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HOST–PARASITE GENETIC INTERACTIONS AND VIRULENCE-TRANSMISSION RELATIONSHIPS IN NATURAL POPULATIONS OF MONARCH BUTTERFLIES

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Evolutionary models predict that parasite virulence (parasite-induced host mortality) can evolve as a consequence of natural selection operating on between-host parasite transmission. Two major assumptions are that virulence and transmission are genetically related and that the relative virulence and transmission of parasite genotypes remain similar across host genotypes. We conducted a cross-infection experiment using monarch butterflies and their protozoan parasites from two populations in eastern and western North America. We tested each of 10 host family lines against each of 18 parasite genotypes and measured virulence (host life span) and parasite transmission potential (spore load). Consistent with virulence evolution theory, we found a positive relationship between virulence and transmission across parasite genotypes. However, the absolute values of virulence and transmission differed among host family lines, as did the rank order of parasite clones along the virulence-transmission relationship. Population-level analyses showed that parasites from western North America caused higher infection levels and virulence, but there was no evidence of local adaptation of parasites on sympatric hosts. Collectively, our results suggest that host genotypes can affect the strength and direction of selection on virulence in natural populations, and that predicting virulence evolution may require building genotype-specific interactions into simpler trade-off models.

KEY WORDS: **Coevolution, G × G interaction, local adaptation, *Ophryocystis elektroscirrha*, tolerance.**

Parasites are ubiquitous in nature (Windsor 1998), and a defining trait of parasitism is the harm caused to hosts following infection. Understanding how parasites evolve to become more or less harmful is of interest to evolutionary biologists, and also has relevance to medical science and conservation biology (Ewald 1994; Daszak et al. 2000; Gandon et al. 2001; Cleaveland et al. 2002; Dieckmann et al. 2002; Lafferty and Gerber 2002; Altizer et al. 2003; Galvani 2003). Many models exist to explain the evolution of parasite virulence (Bull 1994; Levin 1996; Ebert 1999; Margolis and Levin 2008), yet the most popular asserts that virulence evolves as a byproduct of natural selection favoring greater between-host transmission. This trade-off model assumes that parasite genotypes that exploit their hosts more (e.g., via greater within-host replication) obtain a greater rate of transmission to new hosts. However, greater host exploitation comes at the cost of increasing the host's mortality rate as well as the host's clearance rate of the parasite, thereby cutting short the infectious period over which transmission takes place. As a consequence, natural selection should favor parasites with intermediate exploitation—and hence intermediate virulence—at which level lifetime transmission is maximized (Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Pickering 1983; Sasaki and Iwasa

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1991; Antia et al. 1994; Van Baalen and Sabelis 1995; Frank 1996).

A key assumption of this trade-off theory is that virulence and transmission are genetically correlated and linked positively with within-host exploitation. Although empirical support for the trade-off model is still limited (Ebert and Bull 2003; Alizon et al. 2009), several studies have found genetic relationships between subsets of traits, including parasite exploitation/replication rate, virulence, transmission potential, and host recovery rate (Fenner and Ratcliffe 1965; Mead-Briggs and Vaughan 1975; Diffley et al. 1987; Turner et al. 1995; Lipsitch and Moxon 1997; Mackinnon and Read 1999; Messenger et al. 1999). In addition, studies of myxomatosis in rabbits (see Anderson and May 1982; Mackinnon et al. 2008), bacterial parasites in waterfleas (Jensen et al. 2006), HIV in humans (Fraser et al. 2007), and protozoan parasites in monarch butterflies (De Roode et al. 2008b) have shown that maximum parasite fitness can be attained at an intermediate level of exploitation or virulence.

Major limitations of the trade-off model are that virulence is assumed to be a property of the parasite and that virulence-transmission relationships are expected to remain similar across all hosts in the population. In reality, however, virulence depends on host properties, including the host's immune response (Graham et al. 2005; Margolis and Levin 2008). Moreover, because immunity can be host- and parasite genotype-specific, it is conceivable that virulence is determined by specific (G $\times$ G) genetic interactions between hosts and parasites (Salvaudon et al. 2005; Grech et al. 2006; Lambrechts et al. 2006; Lefèvre et al. 2007).

G $\times$ G interactions have been demonstrated for parasite infection probability, i.e., the proportion of hosts that become infected following inoculation (e.g., Carius et al. 2001; Lambrechts et al. 2005), such that no host can resist all parasites equally well, and no parasite can infect all hosts equally well. As a result, frequency-dependent selection can lead to dynamic cycling in host and parasite genotypes (Hamilton 1980; Bell and Maynard Smith 1987; Hamilton et al. 1990; Thompson and Burdon 1992; Lively 1999; Lively and Dybdahl 2000). On a geographic scale, these coevolutionary dynamics can result in adaptation of parasites to locally occurring hosts (Parker 1985; Lively 1989; Manning et al. 1995; McCoy et al. 2002; Thrall et al. 2002; Greischar and Koskella 2007) or adaptation of hosts to local parasites (Kaltz et al. 1999).

The importance of G $\times$ G interactions in host–parasite evolution calls for their consideration for traits other than infection probability. In the case of virulence, it is conceivable that if the virulence level maximizing parasite fitness in one host does not maximize fitness in others, optimal levels of virulence could be harder to achieve. To date, one theoretical study has confirmed that when virulence and transmission are determined by both host and parasite genotype, these traits can evolve in ways different than predicted by simpler models (Restif and Koella 2003). Moreover, some experimental studies are also beginning to reveal that parasite exploitation and virulence can be affected by G $\times$ G interactions (Grech et al. 2006; Salvaudon et al. 2007).

Here, we examine how host and parasite genotypes affect virulence, transmission potential, and virulence-transmission relationships in natural populations of the monarch butterfly (*Danaus plexippus*) and its protozoan parasite (*Ophryocystis elektroscirrha*). Monarchs become infected with *O. elektroscirrha* when larvae ingest dormant parasite spores that are deposited on eggs or host plant leaves by female monarchs during oviposition. Parasite spores release sporozoites that pass through the larval gut to invade the hypodermal tissues (McLaughlin and Myers 1970). The parasite then undergoes asexual and sexual replication, ultimately producing large numbers of spores on the outside of the body of the emerging butterfly. These spores undergo no further replication until they are ingested by another larva. Parasite spores provide the full transmission potential of the parasite, and monarchs with higher spore loads transfer more parasites to eggs, host plant leaves, and mating partners (De Roode et al. 2009). At the same time, higher numbers of spores reduce monarch survival, life span, and mating success, thereby reducing the transmission opportunities for the parasite (Altizer and Oberhauser 1999; De Roode et al. 2007). We have previously shown that parasite genotypes with greater spore loads (transmission potential) also cause stronger reductions in the host's life span (virulence) and that lifetime parasite fitness is maximized at an intermediate spore load and virulence (De Roode et al. 2008a,b).

