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PARASITE-DRIVEN GENETIC CHANGE IN A NATURAL POPULATION OF *DAPHNIA*

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A substantial body of theory indicates that parasites may mould the population genetic structure of their hosts, but few empirical studies have directly linked parasitism to genetic dynamics. We used molecular markers (allozymes) to investigate genotype frequency changes in a natural population of the crustacean *Daphnia magna* in relation to an epidemic of the bacterial pathogen *Pasteuria ramosa.* The population experienced a severe epidemic during the study period in which parasite prevalence reached 100% of the adult portion of the population. The parasite epidemic was associated with genetic change in the host population. Clonal diversity was observed to decrease as parasite prevalence increased in the population, and tests for differences in the clonal composition of the population before, during, and after the epidemic indicated that significant change had occurred. A laboratory infection experiment showed that the genotypes which were more common following the peak of the parasite epidemic were also the most resistant to parasite infection. Thus, this study provides an illustration of parasite-mediated selection in the wild.

KEY WORDS: **Allozymes, epidemic, evolution, natural selection, *Pasteuria ramosa*, resistance, susceptibility.**

Parasites are predicted to have extensive effects on their host populations, driving genetic change, population density changes, and speciation. Genetic variation for infection-related traits, a requirement for parasite-mediated selection, is abundant in natural host populations (Little 2002; Woolhouse et al. 2002; Wolinska et al. 2004). Parasite-mediated selection has been demonstrated in experimental populations (Buckling and Rainey 2002; Capaul and Ebert 2003; Haag and Ebert 2004) and in populations that have been subject to artificial selection pressures (Ibrahim and Barrett 1991). Several studies on wild populations have failed to directly observe genotype frequency change due to parasitism (Henter 1995; Little and Ebert 2001; Mitchell et al. 2004; Siemens and Roy 2005), whereas others have revealed genotype frequency change that is seemingly maladaptive (Burdon and Thompson 1995) or is at least difficult to reconcile with predictions based on patterns of genetic variation (Little and Ebert 2001; Siemens and Roy 2005). Thus, whereas many populations experience strong genotype frequency dynamics, direct links to parasitism have been tenuous, and thus the impact of parasitism on the genetic structure of natural populations remains unresolved.

The lack of observed parasite-mediated dynamics in natural systems is surprising considering the ubiquity of parasites and their often strong detrimental effects. Recent work has sought to gain insight into dynamics by highlighting how environmental variation may interact with infection, and the genetic basis of infection. Environmental variation has been shown to be a major factor determining the ability of a host or its offspring to defend against parasites (Moret and Schmid-Hempel 2001; Little et al. 2003; Mitchell et al. 2005; Robb and Forbes 2005; Sadd et al. 2005) and several studies have demonstrated strong genotype by environment (Ferguson and Read 2002; Mitchell et al. 2005; Fels and Kaltz 2006) or phenotype by environment (Bedhomme et al. 2005) interactions that could make responses to selection difficult to predict. Another important factor that may not have been sufficiently accounted for is the full diversity of strategies used by hosts to evade parasitism. Traditionally, studies that have attempted to

identify host resistance have measured infection intensity and fitness of infected hosts under tightly controlled laboratory conditions. While these studies have successfully demonstrated the fitness costs associated with parasitism and the potentially strong selective forces imposed by parasites, it is becoming apparent that they may paint unrealistic expectations of host–parasite dynamics in the field. Recent studies have demonstrated that behavioral traits (Decaestecker et al. 2002; Karvonen et al. 2004; Behringer et al. 2006), modification to nest environments (Christe et al. 2003), and mutualisms (Arnold et al. 2003; Currie et al. 2006) may all be important factors in determining overall levels of disease. Furthermore, life-history shifts in timing of reproduction (Minchella and Loverde 1981; Krist 2001; Chadwick and Little 2005) and diet alterations (Lee et al. 2005) in response to parasitism have been shown to reduce the costs of infection in a number of taxa. Taking these factors in to account it is easy to see why simple measurements of resistance in the laboratory may not shed sufficient light on the potential for parasite-mediated dynamics.

