A virulent parasite can provide protection against a lethal parasitoid

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ABSTRACT

Hosts often become infected with multiple parasite strains or species. Previous work has shown that the outcome of infections with multiple parasite strains or species often differs significantly from that of single infections, making them a potentially important factor in determining the prevalence and spread of disease. Here we show that infection with a virulent parasite increases host survival during later exposure to a lethal parasitoid. Specifically, when monarch butterfly larvae ( Danaus plexippus ) are inoculated with the virulent protozoan parasite Ophryocystis elektroscirrha and then attacked by the lethal parasitoid fly Lespesia archippivora, survival is higher than when the larvae are exposed to the parasitoid only. This is potentially a result of the protozoan’s requirement for host survival to obtain between-host transmission. Our findings suggest that a virulent parasite can play a protective role for its host and indicate that parasites can act as mutualists depending on the presence of other parasites. We emphasize the importance of considering infection in an ecological context, including the presence of competing parasites.

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1. Introduction

In laboratory studies of infectious diseases, experimental infections typically consist of a single parasite strain or species. However, infection of a host with multiple parasite strains or species occurs frequently outside of the laboratory (Cox, 2001; Petney and Andrews, 1998; Rigaud et al., 2010). In natural populations, the parasites infecting a host can range from multiple strains of the same species (Bharaj et al., 2008; Lord et al., 1999) to different species with varying degrees of taxonomical distance (Cattadori et al., 2007; Craig et al., 2008; Petney and Andrews, 1998; Rutrecht and Brown, 2008). Previous work has demonstrated that the effect of co-occurring parasites on the host is not necessarily the additive effect of each single infection (Druhlke et al., 2005; Haine et al., 2005; Malakar et al., 1998; Pedersen and Fenton, 2006; Thomas et al., 2002). For example, infection with multiple parasite strains or species may benefit the host if the parasites use similar host resources, thus resulting in the competitive suppression of parasite growth (Berchieri and Barrow, 1990; Dobson and Barnes, 1995; Ishii et al., 2002; Read and Taylor, 2001). Alternatively, the host may incur a greater cost from infection with multiple parasites strains or species, for example due to collateral damage from intense competition between parasites (Read and Taylor, 2001; Roper et al., 1998).

From the parasite’s perspective, there can be benefits to infecting a host with other parasites, e.g., if one of the parasites dampens the host immune response (Cattadori et al., 2007; Graham, 2008; Su et al., 2005) or if one of the parasites facilitates infection or transmission of the other parasite (Friedi and Bacher, 2001; Hughes and Boomsma, 2004; Poulin et al., 2003). However, there can also be costs if the parasites overlap extensively in their host resource use (Hochberg, 1991; Ishii et al., 2002) or if one species alters the host environment in a way that makes it inhospitable to another parasite, for example through the activation of the host immune response (Dobson and Barnes, 1995; Lello et al., 2004). Negative interactions can occur between parasitoids and microparasites (e.g., bacteria and viruses), for example when parasite-induced host death occurs too quickly for the second parasite to develop fully, thereby blocking the transmission of the second parasite (Chilcutt and Tabashnik, 1997; Escríbano et al., 2000). In these examples, the amount of time between infections often determines whether the second parasite is able to transmit upon host death. In addition to the temporal spacing of infection, the order of infection can modify the effect of multiple infections on host and parasites. Simultaneous infections may have a different outcome than sequential infections and in some systems (but not all: Lohr et al., 2010), the effect of multiple infection on the parasites depends on which parasite has prior residency in the host (De Roode et al., 2005).

All of the examples above illustrate that the outcome of infection with multiple parasites can diverge greatly from that of single infections, and that the different biological characteristics of the host and parasite species determine the outcome of infection. Furthermore, theoretical work has shown that the impact of
co-occurring parasites extends to the evolution of hosts and parasites (Rigaud et al., 2010), for example, by affecting the selection pressures that drive parasite virulence (Brown et al., 2002; Choisy and De Roode, 2010; Van Baalen and Sabelis, 1995), facilitating the emergence of novel pathogen strains (Lloyd-Smith et al., 2008), and altering the antagonistic co-evolutionary feedback between host and parasite (Mostowy et al., 2010).