To test whether virulence-transmission relationships are affected by genetic host–parasite interactions, we collected host and parasite genotypes from each of two North-American monarch populations: an eastern population in which monarchs migrate annually to overwinter in Mexico, and a western population in which monarchs migrate annually to overwinter along the Californian coast (Urquhart and Urquhart 1978; Brower 1995). We used a cross-inoculation design to infect each host genotype with each parasite genotype, and quantified the probability of infection, spore load, and adult monarch life span. We then tested whether host and parasite genotype and G $\times$ G interactions influence these traits as well as the virulence-transmission relationship. Analyses were performed initially across all host–parasite combinations tested, and were repeated within the context of sympatric host–parasite populations. Although the first analysis shows whether host effects and G $\times$ G interactions can in principle occur in this system, the second analysis demonstrates whether they exist within actual host and parasite populations, which is necessary for coevolution to occur. Finally, we asked whether host and parasite source population influenced the outcome of infection, and tested for possible local adaptation of hosts and parasites.

Materials and Methods

HOST AND PARASITE SOURCES

We collected adult monarch butterflies from overwintering sites of both eastern (Sierra Chincua, Michoacan, Mexico, January 2007) and western North American populations (Pismo Beach, California, February 2007). The F1 offspring were reared in the laboratory using cuttings of greenhouse-raised *Asclepias incarnata* host plants. Newly emerged uninfected adults were selected to produce five noninbred F2 family lines for each monarch population.

Parasites used for this study were clonal isolates derived from nine parasitized monarchs collected from each of the two North American populations. Parasite strains were cloned prior to the experiment by infecting monarch caterpillars with single haploid parasite spores and using their offspring as the sources for this experiment (De Roode et al. 2007). Parasite clones denoted E1, E2, E3, E6, E7, E10, E11, E12, and E13 were derived from the eastern North American monarch population, and clones denoted C2, C8, C10, C12, C13, C14, C15, C16, and C17 from the western North American population (the "C" denoting "California"). Collection sites and dates of wild-infected monarchs used to generate the 18 parasite clones are provided in the Appendix. These parasite clones had previously been used in different experiments, where they successfully infected monarch caterpillars and showed genetic variation in both spore loads and virulence.

EXPERIMENTAL DESIGN

We used a fully factorial experimental design in which replicate larvae from each of the 10 host family lines were infected with one of nine western or nine eastern parasite clones. A total of five monarchs per host family line were infected with each clone, and 10 monarchs of each host family were left uninfected to serve as controls (total $N = 1000$ monarchs). Monarchs were inoculated by feeding them 0.5 cm^2 pieces of milkweed (*A. incarnata*) onto which 10 parasite spores were manually deposited. Larvae were reared singly in 0.94 L containers using cuttings of greenhouse-raised *A. incarnata* following previous methods (De Roode et al. 2007, 2008a). Upon adult emergence, monarchs were sexed, transferred to individual glassine envelopes, held in a 14°C incubator and checked daily to record the date of host death. Following host death, parasite spore load was determined by vortexing monarch bodies and counting spores using a hemocytometer (De Roode et al. 2007, 2008a).

MEASUREMENTS OF INFECTION PROBABILITY, TRANSMISSION POTENTIAL, AND VIRULENCE

Infection probability was recorded as the proportion of parasitized monarchs (within each host–parasite genotypic or population combination depending on the specific analysis). Transmission potential was determined by quantifying the spore load of adult butterflies; this measure is strongly positively correlated with both the level of within-host replication and parasite transmission to eggs, host plant leaves, and mating partners (De Roode et al. 2009). As a measure of virulence, we calculated adult monarch life span as the difference (in days) between adult emergence and death (see De Roode et al. 2007). This measure provides a combined index of adult monarch life span and starvation resistance, which can be crucially important for monarch survival during periods of food limitation such as occurs during the overwintering phase (Alonso-Mejia et al. 1997; Brower 1999). Adult life span can be used as a measure of virulence (rather than the difference in life span between infected and uninfected animals), because there were no host family differences in the life span of uninfected control monarchs ($F_{9,87} = 1.62$, $P = 0.12$).

STATISTICAL ANALYSIS

We carried out two main analyses. The first analysis was aimed at examining the effects of host and parasite genotypes, whereas the second analysis was aimed at studying population-level effects. In both analyses, parasite spore loads were $\log_{10}$-transformed and models were checked to ensure normality of errors and homogeneity of variance.

In the first analysis, we started by testing whether host and parasite genotypes (modeled as random effects) and their two-way (G × G) interaction affect infection probability, transmission potential (spore load), and virulence (host life span). Next, we included spore load as a covariate in the analysis of adult life span to test whether life span differences could be explained principally by differences in spore load, and whether the relationship between spore load and life span was similar across host and parasite genotypes. We also examined the relationship between spore load and adult life span in a second way, using parasite clone means (averaged within each host family line) as the unit of observation. This was done to more directly quantify the genetic correlation between parasite transmission (spore load) and virulence (adult life span); clone means were used to be conservative in this analysis.

In each of these analyses, we started by testing for effects across the full 180 host family-by-parasite clone interactions. We then repeated the analyses for the two sympatric datasets separately (eastern hosts with eastern parasites, western hosts with western parasites). This was done because analyses using the full dataset demonstrate whether host effects and G × G interactions can in principle occur in this system whereas analyses restricted to sympatric hosts and parasites demonstrate whether they actually exist in the context of natural populations. In these analyses, we used a significance level of $\alpha = 0.0125$ to correct for multiple tests, based on a Bonferroni approximation of $\alpha = 0.05/4$ tests.

