (McCune & Grace 2002)]. We used the NMDS coordinates from this analysis to plot the position of plants, butterflies and eggs in multidimensional cardenolide space.

Potential relationships between chemical provisioning of eggs and disease resistance

Cardenolides were extracted from the monarch eggs collected during experiments 1 and 2 (above) and quantified using the same methods outlined in the previous section. Because chemical analysis required destructive sampling, we could not compare the cardenolide profile of the eggs directly with infection of the offspring hatching from those eggs. Instead, we measured cardenolides in one set of eggs, and disease resistance in another set of eggs collected at the same time from the same mated female. We performed two sets of analyses with these data: first using cardenolide measures and pupal scores averaged for each of the four parental food plant groups, and second using cardenolide measures and pupal scores averaged across sibling groups from mating pairs where the mother was reared on *A. incarnata*. In the second analysis, we restricted our analysis to females reared on *A. incarnata* because this is the group in which male diet had a significant effect on egg cardenolide concentration (described below). Because we analysed average values across groups in this section, instead of values for each individual offspring as we did in the above section, there was no random effect corresponding to maternal identity. Therefore, instead of mixed effect models, we used linear models to assess the effects of total cardenolide concentration, cardenolide polarity and cardenolide profile (NMDS axes 1 and 2) on pupal score. We used pupal score as a measure of infection intensity because it shows a strong relationship with parasite burden (De Roode *et al.* 2009) and it was the only measure taken in both experiments. Full models included one of the cardenolide measurements (i.e. concentration, polarity, NMDS axes 1 or axes 2) and experiment as factors, as well as the interaction between the cardenolide measure and experiment. Minimal adequate models were derived by the stepwise removal of terms followed by model comparison using the ANOVA function in R (Crawley 2007).

Results

EFFECTS OF PARENTAL DIET ON PARASITE RESISTANCE

In both experiments, pupal scores tended to be lower when parents had been reared on *A. curassavica* than

when they had been reared on *A. incarnata* (Fig. 1a). Although the effects of paternal food plant ($\chi^2 = 0{\cdot}445$, d.f. = 1, $P = 0{\cdot}505$; Table 1) and maternal food plant ($\chi^2 = 0{\cdot}814$, d.f. = 1, $P = 0{\cdot}367$) on offspring infection score were not significant in the analysis of experiment 1, monarch butterflies in experiment 2 experienced significantly lower pupal infection scores when their fathers had been reared on *A. curassavica* compared to monarchs with fathers reared on *A. incarnata*, regardless of whether their mothers had been reared on *A. curassavica* or *A. incarnata* ($\chi^2 = 4{\cdot}72$, d.f. = 1, $P = 0{\cdot}030$; Table 1). Although the trend was non-significant in experiment 1 (likely due to small sample size), the rank order of infection scores by parental food plant was consistent across both experiments (Fig. 1a) and paternal food plant was also significant ($\chi^2 = 5{\cdot}31$, d.f. = 1, $P = 0{\cdot}021$; Table 2) in the combined analysis of experiments 1 and 2. Pupal score is a combined measure of resistance to becoming infected and resistance to the subsequent reproduction of the parasites in those monarchs that become infected. In the combined analysis, experiment was also significant ($\chi^2 = 9{\cdot}54$, d.f. = 1, $P = 0{\cdot}002$), which is expected given the difference in the inoculation dose; critically, however, there were no significant interactions between experiment and parental food plant, which indicates that the effects of parental diet were consistent across the two experiments. The fixed effects retained in all minimal adequate models are listed in Table 3 (experiments separated) and Table 4 (experiments combined).

In the second experiment, we also measured parasite spore load in infected monarchs more precisely, using a haemocytometer, and we measured larval development, adult mass and adult longevity in both infected and uninfected monarchs as measures of general vigour (Fig. S1, Supporting information). The relationship between pupal score and spore load was highly significant ($F_{1,443} = 906$, $R^2 = 0{\cdot}67$, $P < 0{\cdot}001$), which is consistent with results reported in previous work (De Roode *et al.* 2009). Again, we found lower parasite growth in infected monarchs whose fathers had been reared on *A. curassavica* than in those whose fathers had been reared on *A. incarnata* ($\chi^2 = 5{\cdot}25$, d.f. = 1, $P = 0{\cdot}022$; Fig. 1b; Table 2). We found no significant main effects of either parental food plant species on our measures of general vigour in uninfected monarchs (Table S1, Supporting information), suggesting that the observed effects of parental diet on infection were not due to enhanced general vigour of offspring.