Even with increasing awareness of both the ubiquity and importance of multi-parasite infections, there are relatively few examples of studies in systems where transmission of one parasite is completely blocked by the successful transmission of a second parasite. In the literature that does exist on this subject, the examples predominantly come from systems in which one parasite is transmitted trophically (through predation by a definitive host on the intermediate host) while a second parasite is either not trophically transmitted or requires a different definitive host species (Cezilly et al., 2000; Haine et al., 2005; Rigaud and Haine, 2005; Thomas et al., 2002). In these cases, there is evidence that parasites can rescue their hosts from the potentially lethal effects of a second parasite if host survival is required for transmission of the first parasite (Cezilly et al., 2000; Haine et al., 2005). For example, the amphipod Gammarus roeseli serves as a host for both the trophically transmitted acanthocephalan Polymorphus minutus and the vertically transmitted microsporidian Dicyo corda sp. (roeselum). P. minutus induces behavioral changes in G. roeseli to increase predation by its definitive host; however, in the presence of the microsporidium, this behavioral manipulation is reduced (Haine et al., 2005).

Based on these results, we expect to find a similar outcome in other systems where transmission of one parasite is blocked by a second parasite causing extensive damage to their shared host. This type of interaction occurs between the monarch butterfly (Danais plexippus) and two of its most common parasites, the virulent protozoan Ophryocystis elektroscirrhna and the lethal parasitoid fly Lespesia archippivora. Transmission of the protozoan O. elektroscirrhna occurs exclusively through the transfer of spores from adult butterflies to larvae, and hence depends on the survival of infected monarchs to the adult stage (De Roode et al., 2008b; McLaughlin and Myers, 1970). In contrast, the fly L. archippivora lays eggs onto monarch larvae, after which the eggs hatch and the maggots penetrate the larvae, consume them from the inside out, and emerge at the monarchs’ prepupal or pupal stage, killing the monarchs in the process. These differences in life cycle would suggest that L. archippivora prevents transmission of O. elektroscir rhna when it kills the host during the pre-adult stages. However, as an alternative hypothesis, it is possible that the protozoan parasite reduces the infection success of the lethal fly, and thereby alleviates its own fitness loss as well as that of the host. Here, we address these hypotheses by studying single and multiple infections of these two parasite species in laboratory experiments.

2. Materials and methods

2.1. The host–parasites system

The protozoan O. elektroscirrhna is a parasite that infects monarch butterflies across their natural range (Altizer et al., 2000; Leong et al., 1997; McLaughlin and Myers, 1970). Infection occurs when adult female butterflies shed parasite spores on their eggs or milkweed foliage during oviposition, after which these spores are ingested by hatching larvae; as a result, transmission occurs often from mother to offspring but may also occur from adult butterflies to unrelated larvae (Altizer et al., 2004; McLaughlin and Myers, 1970). Upon ingestion, parasite spores lyse in the larval gut to release sporozoites that invade the hypoderm, replicate asexually, and then form sexual spores on the outside of the developing butterfly. The production of these spores reduces monarch adult lifespan, mating ability and fecundity (De Roode et al., 2009; De Roode et al., 2007; De Roode et al., 2008b). O. elektroscirrhna does not continue to replicate once the adult monarch emerges, so the number of spores present on a newly emerged adult monarch represents the entire transmission potential of that infection. Importantly, O. elektroscirrhna requires its host to reach the adult stage, when the host can lay eggs and transfer these spores to hatching caterpillars (De Roode et al., 2009).

The parasitoid fly (L. archippivora) co-occurs with O. elektroscirrhna in the monarch populations inhabiting North America and Hawaii (Altizer et al., 2000; Etchegaray and Nishida, 1975; Leong et al., 1997; Oberhauser et al., 2007). Female L. archippivora deposits their eggs on the cuticle of larval monarchs and when these eggs hatch, the maggots burrow into the host. Previous work on L. archippivora indicates that this species typically limits its brood size to one to three offspring per host (Etchegaray and Nishida, 1975; Oberhauser et al., 2007; Stapel et al., 1997). When the host reaches its final instar or soon after it pupates, the fly maggots kill the host as they emerge to form pupae (Oberhauser et al., 2007; Stapel et al., 1997; Stireman et al., 2006). Thus, in contrast with the protozoan O. elektroscirrhna, the parasitoid fly L. archippivora kills the host during the pre-adult stage.