To analyze infection probability, we used multinomial regression with backward stepwise selection of significant terms in SPSS 15.0 (SPSS, Chicago, IL). For all other analyses, we used analyses of variance and covariance, also in SPSS 15.0. When analyses of sympatric host and parasite combinations showed a significant G × G interaction term, we partitioned the G × G variance into "responsiveness" and "inconsistency" components following Barrett et al. (2005). Here, responsiveness quantifies the degree to which some parasite genotypes respond more strongly to host lineages than other parasites, whereas inconsistency quantifies the degree to which parasite genotypes switch in rank order of character measures across host family lines.

In the second analysis, we tested whether our data showed evidence for local adaptation of hosts and parasites. We tested whether parasites and hosts from the two populations differed in their infection probability, parasite spore load, and monarch life span, and whether parasites are more infectious, virulent, or transmissible in their sympatric than allopatric hosts. We also tested whether hosts are more resistant to their locally occurring parasites. To analyze infection probability, we used a GLM with quasi-binomial error structure in R 2.7.0, with host family-by-parasite clone combinations as the unit of replication. *F*-values are reported for this analysis. To analyze parasite spore load and host longevity, we used linear mixed effects models in R2.7.0, with monarch population as a fixed effect and parasite clone and host family as random effects nested within population. In the latter analyses, significance was based on model comparison using maximum likelihood; we report the total number of degrees of freedom for the minimal model, as well as the *P*-value associated with model comparison.

Results

A total of 860 of 900 inoculated monarchs (95.6%) and 97 of 100 control monarchs (97%) survived to the adult stage. These survival rates were not significantly different ($P > 0.05$), and all subsequent analyses are restricted to surviving monarchs. Of the 860 surviving monarchs that had been inoculated, a total of 729 became infected (85%) whereas none of the surviving control monarchs ($N = 97$) became infected. Infected monarchs lived significantly shorter than uninfected monarchs (mean ± SE life span: infected 9.8 ± 0.16; uninfected: 21.6 ± 0.22; $P < 0.001$). Aside from analyses of the proportion of infected monarchs, analyses described in the sections below are restricted to the subset of infected monarchs. Host sex had no effect on parasite spore load and had marginal effects on host longevity; it explained 0.48–0.81% of the total variance in genotypic analyses of host longevity and did not alter the effects of host and parasite genotypes. Hence, for simplicity, the analyses described below do not include host sex as a factor.

EFFECTS OF HOST AND PARASITE GENETIC INTERACTIONS

Infection probability

Across all hosts examined, parasite clones varied in the proportion of hosts they infected (Table 1a). Although there was a trend for host families to vary in the probability of infection, this was not significant at the 0.0125 level. There was also no significant parasite clone-by-host family interaction (Table 1a). Similar findings were obtained in separate analyses restricted to hosts and parasites from within the same population (Table 1b,c): in both cases the effect of parasite clone was significant, but host families did not differ in the probability of infection, and there were no host family-by-parasite clone interactions.

Parasite spore load (transmission potential)

Across the whole dataset (Table 1d), parasite spore load varied significantly among parasite clones. There was also a significant host family-by-parasite clone interaction, suggesting that parasite spore loads depended on interactions between parasite and host genotypes (Fig. 1). Analyses restricted to hosts and parasites from the same population (Table 1e,f) showed that parasite clone was significant for eastern hosts and parasites, and the clone × family interaction remained significant for western hosts and parasites. For the interaction involving western hosts and parasites, the fraction of G × G variance attributed to inconsistency was much greater (69.5%) than the fraction attributed to responsiveness (30.5%). Thus, the G × G interaction was largely driven by changes in the rank order of parasite clones among host families.

Host life span (virulence)

Across the whole dataset (Table 1g) adult life span varied significantly among host families, parasite clones, and host family-by-parasite clone combinations (Fig. 2). When we repeated the analysis for hosts and parasites from within the same population (Table 1h,i), effects of host family and parasite clone remained significant, but the interaction between host family and parasite clone was only marginally significant for western hosts and parasites, and was not significant for eastern hosts and parasites. For western hosts and parasites, the G × G variance component was again largely due to inconsistency (75.6%) as compared to differential responsiveness (24.4%) among parasite clones.

Relationship between parasite spore load and host life span

To test whether differences in parasite spore load could explain the observed differences in adult life span, we repeated the above analyses while including spore load as a covariate. When examined across the entire dataset (Table 1j), spore load was the main predictor of life span, having by far the highest effect size. However, host family and parasite clone still had considerable effect

Table 1. Genotype-level analyses on the proportion of animals infected, parasite spore load, host life span, and life span-by-spore load relationships. Significance (α=0.0125) is indicated by asterisks. In d–l significance was based on minimal models; hence, significant terms have more error degrees of freedom than deleted insignificant terms. When the Host family × Parasite clone term was retained in the model, the Host family and Parasite clone main effects were tested against an error term that was weighted 85–96% toward the interaction term, and 4–15% toward the MS error for the entire model. The estimated effect sizes for retained model terms are indicated by partial Eta squared (η_p^2).