This more complex view on natural host–parasite interactions does not necessarily imply that parasite-mediated selection is not important, rather that it may simply be difficult to detect. One hindrance to the study of change over time is the constraints imposed by the feasible size of the common garden infection experiments that are often used to study genetic variation for resistance. This can, for example, affect the possible number of time points over which parasite-mediated selection may be studied. An alternative method to study parasite-mediated selection is to use genetic markers, which enable the processing of a larger number of individuals. Due to their short generation time, invertebrates are often targets for the study of genetic change, but we do not typically know which immune-related genes to use for the tracking of parasite-mediated dynamics. Neutral genetic markers such as microsatellites or allozymes are potentially useful for correlating general levels of diversity with parasitism, but are not expected to be involved in loci associated with, or directly involved in, resistance or even associated with loci. However, in organisms with high levels of linkage disequilibrium (e.g., clonal or highly selfing taxa), associations between neutral loci and loci under selection may occur. This provides the opportunity for intensive sampling of natural populations to reveal parasite-mediated genetic dynamics (Dybdahl and Lively 1998; Little and Ebert 1999).

Dramatic changes in allozyme genotype frequency over time are well documented in natural populations of *Daphnia* (Carvalho 1987; Carvalho and Crisp 1987), which are cyclically parthenogenetic and often show high levels of genotypic disequilibria. Strong associations between allozyme genotypes and infection prevalences (Little and Ebert 1999) or important life-history traits (Carvalho 1987) have been revealed by field studies. However, attempts to link genotypic changes to parasite-mediated selection in *Daphnia* have generated mixed results (Little and Ebert 2001; Mitchell et al. 2004), leading previous researchers to conclude, somewhat unsatisfactorily, that unmeasured environmental variables were overwhelming the effects of parasitism. Two problems with previous studies are that associations between allozyme markers and infection in the field were not verified with controlled laboratory experiments, or that studies were conducted during periods when the impact of parasitism was relatively low. The present study analyzed allozyme variation in a population of *Daphnia magna* over an eight-month period that spanned a very intense epidemic of a bacterial parasite that essentially sterilizes its host. We observed dramatic fluctuations in allozyme genotype frequencies and brought live samples of hosts in to the laboratory to test their susceptibility with controlled infections. This enabled us to confirm whether parasites were indeed responsible for the observed genotypic dynamics.

Methods

ORGANISMS AND FIELD COLLECTIONS

Daphnia magna is a planktonic freshwater crustacean found in still freshwater ponds. It is host to numerous bacterial, fungal and microsporidian parasites (Stirnadel and Ebert 1997; Little and Ebert 1999). Substantial genetic variation for resistance has been observed among genotypes of *D. magna* when exposed to *Pasteuria ramosa* (Carius et al. 2001), a bacterial, spore forming, obligate endoparasite that is the best studied of the *D. magna* parasites. *Pasteuria ramosa* is horizontally transmitted by the release of spores from decomposing cadavers of infected hosts (Ebert 1996). Infection is highly costly, causing dramatic declines in host fecundity, often resulting in complete sterilization.

Daphnia magna were collected in 2003 from a farm pond at Leitholm, in the Scottish Borders (2°20.43′W 55°42.15′N). Samples were taken twice per month between April and September when the *Daphnia* population was large or growing, and then once per month during the colder months of October to December when the population was experiencing little change. Three samples were taken at each collection from different locations around the pond, although the same three locations were always sampled. Variability between samples due to sampling techniques were minimized by always using the same net (opening of 630 cm^2) and sweep length. After each collection live samples were taken back to the laboratory where an estimate of prevalence of the parasite *P. ramosa* was checked in the adult portion of all subsamples. Infected adult *D. magna* are usually distinct making infection easy to detect by eye. Random samples of the host population were frozen in eppendorf tubes at −80°C for later allozyme electrophoresis.

We genotyped an average of 107 host individuals from each of 15 time points using standard methods of cellulose acetate allozyme electrophoresis (Hebert and Beaton 1993). The enzymes studied were mannose-6-phosphate isomerase (MPI), aspartate amino transferase (AAT), and fumerate hydratase (FUM), each of which had just two alleles, and all of which were known to be polymorphic based on a previous study of this population (Mitchell et al. 2004). Allozyme bands with unique electrophoretic mobility were assumed to correspond to unique alleles. Accordingly individuals sharing the same electrophoretic phenotype were regarded as having the same "electrophoretic genotype." However, it is probable that individuals indistinguishable at these loci may differ at other loci not assayed, or possess amino acid substitutions that do not result in detectable mobility differences. This caveat applies to all allozyme studies and a substantial proportion of studies using other molecular markers.