2.2. Host and parasite collection and care

This study consisted of two experiments conducted approximately one year apart. Experimental designs were similar, except for some minor differences as outlined below. All of the monarchs belonged to the migratory eastern North American population, and all of the parasites were isolated from wild-caught monarchs belonging to this population. Thus, both experiments used sympatric host and parasite combinations. The protozoan parasite used for both studies (denoted C1E3-P3-1) was isolated from a monarch belonging to the eastern North American population (Cape May, New Jersey, 2001). A clonal line of the parasite was used to prevent mixed-genotype infections and to provide consistency in protozoan parasite genotype across experiments. To establish the line, a monarch was inoculated with a single spore to produce an infection with genetically identical parasites. The parasite was then passaged through three monarchs and held at 12 °C between infections.

The monarchs used in this study were the grand-progeny of monarchs collected either as overwintering adults in Central Mexico (March 2008; experiment 1) or as larvae in Georgia, USA (September 2009; experiment 2). Unrelated females and males were mated in a design that produced independent families of half- or full siblings. Adults were held in mesh cages at 26 °C on a 16L:8D cycle and fed with a 10% honey water solution. After mating, males were removed and females were provided with greenhouse-grown Asclepias incarnata (swamp milkweed) for ovipositing. The plants were checked daily and those with eggs were replaced with fresh plants. Hatching larvae were pooled by hatch date and family, and transferred into plastic containers (739 mL) with fresh As. incarnata cuttings. Individuals from each of the families were randomly distributed across all treatment groups for both experiments.

The parasitoid flies used for this study came from a laboratory colony descended from maggots that emerged from monarchs collected as larvae in Ohio, USA (June 2008; experiment 1) or Georgia, USA (September 2009; experiment 2). Upon emergence from the monarch larvae, the maggots were transferred either into 1L plastic containers (experiment 1) or into a 50.8 cm × 27.9 cm × 33.0 cm glass terrarium fitted with a screened lid (experiment 2). When adult flies eclosed, they were provided with sugar, dehydrated milk and a moistened cotton ball for water. To establish and maintain the colony,
flies were given ≥24 h to mate, then provided with 3rd instar monarch larvae for ovipositing. Once the monarch larvae had been attacked by flies, they were pooled into plastic containers and provided with fresh A. incarnata cuttings. When maggots emerged from these monarch larvae, they were collected and added to the existing colony.

2.3. Experiment 1

Monarchs (n = 292) received one of four treatments: (1) uninfected control (n = 18); (2) exposure to only the protozoan parasite O. elektroscirrha (n = 38); (3) exposure to only the parasitoid fly L. archippivora (n = 117); and (4) exposure to both the protozoan parasite and the parasitoid fly (n = 119). Larger sample sizes were used for the monarchs that were exposed to the parasitoid fly so that differences in low survival rates could be detected, even in the presence of the highly lethal parasitoid.

To infect monarchs with the protozoan parasite, 2-day-old 2nd instar monarch larvae were placed in individual 10 cm petri dishes with moist filter paper and a disc of A. incarnata leaf (0.8 cm in diameter) on which 10 O. elektroscirrha spores had been deposited manually (De Roode et al., 2007; De Roode et al., 2008a); uninfected controls and monarchs that were infected with the parasitoid fly only were fed a leaf disk without parasite spores. To infect monarchs with the parasitoid fly, we placed 4-day-old 3rd instar monarch larvae in the plastic containers housing the parasitoid flies and observed until a fly was seen approaching a larva and performing ovipositing behaviors. At this point the larva was removed and examined under a dissecting microscope for fly eggs. If fly eggs were not observed, the larva was returned to the container with flies. Once eggs were visible, the number of eggs present was recorded and the larva was not returned to the plastic container with the flies. In the multiple infection treatment, monarchs were first inoculated with the protozoan parasite (2 days post-hatching) and then exposed to the parasitoid (4 days post-hatching).

After treatment, monarch larvae were transferred into individual plastic containers covered with mesh tops. They were provided with fresh cuttings of greenhouse-grown A. incarnata in florist tubes. These containers were kept in a climate-controlled room (26 °C, 16L:8D) and checked daily. Fresh plant cuttings were added as needed. Monarch larvae that died prior to pupation were monitored for signs of parasitoid maggots. If maggots emerged, the number of maggots per host was recorded. If the monarch larvae survived to pupation, they were transferred into clean plastic containers and kept in the climate controlled room for an additional 7 days. Monarch pupae were also checked daily for signs of fly maggots. After 7 days, the monarch pupae were transferred to a separate room to prevent cross-contamination with spores from the protozoan parasite. When the adult monarchs eclosed, they were sexed and weighed and then transferred into individual glassine envelopes and kept at 12 °C. Adult monarchs were checked daily for mortality to measure post-eclosion longevity. This measure of lifespan provides a combined index of adult monarch life span and starvation resistance and responds to parasite infection and increasing parasite numbers in a similar way as lifespan under more natural conditions (De Roode et al., 2009).