Infection probability		χ^2	df	P
(a) Whole data set	Host family	21	9	P=0.013
	Parasite clone	160	17	P<0.001*
	Host family × Parasite clone	138	153	P=0.81
(b) Eastern hosts with eastern parasites	Host family	4.9	4	P=0.30
	Parasite clone	44	8	P<0.001*
	Host family × Parasite clone	35	32	P=0.31
(c) Western hosts with western parasites	Host family	6.8	4	P=0.14
	Parasite clone	23	8	P=0.003*
	Host family × Parasite clone	34	32	P=0.38
Parasite spore load		F	P	η_p^2
(d) Whole data set	Host family	$F_{9,179}$=1.65	P=0.103	0.077
	Parasite clone	$F_{17,165}$=4.46	P<0.001*	0.315
	Host family × Parasite clone	$F_{152,549}$=1.47	P<0.001*	0.290
(e) Eastern hosts with eastern parasites	Host family	$F_{4,158}$=3.40	P=0.011*	0.079
	Parasite clone	$F_{8,158}$=1.14	P<0.001*	0.207
	Host family × Parasite clone	$F_{32,126}$=0.91	P=0.61	–
(f) Western hosts with western parasites	Host family	$F_{4,34}$=2.41	P=0.068	0.222
	Parasite clone	$F_{8,33}$=1.42	P=0.224	0.255
	Host family × Parasite clone	$F_{32,146}$=2.18	P=0.001*	0.324
Host life span		F	P	η_p^2
(g) Whole data set	Host family	$F_{9,177}$=4.94	P<0.001*	0.201
	Parasite clone	$F_{17,164}$=8.24	P<0.001*	0.460
	Host family × Parasite clone	$F_{152,554}$=1.49	P=0.001*	0.291
(h) Eastern hosts with eastern parasites	Host family	$F_{4,159}$=7.75	P<0.001*	0.163
	Parasite clone	$F_{8,159}$=4.98	P<0.001*	0.200
	Host family × Parasite clone	$F_{32,127}$=0.76	P=0.81	–
(i) Western hosts with western parasites	Host family	$F_{4,179}$=6.88	P<0.001*	0.133
	Parasite clone	$F_{8,179}$=5.51	P<0.001*	0.198
	Host family × Parasite clone	$F_{32,147}$=1.75	P=0.014	–
Host life span × parasite spore load relationships		F	P	η_p^2
(j) Whole data set	Host family	$F_{9,690}$=5.72	P<0.001*	0.069
	Parasite clone	$F_{17,690}$=6.35	P<0.001*	0.135
	Spore load	$F_{1,690}$=882	P<0.001*	0.561
	Host family × Spore load	$F_{9,690}$=5.10	P<0.001*	0.062
	Host family × Parasite clone	$F_{152,538}$=1.17	P=0.103	–
(k) Eastern hosts with eastern parasites	Host family	$F_{4,165}$=10.5	$P\leq$0.001*	0.203
	Parasite clone	$F_{8,157}$=1.69	P=0.11	–
	Spore load	$F_{1,165}$=297	P<0.001*	0.643
	Host family × Parasite clone	$F_{32,125}$=1.11	P=0.33	–
(l) Western hosts with western parasites	Host family	$F_{4,176}$=2.32	P=0.059	–
	Parasite clone	$F_{8,180}$=2.55	P=0.012*	0.102
	Spore load	$F_{1,180}$=197	P<0.001*	0.523
	Host family × Parasite clone	$F_{32,144}$=1.12	P=0.32	–

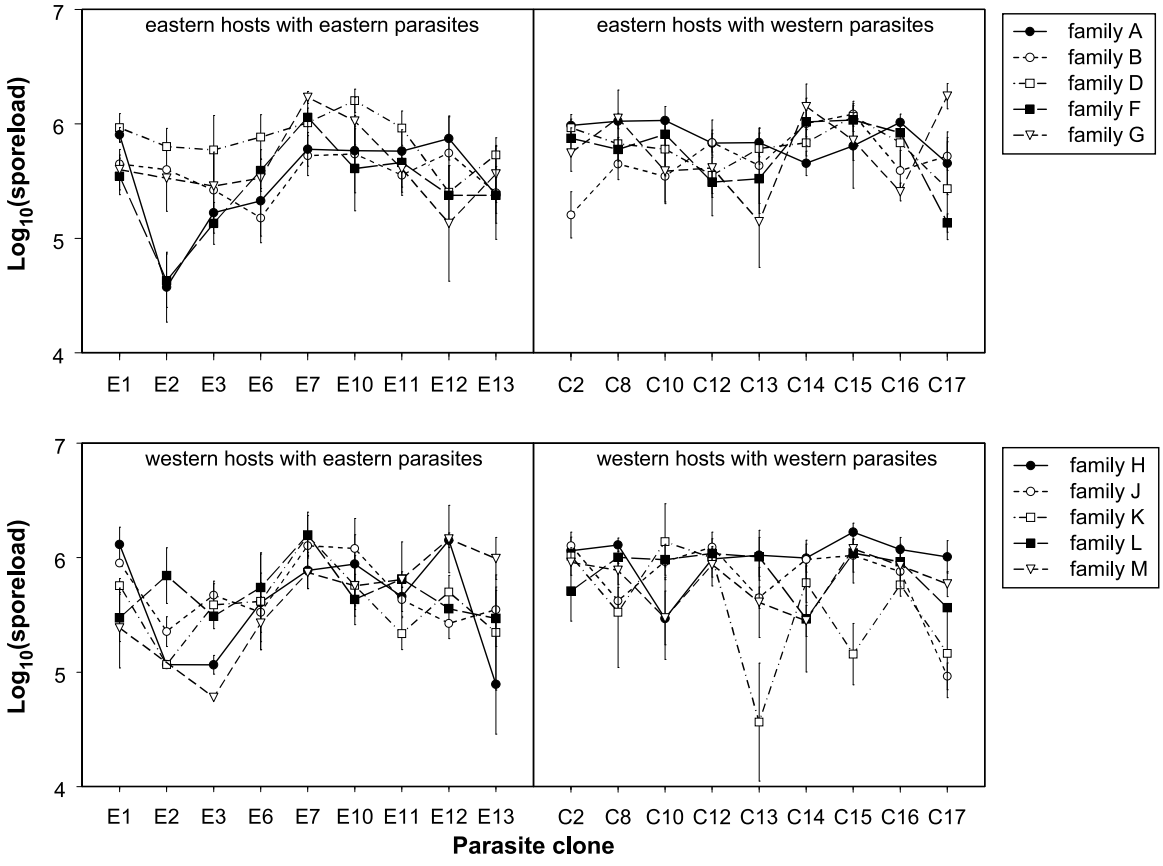

Figure 1. **Parasite spore load as a function of host family and parasite clone (mean ± SEM). Parasite clones E1–E13 are from the eastern population, and clones C2–C17 are from the western population; host families A–G are from the eastern population and host families H–M from the western population. Data are based on infected animals only. No datapoint is shown for parasite E2 in family M, because none of these individuals became infected.**

sizes, suggesting that different host families and parasite clones suffered or caused more virulence on a per-parasite basis. The G × G interaction became nonsignificant when correcting for spore load, suggesting that the observed G × G interactions on longevity were due to their effects on spore load. When we restricted this analysis to hosts and parasites from within the same population (Table 1k,l), we again found that spore load was the main predictor of adult life span. Indeed, correcting for spore load made parasite clone and host family insignificant factors in the analysis within the eastern and western population respectively, suggesting that the effects of these factors on longevity were due to their effects on spore load. However, host family and parasite clone remained significant factors in the analysis within eastern and western populations, respectively, again suggesting that different host families and parasite clones suffered or caused more virulence on a per-parasite basis.