INFECTION EXPERIMENT

Live samples from before the peak of the parasite epidemic (30 individuals from May 14, 2003, 30 individuals from June 27, 2003), and after the epidemic had abated (36 individuals from November 21, 2003), were isolated and then maintained clonally as isofemale lines in the laboratory for use in later experimentation (Duncan et al. 2006). Using methods identical to those described above, we allozyme genotyped clonal copies of these live *Daphnia*. We then performed an infection experiment on these host lines to test for susceptibility differences between electrophoretic genotypes under controlled conditions.

The experimental infection protocols are described in Duncan et al. (2006). Briefly, to equilibrate environmental variation among the lines prior to experimentation effects, three replicates of each iso-female line were kept under experimental conditions for three generations. Replicates contained five females all from the same clutch in a 200-ml jar of *Daphnia* medium (Klüttgen et al. 1994). A suspension of *P. ramosa* transmission spores that had been frozen at −20°C was used for the infection experiment. The spores in suspension originated from a large mixture of *D. magna* infected with *P. ramosa* collected from the same pond in 2000 (Mitchell et al. 2004). Creating the solution involved infecting a mixture of *Daphnia* individuals (from 15 clones taken from the same population) with *P. ramosa*. Infected individuals were frozen, eventually being crushed together to form the spore solution. Mitchell et al. (2004) confirmed in a pilot study that there is no significant difference in infection rates between spores collected in different years, and consistent with this Little and Ebert (2001) found no difference in the infective properties of mixed spore solutions applied to diverse host collections.

From each replicate, five female offspring less than 24 h old was placed in a jar containing 50 ml of *Daphnia* medium, with purified sand at the bottom. The infection experiment was set up over four days. To each jar, 1×10^5 *P. ramosa* transmission spores were added. Everyday, until day 8, each jar was stirred with a glass rod to increase chances of contact with parasite spores. During the infection period *Daphnia* were fed 1×10^7 algae cells per jar on day 1, and 5×10^6 algae cells on days 3 and 6. This comparatively low level of food encourages the *Daphnia* to graze the sand, increasing contact with the parasite. Throughout the experiment all *Daphnia* were kept at 20°C, and experienced a light:dark cycle of 16:8 h.

On day 8 each group of five *Daphnia* were transferred to a jar containing 200 ml of *Daphnia* medium and fed 1.75×10^7 algae cells per day until the end of the experiment. Each jar was checked for newborn daily. When newborn were present the adult females were moved to a new jar. In the absence of any clutches *Daphnia* were transferred to a new jar with fresh medium every three days. The experiment finished on day 25 at which time each individual *D. magna* was frozen in a 1.5 ml eppendorf tube. Frozen *Daphnia* were later crushed in 100 μL of water, and then 8 μL of this was placed on to a Nebauer haemocytometer where we could confirm infection and count transmission spores (an estimate of parasite fitness).

ANALYSIS

For analysis we classified our field data into three sampling periods: before, during, and after the epidemic. The parasite epidemic was considered to be the period when prevalence was greater than 0.1, thus the epidemic spans from June 13, 2003, to October 17, 2003. Differences in the frequencies of the different multilocus electrophoretic genotypes collected before (the period April 25 to June 13), during (within the period June 27 to October 17), and after (the period from November 6) the parasite epidemic were analyzed using contingency table analysis. Fifteen "electrophoretic genotypes" were identified throughout the study period, but 10 that were present in low numbers were pooled into a separate "rare" group. Criteria for the rare group entailed those genotypes that had a count less than five in the contingency table analysis in either the before, during or after category of the epidemic. Similarly, we investigated allele frequency change over time.

Clonal diversity at each collection date was estimated using Simpsons diversity index, corrected for sample size (Rosenzweig 1997). Samples collected on the July 25, 2003, and December 15, 2003, were excluded as less than 10 daphnia were sampled on these dates. All 15 detected electrophoretic genotypes were used for this estimate of diversity. Each estimate of diversity was subtracted from 1 to obtain a value that increased with increasing diversity.

Conformance to Hardy–Weinberg equilibrium was determined at each locus for each sampling date also using chi-square

analysis. Samples collected on the July 27 and December 15 were excluded from this analysis as too few *Daphnia* were collected on these dates. Similarly samples that had expected values less than 5, and dates when the frequency of the most common allele was greater than 95%, were also excluded from this analysis. We used analysis of variance to see if there was a difference in observed and expected heterozygosities before, during and after the parasite epidemic. Expected heterozygosities were calculated at each of the three loci using expectations of the Hardy–Weinberg equation.