In addition to parasite growth within monarchs, we also compared the probability of parasite infection among parental diet treatments. Although there was a trend that fewer monarchs became infected when their fathers had been reared on *A. curassavica*, this trend was non-significant in the fully factorial model for experiment 1 and marginally non-significant in the fully factorial model for experiment 2 and in the combined analysis of both experiments (Tables 1 and 2).

Table 3. Parameter estimates and intercepts for fixed effects remaining in minimal adequate models for three measures of disease resistance, separated by experiment. Models were fitted using the LME4 package in R with restricted maximum likelihood (REML) for pupal score and spore load, and Laplace approximation for infection probability

Experiment 1			
Pupal score	Estimate	SE	*t* value
Intercept	0·825	0·157	5·26
Infection probability	Estimate	SE	*z* value
Intercept	0·078	0·247	0·315
Experiment 2			
Pupal score	Estimate	SE	*t* value
Intercept	1·23	0·140	8·83
Paternal food plant	0·434	0·198	2·19
Infection probability	Estimate	SE	*z* value
Intercept	0·571	0·171	3·34

Table 4. Parameter estimates and intercepts for fixed effects in minimal adequate models for three measures of disease resistance, experiments 1 and 2 combined with experiment included in the models as a fixed effect. Models were fitted using the LME4 package in R with restricted maximum likelihood (REML) for pupal score and spore load, and Laplace approximation for infection probability

	Estimate	SE	*t* value
Pupal score			
Intercept	1·26	0·130	9·64
Paternal food plant	0·391	0·169	2·31
Experiment	−0·656	0·201	−3·26
Log_{10}(spore load)			
Intercept	5·20	0·048	109
Paternal food plant	0·143	0·062	2·29
	Estimate	**SE**	***z* value**
Infection probability			
Intercept	0·454	0·143	3·18

SECONDARY CHEMICALS IN PLANTS, MONARCHS AND EGGS

As expected based on the previous studies (Agrawal & Fishbein 2008; De Roode *et al.* 2008), we found much higher concentrations of cardenolides in foliage from *A. curassavica* than from *A. incarnata* ($F_{1,44} = 241{\cdot}61$, $P < 0{\cdot}0001$). Total foliar cardenolide concentrations were over 4·5-fold higher in *A. curassavica* ($1{\cdot}67 \pm 0{\cdot}15$ mg g^{-1} dry leaf weight) than in *A. incarnata* ($0{\cdot}36 \pm 0{\cdot}02$ mg g^{-1}). Consistent with foliar cardenolide concentrations, butterflies reared on *A. curassavica* sequestered higher concentrations of cardenolides than did monarchs reared on *A. incarnata* (Fig. 2a; $F_{1,88} = 506{\cdot}73$, $P < 0{\cdot}0001$), with no significant difference between male and female monarchs reared on the same milkweed species ($F_{1,88} = 1{\cdot}00$, $P = 0{\cdot}3198$).