2.4. Experiment 2

As with experiment 1, monarchs (n = 351) received one of four treatments: (1) uninfected control (n = 25); (2) exposure to only the protozoan parasite O. elektroscirrha (n = 32); (3) exposure to only the parasitoid fly L. archippivora (n = 155); and (4) exposure to both the protozoan parasite and the parasitoid fly (n = 139). Again, larger sample sizes were used for groups exposed to the parasitoid fly to enable the detection of differences in the low survival rates of fly-exposed monarchs.

Procedures for experiment 2 were similar to those for experiment 1, except for the following. First, to infect monarchs with parasitoid flies, 3rd instar monarch larvae were placed in the terrarium housing the L. archippivora colony and observed until a fly was seen approaching a larva and performing characteristic ovipositing behaviors, at which point the larva was removed from the terrarium. The larva was then examined under a dissecting microscope and the number of parasitoid fly eggs present recorded. Unlike experiment 1, larvae were not returned to the container with the flies even if there were no visible eggs. The results from experiment 1 had shown to us that we often missed eggs, as evidenced by the fact that some monarchs produced more maggots than the recorded number of eggs. This modified protocol was used to more accurately mimic the natural numbers of flies per infected monarch (in experiment 1 they exceeded those numbers). Second, larvae were reared as in experiment 1, except that Asclepias curassavica (tropical milkweed) was used in addition to A. incarnata to feed later stage caterpillars; the use of a second species of milkweed at this stage has no effects on the protozoan parasite (De Roode et al., in press) and parasitoid fly post-inoculation (M. Solensky, unpublished data). The host plant species that was provided was randomly allocated across treatment groups. Third, if fly maggots emerged from monarch larvae or pupae, the number of maggots per host and the mass of each maggot once it pupated were recorded, after which the fly pupae were placed in individual plastic containers. If a fly eclosed from its pupa, it was recorded to measure the proportion of flies that survived to adulthood. Adult flies were provided with sugar, dehydrated milk, and a moist cotton ball. Flies were checked daily for mortality to measure adult longevity. Fourth, after monarchs died, their bodies were vortexed and the protozoan parasite spore load was measured with a haemocytometer as described in De Roode et al. (2007, 2008a). Since the protozoan does not replicate on the adult monarch, this was used as a measure of parasite replication and transmission potential.

2.5. Statistics

All analyses were carried out in R version 2.7.1 (R Development Core Team, 2006). Logistic regression by Generalized Linear Model (GLM, binomial error distribution, logit link) was used to investigate the effect of treatment, number of parasitoid fly eggs, and experimental block (experiment 1 vs. 2) on the proportion of monarch hosts that survived to adulthood. A multiway Analysis of Variance (ANOVA) was used to analyze the effect of treatment and experimental block on monarch host longevity and in experiment 2 protozoan parasite spore load. Logistic regression (GLM, quasi-binomial distribution) was used to assess the effect of treatment and experimental block on the proportion of monarch hosts that produced fly parasitoid maggots. A GLM with a quasi-Poisson error distribution was used to analyze the effect of treatment on the number of fly parasitoid maggots that emerged from monarch hosts. For the data from experiment 2, a Generalized Linear Mixed Model (GLMM) with a normal error distribution was used to analyze the effect of treatment (fixed effect) and monarch host (random effect) on fly parasitoid mass and longevity. An Analysis of Covariance (ANCOVA) was used to test for a relationship between fly parasitoid pupal mass, adult longevity, and for an effect of treatment on this relationship. Protozoan parasite load and monarch host longevity were Log_e-transformed prior to analyses and models were checked for homogeneity of variance by using the Fligner–Killeen test (Crawley, 2007). In these analyses, treatment and experimental block were treated as categorical explanatory
variables. Full models included treatment, experimental block and the interaction between them as explanatory variables. Minimal models were derived by removing model terms followed by model comparison. Only terms for which removal significantly ($P < 0.05$) reduced the explanatory power of the model were retained in the minimal model (Crawley, 2007).