For the experimental data we used general linear models to study proportion of each genotype that became infected, infected offspring production, and parasite transmission spore production. Proportion data was arcsine square root transformed, offspring counts were square root transformed, and transmission spore counts were log transformed to meet the assumptions of ANOVA. Iso-female line was included in each model as a random effect, nested within "genotype." The experiment was set up over four days and thus "set up day" was included as a random effect in the models investigating proportion infected and offspring production. "Set up day" was not included in the model investigating parasite transmission spore production because each genotype did not contribute to each of the set up days. In this analysis we kept the group of rare genotypes the same for consistency. To relate genotype frequency changes in the field to susceptibility in the laboratory we calculated the percent change in frequency of each of the different genotypes from the beginning of the epidemic to the end. We then performed a Spearman's rank correlation to relate this change in frequency to mean proportion infected, mean offspring production and mean spore production of each of these genotypes in the laboratory infection experiment. All analyses were done using JMP 5.1.

Results

ALLOZYME VARIATION IN THE FIELD

Pasteuria ramosa first appeared in the population in late June, briefly reached 100% prevalence in the adult portion of the population in late July, and then declined until it was absent from the population by late November (Fig. 1). Figure 1 shows that the peak of the parasite epidemic does coincide with a drop in *Daphnia* abundance. There was, however, also a drop in *Daphnia* abundance before the parasite epidemic was present in the population thus emphasizing that changes in host population dynamics may be affected by a number of environmental factors. Clonal diversity ranged over time from 0.46 to 0.82 with a mean value of 0.66. Clonal diversity declined as parasite prevalence increased in the population (Fig. 2). However, as the epidemic abated, clonal diversity increased once again to preepidemic levels. It should though be noted that clonal diversity does decline again in late November. This emphasizes further that population dynamics would have been influenced by a variety of factors such as competition for food or temperature. Contingency table analysis to test for heterogeneity in the composition of electrophoretic genotypes collected before, during, and after the parasite epidemic indicated strong genetic change over time ($\chi^2 = 141.25$, df $= 8$, $P < 0.0001$; Fig. 3). Allele frequencies were found to change at loci AAT ($\chi^2 = 56.77$, df $= 2$, $P < 0.001$) and FUM ($\chi^2 = 12.82$, df $= 2$, $P = 0.0016$) before, during, and after the parasite epidemic. Allele frequencies were not observed to differ significantly throughout the study period at locus MPI ($\chi^2 = 2.27$, df $= 2$, $P = 0.32$) (Fig. 4).

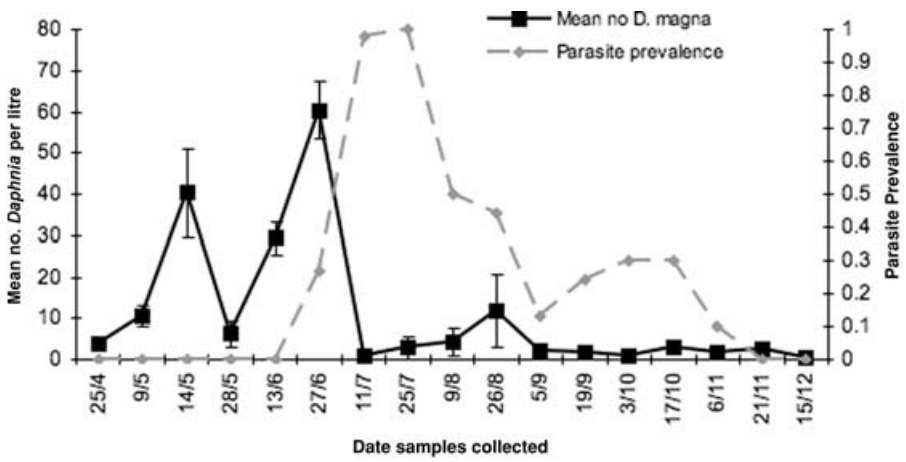

Figure 1. **Mean number of *Daphnia* per liter and proportion of population infected with *Pasteuria ramosa*. (± standard error) in collections from the Leitholm population in 2003.**