Genetic relationships between parasite spore load and host life span

We examined the genetic relationship between spore load and life span using clone means (tested within each host family) as the unit of observation. Across the whole dataset (Table 2a), spore load was the main predictor of monarch life span, having by far the largest effect size (and thus suggesting a strong genetic correlation). Host family remained significant in this analysis, suggesting that host families differ in the virulence they suffer for a given spore load. When analyses were restricted to hosts and parasites from within the same population (Table 2b,c), we again found spore load to be the main predictor of virulence. In the eastern population, host family remained significant, again suggesting that host families differ in the virulence they suffer on a per-parasite basis (i.e., differences in *y*-intercept of the sporeload—life span relationship; Fig. 3). There were no significant host family × spore load interactions in any of the analyses, suggesting that the spore load–life span relationships did not have different slopes for different host families.

The results of the above analyses are summarized in Figure 3, which for each host family shows the relationship between host life span and parasite spore load across the nine sympatric clones tested. A comparison of host families and parasite clones readily shows their variation in average life span (relative position of datapoints on *y*-axis) and spore load (relative position on *x*-axis).

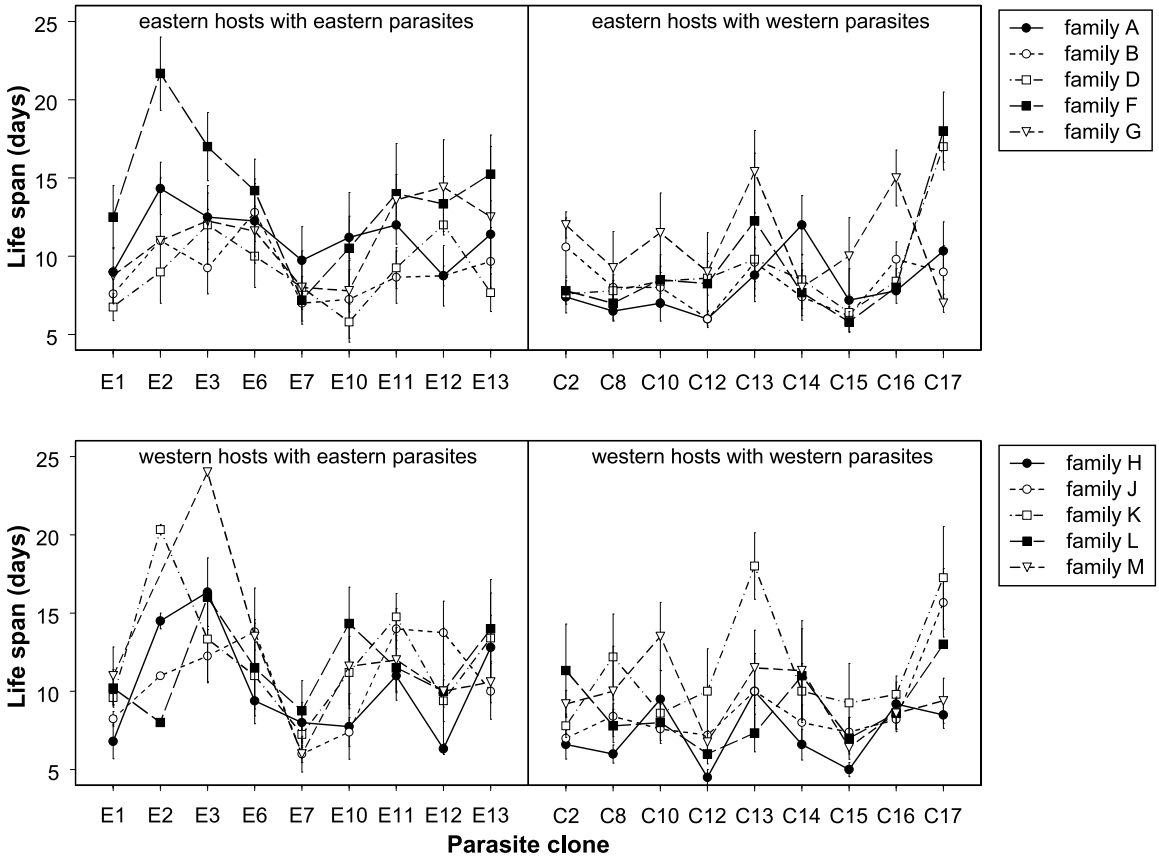

Figure 2. Host life span as a function of host family and parasite clone (mean ± SEM). Parasite clones E1–E13 are from the eastern population, and clones C2–C17 are from the western population; host families A–G are from the eastern population and host families H–M from the western population. Data are based on infected animals only. No datapoint is shown for parasite E2 in family M (none of these individuals became infected).

It is worth noting that for several host family lines (e.g., B, D, G, H, L, and M) most datapoints are clustered in the lower right portion of the graph, indicating that the majority of parasite clones produced high parasite loads and high virulence. However, a few host families (e.g., F, K) showed a wider response across parasite clones, escaping heavy infection by some clones and thereby experiencing greater longevity.

Moreover, the G × G interactions on spore load and life span for western hosts with western parasites as described above (subsections "Infection probability" and "Parasite spore load (transmission potential)"; Table 1) are visualized graphically by changes in the rank order of parasite clones in different host families. For example, although clone C17 obtained the lowest spore load and caused least virulence

Table 2. Analyses on genetic virulence-transmission relationships. Annotations as in Table 1.

		F	P	η_p^2
Genetic virulence (life span)—transmission (spore load) relationships				
(a) Whole data set	Host family	$F_{9,168}=4.9$	$P<0.001^*$	0.208
	Spore load	$F_{1,168}=375$	$P<0.001^*$	0.691
	Host family × spore load	$F_{9,159}=2.34$	$P=0.017$	–
(b) Eastern hosts with eastern parasites	Host family	$F_{4,39}=8.74$	$P<0.001^*$	0.473
	Spore load	$F_{1,39}=82.4$	$P<0.001^*$	0.679
	Host family × spore load	$F_{4,35}=3.67$	$P=0.014$	–
(c) Western hosts with western parasites	Host family	$F_{4,39}=0.84$	$P=0.51$	–
	Spore load	$F_{1,43}=118$	$P<0.001^*$	0.73
	Host family × spore load	$F_{4,35}=0.90$	$P=0.48$	–