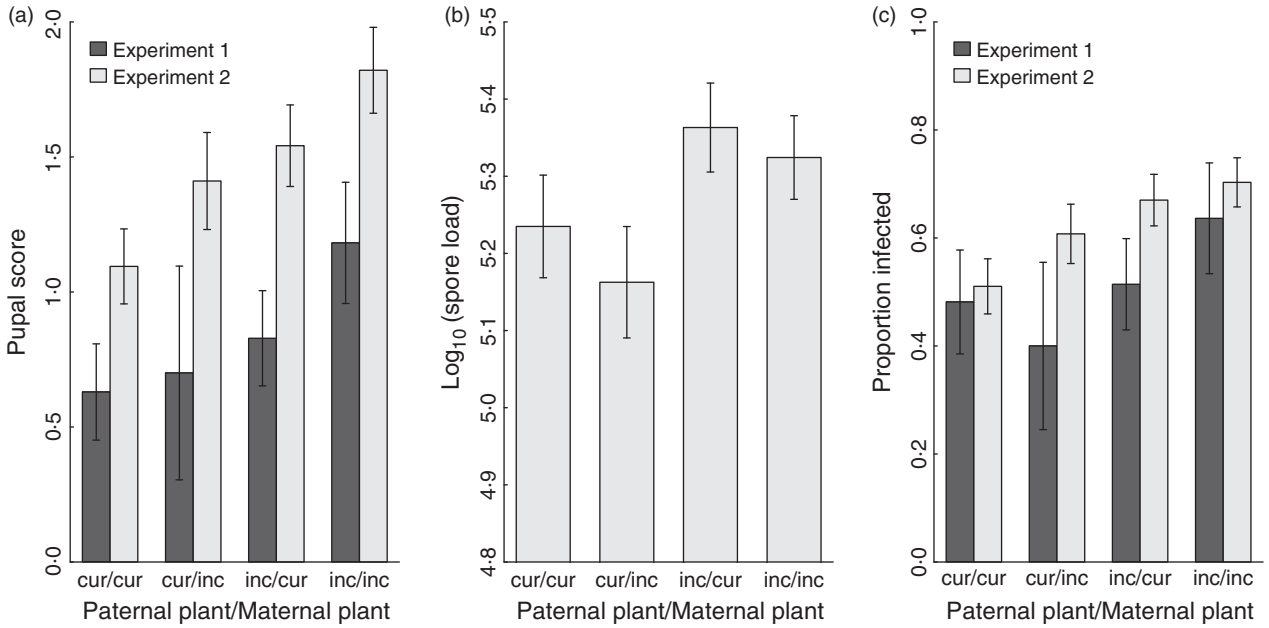

Fig. 1. (a) Pupal score (a combined measure of parasite infection probability and subsequent parasite growth) of monarch butterflies whose parents had been reared on *Asclepias curassavica* (cur; medicinal) and *Asclepias incarnata* (inc; non-medicinal). There was a 1·4-fold (experiment 1) to 1·5-fold (experiment 2) increase in pupal score for monarchs if their father was reared on *A. incarnata* relative to monarchs with fathers reared on *A. curassavica*. If both parents were reared on *A. incarnata*, there was a 1·7-fold increase (experiment 2) to 1·9-fold increase compared to monarchs with both parents reared on *A. curassavica*. (b) Log_{10} spore load (a measure of parasite growth) for infected monarchs whose parents had been reared on *A. curassavica* and *A. incarnata* (experiment 2 only). There was a 1·4-fold increase in untransformed spore load when fathers were reared on *A. incarnata* compared to monarchs with fathers reared on *A. curassavica* and a 1·2-fold increase if both parents were reared on *A. curassavica* compared to *A. incarnata*. (c) Proportion of monarchs that became infected, depending on whether their parents had been reared on *A. curassavica* or *A. incarnata*. For both experiments, monarchs with fathers reared on *A. incarnata* had a 1·2-fold increase in the probability of infection compared to monarchs with fathers reared on *A. curassavica*. Monarchs with both parents reared on *A. incarnata* had a 1·3-fold (experiment 1) to 1·4-fold (experiment 2) increase in the probability of infection, compared to monarchs with both parents reared on *A. curassavica*. Dark and light grey bars represent experiments 1 and 2, respectively; spore load was measured only in the second experiment; all figures show mean ± SE.

Both maternal and paternal diets influenced the cardenolide composition of monarch eggs. Total cardenolide concentrations in the eggs were significantly higher when either the mother ($F_{1,43} = 142{\cdot}18$, $P < 0{\cdot}0001$) or father ($F_{1,43} = 5{\cdot}36$, $P = 0{\cdot}0254$) had fed on *A. curassavica* (Fig. 2b). Moreover, the relative contribution to egg cardenolides from fathers fed on *A. curassavica* was much greater when mothers had fed on the low-cardenolide *A. incarnata* (significant paternal plant by maternal plant interaction: $F_{1,43} = 4{\cdot}78$, $P = 0{\cdot}0343$, Fig. 2b). In addition to influencing the total concentration of egg cardenolides, maternal ($F_{1,39} = 26{\cdot}10$, $P < 0{\cdot}001$) and paternal ($F_{1,39} = 4{\cdot}13$, $P = 0{\cdot}009$) diet influenced the composition, that is peak identity and relative concentration, of egg cardenolides (Fig. 2c). Lastly, the composition of cardenolides differed significantly between the milkweed plants, adult butterflies and eggs that we sampled in this experiment (Fig. 2c; $F_{2,185} = 42{\cdot}25$, $P < 0{\cdot}001$).