3. Results

3.1. Monarch host pre-adult survival

Parasitoid flies dramatically reduced the survival to adulthood of their monarch hosts, both in the presence of the protozoan parasite (Fig. 1; Odds Ratio ($OR = 25.0$, 95% Confidence Interval ($CI) = [24.1, 26.0]$, $P < 0.001$) and in single infections ($OR = 41.9$, $CI = [40.9, 42.9]$, $P < 0.001$). The protozoan parasite alone did not affect monarch host survival to adulthood; however, it did increase survival of monarchs that were also infected with the parasitoid fly (Fig. 1; $OR = 1.8$, $CI = [1.28, 2.27]$, $P = 0.0239$). Only 12% and 17% (experiments 1 and 2 respectively) of monarchs survived to adulthood when infected with the parasitoid fly alone, but when the protozoan parasite was also present, survival increased to 18% and 27% (experiments 1 and 2 respectively). The number of eggs laid by the parasitoid fly also had a significant effect on survival in monarchs that were exposed to the parasitoid fly ($OR = 4.12$, $CI = [3.72, 4.52]$, $P < 0.001$), with higher numbers resulting in lower survival. There were no significant interactions between the number of eggs and the treatment group. Overall, monarch pre-adult survival differed significantly between experiments ($OR = 1.9$, $CI = [1.34, 2.50]$, $P = 0.0281$) but there were no significant interactions between experiment and treatment or the number of eggs present.

3.2. Monarch host adult longevity

Analysis of monarch host longevity was performed on the monarchs that survived to adulthood. For experiment 1, there were 89 surviving monarchs (18 of 18 in the control group, 34 of 38 in the protozoan only group, 15 of 117 in the fly only group, and 22 of 119 in the protozoan and fly group). For experiment 2, there were 111 surviving monarchs (20 of 25 in the control group, 26 of 32 in the protozoan only group, 27 of 155 in the fly only group, and 38 of 139 in the protozoan and fly group).

The presence of the protozoan parasite had a significant effect on mean longevity (Fig. 2; $F_{1,185} = 695$, $P < 0.001$): both singly protozoan parasite-infected and multiply-infected monarchs lived much shorter as adults than control monarchs and monarchs that had survived single infection with the parasitoid fly. There was no significant effect of larval exposure to the parasitoid fly on adult longevity across all treatment groups ($F_{1,185} = 1.2$, $P = 0.271$). A significant interaction between experiment and treatment ($F_{1,185} = 62.0$, $P < 0.001$) arose from the fact that the protozoan parasite reduced adult monarch lifespan more strongly in experiment 1 than 2.

3.3. Protozoan parasite spore load

Analysis of the protozoan parasite spore load was carried out for the monarchs in experiment 2 that survived to adulthood ($n = 64$). All of the surviving monarchs that were inoculated with the protozoan parasite were infected. There was no significant difference in the mean spore loads of monarchs that survived attack by the parasitoid fly compared to monarchs that had been exposed to the protozoan parasite only ($F_{1,62} = 0.036$, $P = 0.85$).

Fig. 1. Monarch survival to adulthood by experiment and treatment group. The presence of the parasitoid fly resulted in a large decrease in survival for both the singly and multiply infected monarchs, but there was a higher percent survival for monarchs infected with the fly and the protozoan together compared to those that were infected with the fly only. Data are presented as percent survival ±SE.

Fig. 2. Mean adult longevity in days for monarchs that survived the larval stage, by experiment and treatment group. The presence of the protozoan parasite had a significant effect on host longevity in both single and multiple infection groups; however, in monarchs that survived attack by the parasitoid fly there was no additional decrease in adult longevity. Data are presented as mean adult longevity ±SE.
eggs and experiment, indicating that the proportion of monarchs that produced maggots increased more quickly with increasing numbers of eggs in experiment 1 than experiment 2 (OR = 3.78, CI = [3.06, 4.50], \(P < 0.001\)). The interaction between treatment and experiment was not significant.

As with the proportion of monarchs that produced maggots, the protozoan parasite did not significantly reduce the numbers of maggots that emerged from monarchs exposed to the parasitoid fly (Fig. 4; \(F_{1,525} = 0.423, P = 0.52\)). As expected, however, monarchs produced fewer maggots in experiment 2 than experiment 1 (\(F_{1,526} = 231, P < 0.001\)). There was no significant interaction between treatment and experiment.