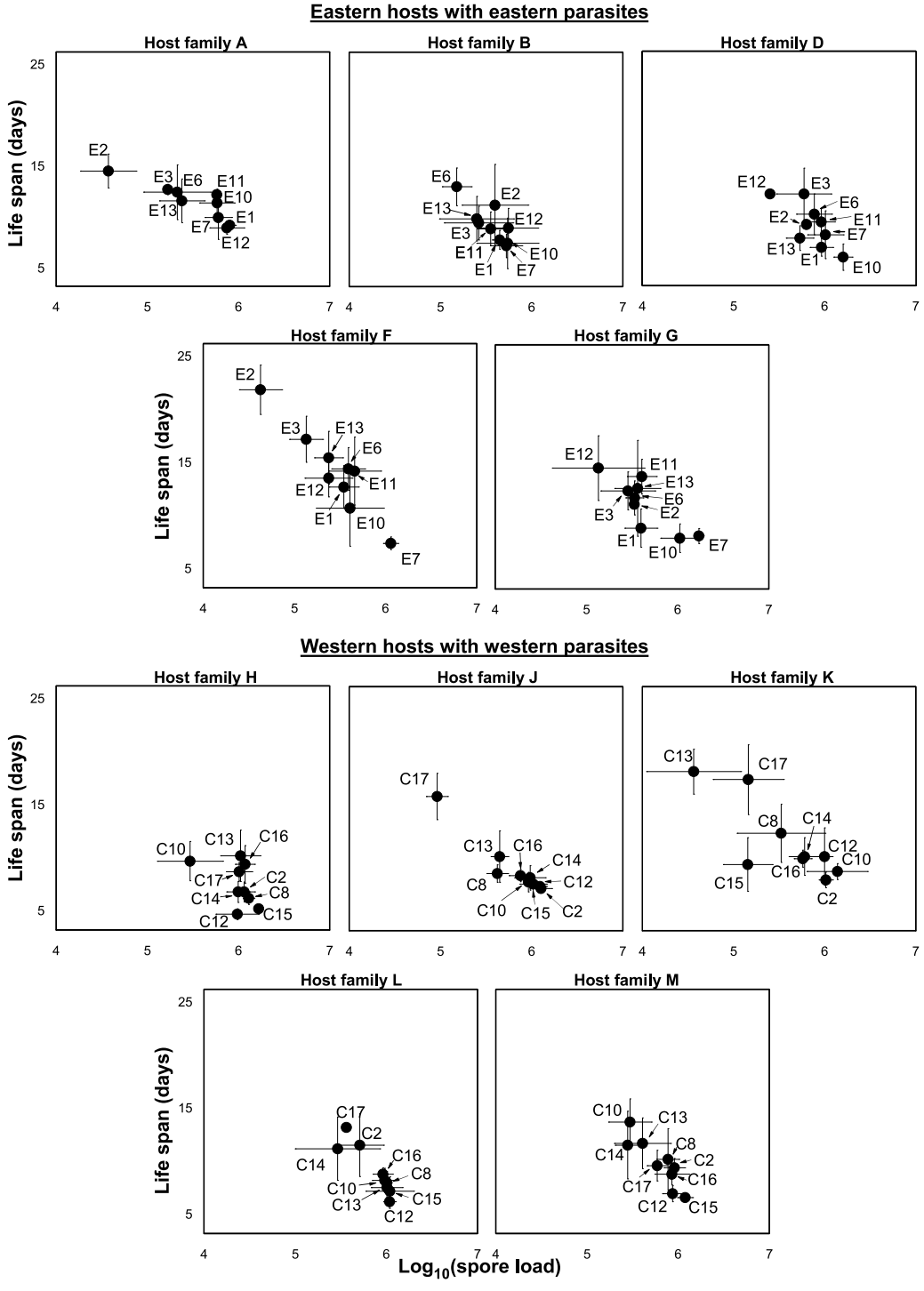

Figure 3. **Virulence-transmission relationships. Panels show virulence (life span)—transmission (spore load) relationships for parasite clones (indicated with their names) tested in each of their sympatric host families. *y*- and *x*-axis scales are the same in each panel to facilitate the observation that host families vary in their average life span and spore load. Datapoints are clone means ± SEM.**

(highest life span) in host family J, it obtained intermediate spore load and caused intermediate virulence in host family M. Similar rank order changes appear apparent with eastern parasites in eastern hosts, yet these were not significant (Table 1).

POPULATION-LEVEL EFFECTS AND EVIDENCE FOR LOCAL ADAPTATION

Infection probability and spore load

Parasites from the two populations varied in their infectivity ($F_{1,178} = 15.3$, $P < 0.001$), with western parasites infecting a

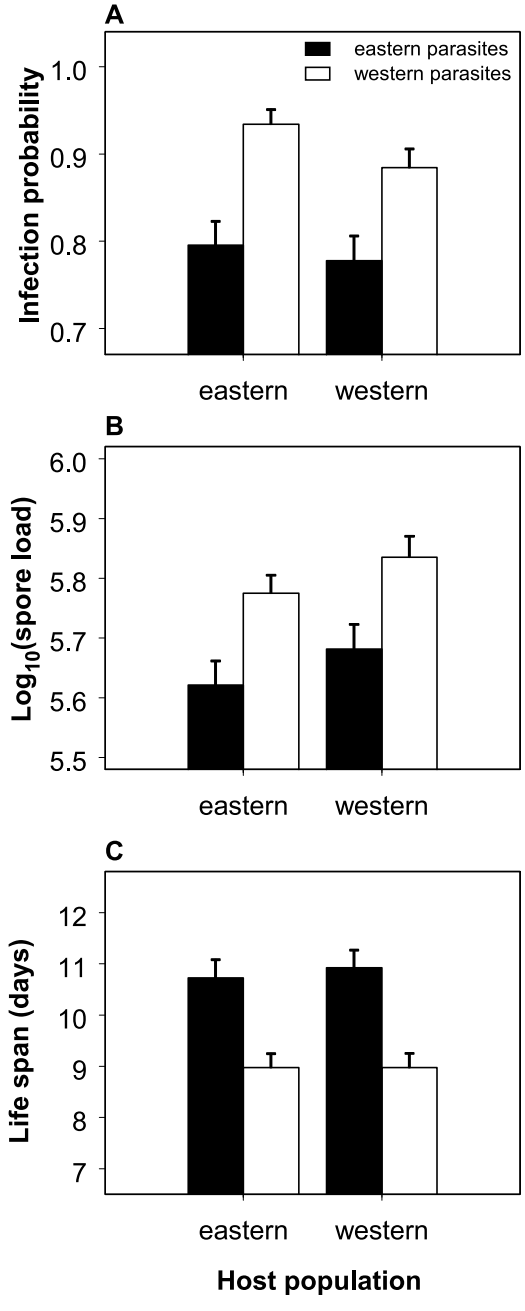

Figure 4. Effects of host and parasite population on infection probability (A), parasite spore load (B), and monarch life span (C). Bars in panels A-C show mean +1 SEM.