POTENTIAL RELATIONSHIPS BETWEEN CHEMICAL PROVISIONING OF EGGS AND DISEASE RESISTANCE

Across the experiments described above, monarch eggs contained the highest concentrations of cardenolides and larvae experienced the lowest risk of parasite infection when both parents had been fed on *A. curassavica*. Likewise, eggs contained the lowest concentrations of cardenolides and larvae experienced the highest risk of parasite infection when both parents had been fed on *A. incarnata*. These results suggest that the transfer of the medicinal properties of milkweeds to monarch offspring could be explained through the provisioning of eggs with cardenolides.

Indeed, across treatment means, the cardenolide profile of monarch eggs was a significant predictor of pupal infection score (Fig. 3c, NMDS1 association with average pupal score $F_{1,6} = 6{\cdot}96$, $P = 0{\cdot}046$, with experiment in the model). However, despite some apparent trends in the data (Fig. 3a,d), there were no other significant relationships between egg cardenolides and average pupal infection score (total cardenolides: $F_{1,6} = 4{\cdot}28$, $P = 0{\cdot}093$; polarity: $F_{1,6} = 4{\cdot}85$, $P = 0{\cdot}079$; NMDS2: $F_{1,6} = 0{\cdot}150$, $P = 0{\cdot}715$). Moreover, when we analysed these data at the level of the individual females reared on *A. incarnata* using pupal scores averaged across sibling groups, we found no significant relationships between pupal infection scores and measures of egg chemistry (Fig. 3e–h). In all the analyses, experiment was a significant predictor of

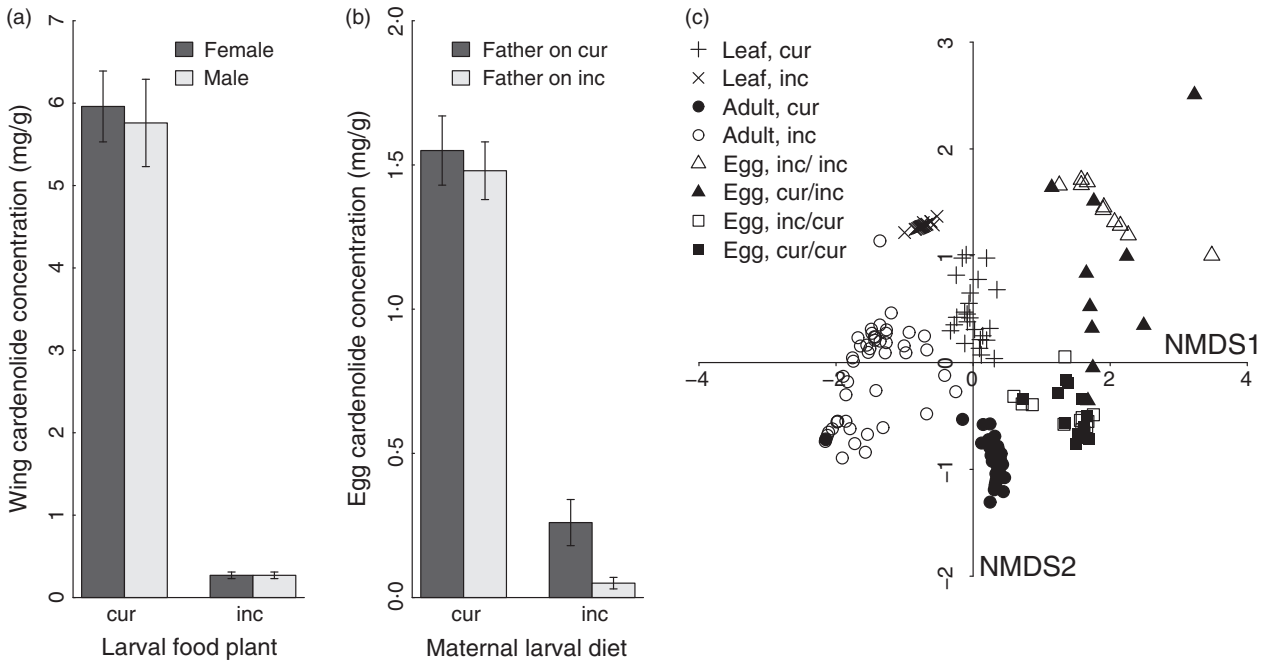

Fig. 2. (a) Cardenolide concentrations in the wings of adult monarch butterflies that were fed either *Asclepias curassavica* (cur) or *Asclepias incarnata* as larvae (inc). (b) Total concentration of cardenolides in the eggs of monarch butterflies fed either *A. curassavica* or *A. incarnata* as larvae. (c) Nonmetric Multidimensional Scaling (NMDS) plots of the cardenolide chemistry of milkweed plants (leaf), adult monarch wings (adult) and monarch eggs (egg). Notations 'inc' and 'cur' represent the milkweed species, larval food plant and parental larval food plant respectively; parental food plant is given as paternal/maternal food plant. (a) and (b) show mean ± SE.