3.5. Parasitoid fly pupal mass and adult longevity

Data on fly pupal mass and adult longevity were obtained for 249 flies (142 maggots from monarchs singly infected with the fly and 107 maggots from multiply-infected monarchs); 3 maggots in the protozoan and fly multiple infection group were removed from analysis due to incomplete data.

Comparison between fly single infections and protozoan and fly multiple infections showed that the protozoan parasite had no effect on the mean fly pupal mass (mean ± SE: 26.2 ± 1.1 and 27.0 ± 1.2 mg in single and multiple infections respectively; d.f. = 1, \(P = 0.851\)), on the proportion of fly pupae that successfully eclosed as adults (73.9% and 78.8% from single and multiple infection respectively; \(X^2 = 0.517, d.f. = 1, P = 0.472\)), or on the adult longevity of flies that eclosed successfully (18 ± 1 days for single and multiple infections; d.f. = 1, \(P = 0.83\)). Higher fly pupal mass resulted in greater fly adult longevity (Fig. 5; \(F_{1,185} = 22.4, R^2 = 0.103, P < 0.001\)) but there was no significant interaction with treatment group.

4. Discussion

Our results clearly demonstrate that infection of hosts with multiple parasite species may have important consequences for host and parasite fitness. Overall, the parasitoid fly *L. archippivora* had strong detrimental effects on its monarch host as well as on the co-occurring protozoan parasite *O. elektroscirrha*. *L. archippivora* caused dramatic pre-adult mortality of monarch butterflies and thereby strongly reduced *O. elektroscirrha*'s fitness as the latter parasite requires the monarch to reach adulthood for its transmission. However, despite the parasitoid’s overwhelming effects, we found that the protozoan parasite reduced the mortality caused by the parasitoid fly, with more monarchs surviving multiple infection with the protozoan and fly than infection with the fly alone (Fig. 1). This effect is beneficial to the host and resembles the protective effects that mutualists and commensals can confer. For example, previous research has shown that mutualistic bacterial symbionts of aphids can protect their host against parasitoid wasps and pathogenic fungi (Oliver et al., 2003; Scarborough et al., 2005; Vorburger et al., 2010), that Wolbachia bacteria can protect *Drosophila melanogaster* against viral infections (Hedges et al., 2008) and that *Spiroplasma* bacteria confer protection against a sterilizing nematode in *D. neotestacea* (Jaenike et al., 2010). Like symbionts, transmission of the monarch’s protozoan parasite depends on the survival of its host but unlike these bacterial symbionts, *O. elektroscirrha* is highly
virulent to monarchs, reducing adult longevity, mating ability, fecundity and flight ability (De Roode et al., 2007, 2008b; 2009; Bradley and Altizer, 2005). Thus, our results suggest that infection with a virulent parasite can be beneficial for a host when it confers protection against a parasite that is even more detrimental. However, this protection appears to be weaker than that provided by beneficial symbionts, perhaps because parasites face different constraints than symbionts. For instance, it is likely that a beneficial symbiont and host cooperate to resist lethal parasitoids; in contrast, although it may be beneficial to one parasite to reduce the virulence induced by another, it is in the host’s interest to resist both.

The increased survival of monarchs infected with both parasites is not only beneficial to the host, but also to the protozoan parasite, suggesting that this effect may be an adaptive parasite trait. Parasites are well known to change the phenotypes of their hosts in ways that serve a specific adaptive function for the parasite (Lefèvre et al., 2009; Moore, 2002; Poulin, 2010; Thomas et al., 2005). For example, parasites often modify the behaviors of their host to prevent infection with a competing parasite (Brodeur and McNeil, 1992), to prevent predation (Grosman et al., 2008); or to increase transmission to the definitive host (Lagruè et al., 2007). Based on these studies, it is possible that the increase in host survival that we observed may also serve an adaptive function for the protozoan parasite due to the protozoan parasite’s requirement that the monarch host survive to adulthood for transmission to occur. If this is the case, we would expect that the parasite’s ability to increase host survival is selected for in populations where the parasitoid occurs and is highly prevalent. Since this study tested the effect of a single genotype of the protozoan parasite, future experiments are necessary to determine if there is variation among protozoan parasite genotypes in their protective effect. However, since all genotypes of the protozoan parasite require host survival to the adult stage for transmission to occur, we expect to see a similar effect with other parasite genotypes.