higher proportion of monarchs than eastern parasites (Fig. 4A). Western parasites also achieved higher average spore loads than eastern parasites (df = 4, $P = 0.046$; Fig. 4B). Hosts from both populations showed similar susceptibility to infection, with no significant main effect of host origin on infection probability ($F_{1,177} = 1.2$, $P = 0.28$; Fig. 4A) or on average spore loads (df = 3, $P = 0.27$; Fig. 4B). Parasites did not perform better (i.e., achieve more infections or higher spore loads) on sympatric versus allopatric hosts; similarly, hosts were not more susceptible to infection by allopatric versus sympatric parasites (Fig. 4A,B).

Host life span

Parasites from the two populations differed in the virulence they caused, with western parasites generally causing shorter adult life span relative to eastern parasites (df = 4, $P = 0.036$; Fig. 4C). In contrast, hosts from both populations did not differ in the virulence they suffered (df = 3, $P = 0.92$; Fig. 4C). There was also no indication that parasites caused greater harm to sympatric than allopatric hosts (Fig. 4C).

Relationship between parasite spore load and host life span

Spore load was a significant predictor of adult life span ($P < 0.001$), suggesting that the observed higher virulence of western parasites was to a large extent caused by their higher spore loads (Fig. 4). However, parasite origin remained significant in the analysis (df = 5, $P = 0.036$) with spore load as a covariate, suggesting that western parasites were also more virulent on a per-parasite basis.

Discussion

Our results provide evidence for virulence-transmission relationships in naturally occurring populations, and show that these relationships can be affected by host genotypes. The first finding confirms a major assumption of virulence evolution theory (Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Pickering 1983; Sasaki and Iwasa 1991; Antia et al. 1994; Van Baalen and Sabelis 1995; Frank 1996). The second finding suggests that hosts can provide a variable backdrop for parasite evolution, such that selection on virulence will be complicated by variation among host genotypes. This result is consistent with recent work showing that parasite virulence can depend on host properties (Graham et al. 2005; Grech et al. 2006; Salvaudon et al. 2007) and indicates that predictions regarding virulence evolution may need to account for genotype-specific interactions.

We have previously calculated that for *O. elektroscirrha*, a spore load of approximately 5.70 ($\log_{10}$-scale) maximizes parasite life-time fitness based on a trade-off between virulence and transmission (De Roode et al. 2008b). This trade-off appears because increasing spore loads on adult monarch butterflies not only increase the number of spores transmitted to monarch offspring per transmission event, but also reduce monarch survival, life span, and mating opportunities, thereby reducing the number of lifetime transmission events. Based on this trade-off it follows that any factor that directly changes (1) the realized spore load of a parasite in its host, or (2) the relative balance between the costs (virulence) and benefits (transmission) of parasite spore production can affect virulence evolution (e.g., Mackinnon et al. 2008).

The data presented here suggest that host genotypes can have both of these effects, through three different mechanisms.

First, monarchs varied in the average spore loads produced by parasite clones, thus demonstrating differential host resistance. For example, in the eastern population, most parasites obtained higher average spore loads in host family line D than in other families whereas in the western population, some parasites obtained lower spore loads in family K than in other families (Fig. 3). By reducing realized spore loads, more resistant hosts could select for parasites with higher levels of intrinsic spore production that can overcome this reduction. Because strains of *O. elektroscirrha* with higher spore production also cause higher virulence (De Roode et al. 2007, 2008a,b), more resistant hosts may select for higher virulence, as has been suggested by other studies (Gandon and Michalakis 2000; Gandon et al. 2001; Mackinnon and Read 2004).

Second, for a given spore load, some monarchs maintained a greater life span than others, thus demonstrating variation in tolerance (i.e., the ability to maintain relatively high fitness when infected). For example, in the eastern population, monarchs in family B suffered greater life span reductions for a given spore load than those from family F, and in the western population monarchs in family H suffered greater life span reductions for a given spore load than those from family K (Fig. 3). More tolerant hosts also have the potential to select for more virulent parasites, because they reduce the costs of spore production whereas parasites maintain the benefits of higher transmission (Restif and Koella 2003; Miller et al. 2006).

Third, host genotypes interacted with parasite genotypes to determine realized spore load and host life span. These G $\times$ G interactions occurred across the full dataset, which includes natural and unnatural host–parasite combinations. However, when tested for within populations, they were only significant in the western, but not in the eastern population. As a result of these G $\times$ G interactions, monarch families changed the relative rank order of some parasite clones across the virulence-transmission relationship. For example, clone C17 obtained the lowest spore load and caused least virulence (highest life span) in host family J, but obtained intermediate spore load and virulence in host family M. Thus, parasites that achieve high fitness in some hosts (i.e., they obtain spore loads closer to the calculated optimum) may be less well-adapted in other hosts.

Effects of host genotypes on virulence-transmission relationships indicate that host variation could constrain virulence evolution in this system. Because monarchs examined here consist of more and less resistant and tolerant hosts, and because hosts can change the rank order of parasite spore load and virulence, it follows that no single parasite strain can achieve maximum fitness in wild populations. Instead, genotype-specific interactions could generate frequency-dependent selection, with parasites evolving optimal virulence in the most common host genotypes. This could ultimately produce coevolutionary dynamics similar to systems in which parasite infectivity depends on genotype interactions (Lively 1999; Lively and Dybdahl 2000). On the other hand, we note that the average spore load of all eastern host–parasite combinations was 5.62 ± 0.04 ($\log_{10}$-scale), and that of western host–parasite combinations was 5.84 ± 0.04 ($\log_{10}$-scale). These spore loads are very close to approximately 5.70, the $\log_{10}$-spore load predicted to maximize parasite fitness in this system based on a simple trade-off model (De Roode et al. 2008b). Thus, although G $\times$ G interactions may increase genetic variance around optimum levels of virulence, they could also operate in tandem with simple models based on virulence-transmission trade-offs.