pupal infection score, which was expected given the difference in inoculation doses between experiments 1 and 2.

Discussion

Until recently, trans-generational immunity was deemed unlikely in invertebrates because of their lack of antibodies (Little *et al.* 2003). However, our results add to a growing body of work demonstrating that invertebrates can still provide their offspring with protection against parasites (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006; Sadd & Schmid-Hempel 2007; Freitek, Heckel & Vogel 2009; Tidbury, Pedersen & Boots 2012). Importantly, our results demonstrate that trans-generational parasite protection is not exclusively maternal and that males may play a role in providing this protection. Paternal effects could be particularly important in species where males can have a large effect on the offspring during their initial development, for example sea horses and related species which have male pregnancy, or insects where males transfer large spermatophores during mating (Roth *et al.* 2010, 2012; Zanchi *et al.* 2011; Triggs & Knell 2012). Moreover, our study illustrates that trans-generational parasite protection could be based on the medicinal properties of parental diet, whether based on defensive chemicals or nutritional properties, thereby suggesting a novel mechanism for the trans-generational transfer of parasite resistance.

These results lay the groundwork for future studies of infection-dependent allocation of chemical defences against parasites, particularly by fathers. Many studies on immune priming have shown that infection with parasites alters the amount and specificity of immunity that parents transfer to their offspring (Little *et al.* 2003; Moret 2006; Sadd & Schmid-Hempel 2007). Further experiments are necessary to test whether parasite exposure increases the transfer of environmentally obtained protective chemicals in this and other systems. Alternatively, such environmentally obtained protection might be transferred passively. As part of such experiments, it will also be important to determine the potential costs to offspring fitness from such transfers. For example, in the monarch–parasite system, increasing cardenolide concentrations provides greater resistance to parasites up to a certain level, but above that level, concentrations can be so high that they start to be detrimental to monarch larvae and adults (Zalucki, Brower & Malcolm 1990; Zalucki, Brower & Alonso 2001; Zalucki *et al.* 2001; Sternberg *et al.* 2012). Here, we did not find evidence of such costs when we considered measures of general vigour in both infected and uninfected offspring (Supporting Information), which suggests that it might be beneficial for male monarchs to always transfer protective chemicals, regardless of the potential for exposure to parasites. Alternatively, our three measures of general vigour might not reflect the costs associated with different parental diets.

Theory predicts that immune priming in invertebrates has important implications for population dynamics in host–parasite systems. Simple epidemiological models that include primed hosts produce cycles in the densities of