One challenge to understanding the protozoan parasite’s protective effect will be to explain how the protozoan reduced monarch pre-adult mortality without also affecting the parasitoid fly’s fitness. Although the protozoan reduced the mortality of fly-infected monarchs (Fig. 1), this did not reduce the proportion of fly-infected monarchs that produced maggots (Fig. 3), nor did it reduce the average number of maggots produced per monarch (Fig. 4). It is known that in some infections, fly maggots do not develop successfully into pupae, but still end up killing the monarch butterfly larva (Oberhauser et al., 2007). This finding, in combination with our results, suggests that the protozoan parasite reduced monarch mortality caused by unsuccessful, rather than successful, parasitoid flies. There are two potential explanations for such a scenario. First, the protozoan parasite may enhance the monarch’s clearance of dying parasitoids inside its body. Previous research on multiple infections has suggested that parasites can limit the growth and transmission of a competing parasite by eliciting a host immune response (Lello et al., 2004; Meister et al., 2009; Pedersen and Fenton, 2006; Räberg et al., 2006), and it is possible that the protozoan parasite increases the immune response against the parasitoid fly. It is certainly likely that the monarch host mounts an immune response against the fly since in our experiments there were several cases in which monarchs were infected with the fly, yet did not produce any maggots. As an alternative explanation, it is possible that the protozoan parasite increases host survival by dampening the host immune response, and that this reduced immune response results in a lower amount of immunopathology. Immunopathology occurs in many vertebrate and invertebrate species (Brandt et al., 2004; Graham et al., 2005; Sadd and Siva-Jothy, 2006; Shi et al., 2001) and may be especially relevant in systems where an insect host must defend itself against insect parasites, such as parasitoid flies. If the parasitoid fly elicits a monarch immune response that damages both monarch and fly, and if the protozoan parasite decreases this response, then this may result in increased host survival. Clearly, we must advance our understanding of the monarch’s immune system to test these hypotheses.

Our results showed that multiple infection with the protozoan and parasitoid fly affected host and parasite fitness only on the basis of parasite interactions in the monarch’s larval stage, and that these interactions did not lead to further effects during the adult stage. From the monarch host’s perspective, adult longevity was not significantly different between uninfected monarchs and monarchs that survived attack by the parasitoid fly, and there was no difference in longevity between monarchs infected with the protozoan alone or those who were also exposed to the parasitoid fly. Thus, the parasitoid fly does not appear to have a long-lasting effect on the monarch if the monarch is able to successfully defend itself against fly infection.

As for the protozoan parasite, infecting a host that was later infected with the parasitoid fly severely reduced its fitness, since most monarchs were killed before they reached adulthood and transmitted the protozoan. However, we found no significant differences between the mean spore loads of surviving monarchs infected only with the protozoan compared to monarchs that were also attacked by the parasitoid fly. Spore load is a measure of replication for the protozoan and it is also associated with virulence and transmission probability, which makes it an appropriate measure of parasite fitness (De Roode et al., 2008b). These results further support the conclusion that if the monarch succeeds in defending itself against the parasitoid, there are no long-term costs to either the host or the protozoan parasite resulting from exposure to the parasitoid fly. Finally, we also found no evidence for an effect of multiple infection on the parasitoid fly beyond its development inside of the host. When we compared the flies that developed in the presence of the protozoan to those that developed alone, we found no significant difference in the pupal mass of the flies, the proportion of flies that successfully eclosed or the longevity of the flies that survived to adulthood. We did find a positive correlation between pupal mass and longevity in the parasitoid fly, but this relationship did not differ between flies that developed from monarchs infected or not infected with the protozoan parasite (Fig. 5). Thus, while the protozoan parasite can reduce the host mortality caused by exposure to the parasitoid fly, it does not appear to do so in a way that affects any of the fly fitness components that we measured.

Overall, our results underscore the need to consider infection with multiple parasites as a major determinant of parasite and host fitness, particularly in systems where one parasite’s success results in a loss of transmission for another one. We emphasize the importance of the host and parasite life cycles and biological characteristics in determining how multiple infection differs from single infection. Finally we suggest that in systems where one parasite is extremely deadly, the host may actually benefit from a second, more benign parasite if that parasite can increase its own transmission by increasing host survival. As such, our results support the view that parasitism is a context-dependent phenomenon (Fellous and Salvadon, 2009; Michalakis et al., 1992; Thomas et al., 2000; Vale et al., 2008; Wolinska and King, 2009), and that parasites can act as mutualists in the presence of more deadly natural enemies.

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