Some host–parasite systems characterized by G $\times$ G interactions show evidence for local adaptation of hosts and parasites (Parker 1985; Lively 1989; Manning et al. 1995; Kaltz and Shykoff 1998; Lively 1999; McCoy et al. 2002; Thrall et al. 2002). However, our data provided no evidence for local adaptation in the *O. elektroscirrha*-monarch system. Parasites were no better at infecting hosts from sympatric versus allopatric populations, and hosts were no more resistant (in qualitative or quantitative terms) or tolerant to sympatric than allopatric parasites. Previous work on host–pathogen coevolution indicates that local adaptation might not be detected under circumstances of high between-population gene-flow, low parasite specificity for host genotypes, low parasite virulence, time-lagged coevolutionary dynamics, or too few studied populations (Imhoof and Schmid-Hempel 1998; Burdon and Thrall 1999; Kaltz et al. 1999; Lively 1999; Thompson 1999; Gandon 2002; Dybdahl and Storfer 2003; Greischar and Koskella 2007). Levels of gene flow between the eastern and western monarch population are currently unknown, but a high level of interpopulation movement appears unlikely based on divergent migratory patterns and geographic barriers (Brower 1995). Therefore, more likely reasons for an observed lack of local adaptation in this study are time-lagged dynamics, too few populations studied, and relatively low parasite specificity for host genotypes.

Another possible explanation is that hosts and parasites might be locally adapted to environmental variables, such as the host plant species on which monarchs feed. Monarch larvae consume species of milkweed (Asclepiadaceae), and different milkweed species can strongly affect infection probability, parasite spore load, and host life span (De Roode et al. 2008a). Because different milkweed species occur in different monarch populations (Woodson 1954; Malcolm and Brower 1989), locally abundant host plants could be an important selective force in host–parasite evolution. Hence, evaluating hosts and parasites on a single host plant species—as done here—could have limited our ability to detect local adaptation (Nuismer and Gandon 2008). Indeed, studies on a fungal pathogen of the plant *Plantago*

lanceolata had to account for geographic variation in temperature to detect adaptation (Laine 2007, 2008). In the case of monarchs, if western monarchs rely on host plants that confer a greater level of resistance to infection, higher levels of spore production and virulence (as reported in this study) may be favored to overcome this environmental resistance. Whether this is the case, is currently unknown; however, it is known that monarchs in the western and eastern North American populations encounter different species of milkweed as their primary larval host plants (Woodson 1954; Malcolm and Brower 1989).

In contrast with other studies (Carius et al. 2001; Lambrechts et al. 2005), we found no effect of host genetic variation on the proportion of monarchs becoming infected (i.e., on qualitative resistance). Instead, host family lines expressed variation in quantitative resistance (based on spore loads produced) and tolerance to infection (based on life span reductions suffered). Although population-level variation in resistance is a common outcome of theoretical models of host–pathogen evolution, variation in tolerance is not. In other words, rather than selection maintaining a variety of tolerance alleles, genotypes with high tolerance are expected to become fixed in the population (Roy and Kirchner 2000; Rausher 2001; Miller et al. 2005). This is because tolerant hosts can produce relatively more infective particles without suffering as high costs, thereby increasing population-level transmission and hence the selective pressure for further tolerance. Although some studies on plant tolerance to herbivory suggest that costs of tolerance can maintain variation (Simms and Triplett 1994; Fineblum and Rausher 1995; Tiffin and Rausher 1999; Koskela et al. 2002), such costs are not always apparent (Simms and Triplett 1994; Mauricio et al. 1997; Tiffin and Rausher 1999; Stowe et al. 2000). In our study, we found no obvious costs of tolerance: across monarch family lines, there were no negative correlations between tolerance and quantitative resistance ($F_{1,8} = 0.014$, $P = 0.91$) or between tolerance and life span in the absence of infection ($F_{1,8} = 0.22$, $P = 0.65$). On the other hand, because parasite genotypes also affected host tolerance, it is possible that host–parasite genetic interactions provide a mechanism to support variation in tolerance. Specifically, if host tolerance depends on the parasite genotype with which it is infected, then evolving tolerance to one parasite genotype may not provide tolerance to another.

In conclusion, our study indicates that expressed levels of virulence and transmission potential, in addition to virulence-transmission relationships, can be affected by host genotypes and host–parasite genetic interactions. Despite these interactions, our results also suggest that average virulence at the population-level could still evolve to values predicted by simple trade-off models. Collectively, these findings suggest that to understand virulence evolution in some host–parasite systems, a comprehensive view that combines assumptions from both trade-off theory and genotype-specific coevolutionary interactions could be highly informative. Thus, mathematical models may be required to predict whether G × G interactions merely increase the variation around predicted optima that maximize parasite fitness, or whether they can direct virulence evolution into directions not envisaged by existing models.

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Appendix

Table A1. Site and date of original capture of wild-infected monarchs from which each of the 18 parasite clones used in the experiment was obtained. Eastern parasite clones were collected from sites widely distributed across the monarchs' breeding range during the summer months and into the fall migration period whereas western clones were collected from monarchs captured at or near overwintering sites in coastal California (thus representing a mix of genotypes originating from across the breeding range).

Clone ID	Capture site	Capture date	Number of generations passed in laboratory prior to experiment
Eastern population			
E1	Ithaca, NY	9/2003	3
E2	Clarkston, GA	9/2004	3
E3	Cape May, NJ	10/2001	3
E6	Charlottesville, VA	9/2004	3
E7	Charlottesville, VA	9/2004	3
E10	St. Paul, MN	7/2005	2
E11	Pembroke, VA	6/2005	2
E12	Sweet Briar, VA	7/2005	2
E13	Sweet Briar, VA	7/2005	2
Western population			
C2	Santa Barbara, CA	4/2003	3
C8	Pismo Beach, CA	5/2003	3
C10	Pismo Beach, CA	2/2005	3
C12	Santa Cruz, CA	3/2005	3
C13	Big Sur, CA	3/2005	3
C14	Santa Cruz, CA	3/2005	3
C15	Big Sur, CA	3/2005	3
C16	Big Sur, CA	3/2005	3
C17	Big Sur, CA	3/2005	2