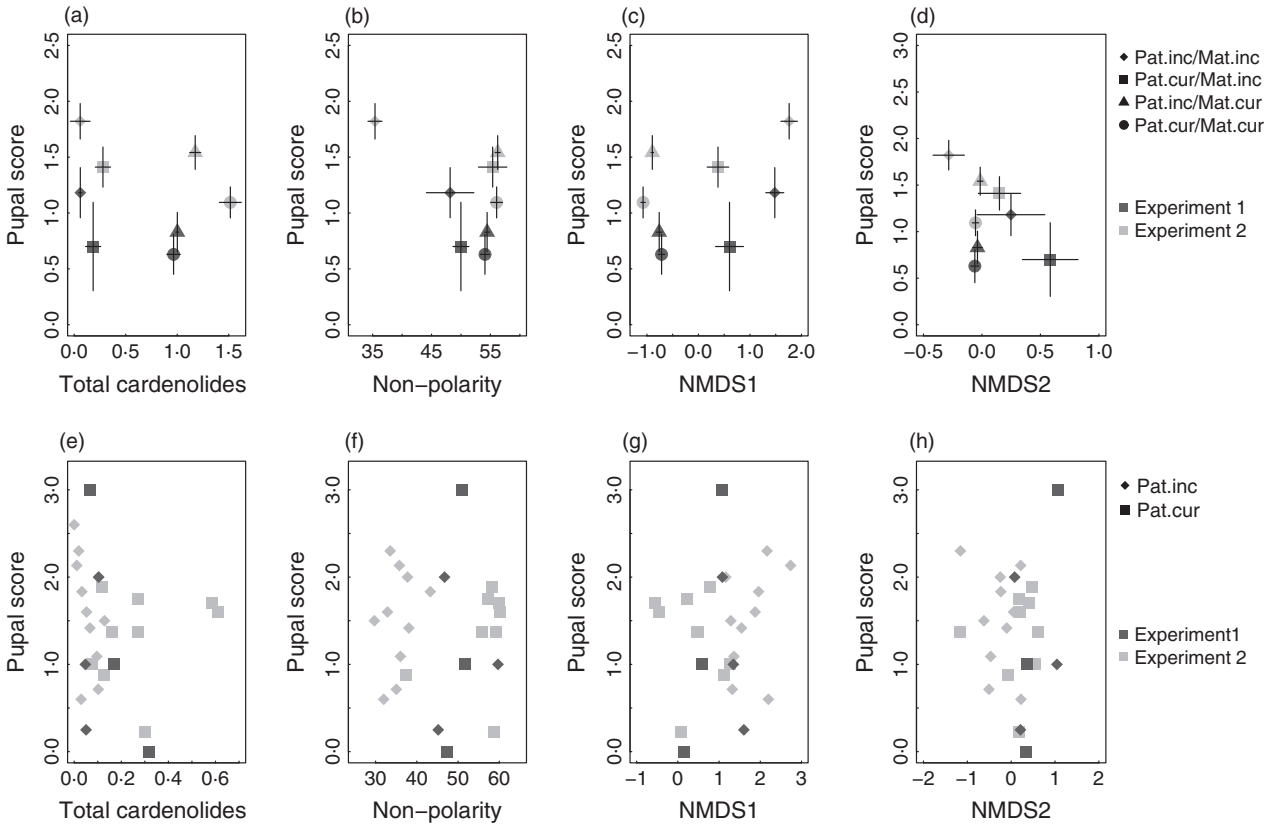

Fig. 3. Relationships between egg chemistry and parasite resistance. Panels a–d show the relationships between the mean pupal scores by parental food treatment and (a) total cardenolide concentration, (b) non-polarity, (c) Nonmetric Multidimensional Scaling (NMDS) axis 1 and (d) NMDS axis 2. Panels e–h show the relationships between the mean pupal scores by sibling groups from mothers reared on *A. incarnata* and (e) total cardenolide concentration, (f) non-polarity, (g) NMDS axis 1 and (h) NMDS axis 2.

infected individuals, while models with few or no primed hosts reach stable equilibria. As a result, primed individuals may act to destabilize populations and prolong the persistence of pathogens (Tidbury, Best & Boots 2012). In contrast, models that include stage-specific, within-generation and trans-generational immune priming produce dramatic reductions in disease prevalence under equilibrium conditions. Moreover, in models where larval stages are disproportionately affected by pathogens (i.e. transmission is greater in the larval stage), trans-generational immune priming reduces infection prevalence in the population relative to models with equivalent levels of within-generation immune priming (Tate & Rudolph 2012). Although the monarch system differs from these simulations in that transmission to larvae depends on the presence of infected adults and not infected larvae, these results suggest that the effects of within-generation vs. trans-generational immune priming are not symmetrical if one life stage is disproportionally affected by infection, as is the case in the monarch system. Demonstrating that parents can acquire parasite protection from their environment and pass it on to their offspring opens the door more widely to potential mechanisms for trans-generational protection. It will be important to explore how such trans-generational protection affects host–parasite dynamics and consequently co-evolution. It will also be important to explore how passive transfer, as shown here, compares to active priming, resulting from infection, in its effect on ecological and evolutionary dynamics.

While our second, larger experiment shows that paternal diet significantly influences the parasite resistance of monarch offspring, consistent with the trend suggested in our initial, pilot experiment (Fig. 1), and that males contribute to the cardenolide content of eggs (Fig. 2), the link between egg cardenolides and subsequent parasite growth in offspring is equivocal (Fig. 3). Cardenolides have been implicated strongly in the resistance of monarch larvae to parasites in multiple previous studies (De Roode *et al.* 2008, 2011b; Lefèvre *et al.* 2010, 2012; Sternberg *et al.* 2012) and, in support of the link, we found a trend of decreasing pupal infection score with increasing egg cardenolide concentrations across the four parental diet treatments. When both parents were fed on *A. curassavica*, eggs had the highest cardenolide content and the greatest offspring protection. Likewise, when both parents fed on *A. incarnata*, eggs had the lowest cardenolide content and the lowest offspring protection. Additionally, we observed a significant relationship between the cardenolide profile of eggs and the subsequent pupal infection score of monarchs across treatment groups (Fig. 3c). However, we

found no relationship between egg cardenolide content and offspring resistance across individual mothers (Fig. 3e–h). Moreover, maternal diet had a greater impact on egg cardenolide concentration than did paternal diet (Fig. 2), yet paternal diet had a greater influence on offspring resistance than did maternal diet (Fig. 1).

There are at least three – non-mutually exclusive – explanations for the equivocal relationships between egg cardenolides and offspring protection. First, it is important to note that we were unable to directly compare the cardenolide concentration of a single egg with subsequent infection of the monarch hatching from that egg because quantification of egg cardenolides is a destructive process. Instead, we measured average cardenolide concentrations across 50 eggs laid by each female and the average pupal score across a different subset of her offspring. As cardenolide concentrations vary among eggs and because monarch offspring vary in their genetic resistance to parasitism (De Roode & Altizer 2010; Lefèvre, Williams & De Roode 2011), it is possible that the averaging of our data at the level of individual mothers obscured any significant association. Second, it is possible that although monarch eggs contained higher overall concentrations of cardenolides when parents were reared on *A. curassavica*, they did not contain the specific cardenolides that maximize antiparasitic resistance. Previous studies have implicated non-polar cardenolides as the most toxic (Fordyce & Malcolm 2000) and pharmaceutically active against monarch parasites (De Roode *et al.* 2011b; Sternberg *et al.* 2012), and the most non-polar of the cardenolides in the leaves of *A. curassavica* were not found in the monarch eggs that we analysed. Very high concentrations of non-polar cardenolides are detrimental to larval performance (Sternberg *et al.* 2012), and the trade-off between antiparasitic activity and toxicity may explain why monarchs do not sequester these cardenolides in their eggs. Finally, cardenolides in monarch eggs may interact with other components of parental diet that influence the susceptibility of their offspring to parasites (Triggs & Knell 2012). Further, studies – including those with artificial diets in which cardenolide concentrations can be manipulated directly – are needed to clarify the mechanism(s) underlying the trans-generational protection observed in our study. Although we observed the same pattern in offspring disease resistance across two experiments, the observed effect size was relatively modest. Experimental manipulation of cardenolide concentrations via artificial diet will be useful to strengthen and expand our results.

Regardless of the exact mechanism, our study shows that trans-generational protection from parental diet is not exclusively maternal and that males play a pivotal role in providing this protection. A few recent studies have provided evidence that immune priming can be passed on through the father (Roth *et al.* 2010; Zanchi *et al.* 2011), which has been termed 'cryptic parental care' (Jokela 2010). Selection may act to maintain paternally derived protection because of a positive impact on offspring fitness and hence the inclusive fitness of males. In addition, trans-generational protection may be maintained through sexual selection. Females may select males based on the nutritive qualities of their spermatophores (Thornhill 1976; Eisner & Meinwald 1995; Vahed 1998; Stålhandske 2001), and we suggest that females may also select for males that are able to provision their offspring with protection from parasites. Alternatively, or additionally, females may be able to adjust their investment in offspring dependent on the paternal transfer of protective chemicals. Female choice or differential investment may be particularly important in the monarch system because *O. elektroscirrha* is often transmitted from parents to offspring. Thus, infection risk in the next generation of monarch butterflies is tightly linked to infection in the previous generation, creating the potential for strong selection for trans-generational protection. Beyond monarch butterflies, parents from many phyla of organisms transmit pathogenic and non-pathogenic microbes to their offspring (Funkhouser & Bordenstein 2013). As such, paternal trans-generational protection from parasites might be selectively advantageous in a variety of systems and more widespread than previously thought.

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Data accessibility

Data available from the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.75c77 (Sternberg, de Roode & Hunter 2014).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Measures of general vigor for offspring in experiment 2.

Table S1. Analysis of variance in three measures of general vigor using mixed effects models.