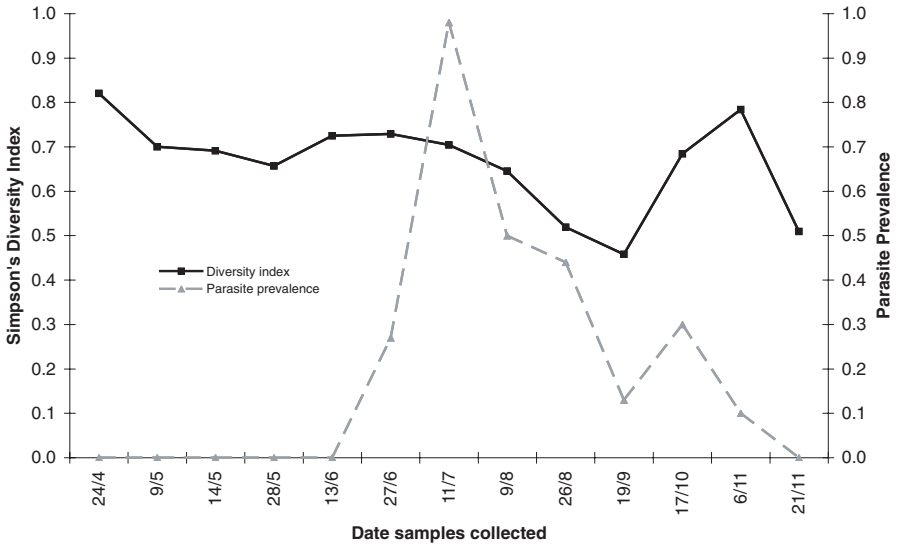

Figure 2. **Clonal diversity, based on Simpsons diversity index, in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* in 2003. (Samples collected on the July 27 and December 15 are excluded from Fig. 2.)**

Deviations from Hardy–Weinberg were consistently observed at locus MPI, frequently observed at locus FUM, and only once observed at locus AAT (Fig. 5). Neither observed heterozygosity ($F_{2,10} = 1.36, P = 0.30$) nor expected heterozygosity ($F_{2,10} = 2.93, P = 0.10$) was found to change significantly before, during, or after the parasite epidemic (Fig. 5).

EXPERIMENTAL INFECTIONS

We attempted to link the parasite epidemic to genotype changes observed in the field using a controlled infection experiment. Parasite growth, measured as mean number of transmission spores per milliliter from each host, was found to differ significantly on the different allozyme genotypes ($F_{4,81} = 10.43$, $P < 0.0001$). Although not significant, there was a trend for levels of infection to differ among the different allozyme genotypes ($F_{4,86} = 2.05$, $P = 0.095$). The different allozyme genotypes did not, however, differ in offspring production ($F_{4,86} = 0.45$, $P = 0.771$). There was a perfect match in the ranking for each electrophoretic genotype, in terms of percent change in frequency over the course of the epidemic, and mean proportion that became infected in the laboratory infection experiment (Fig. 6; $r = -1$, $P < 0.001$; see Neave and Worthington (1988) for discussion of significance

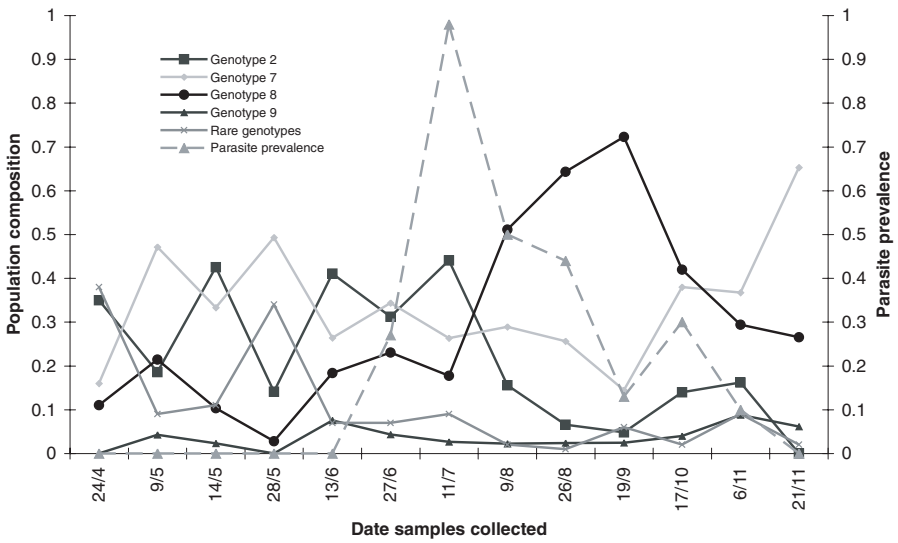

Figure 3. **Genotype frequency changes in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* in 2003. (Samples collected on the July 27 and December 15 are excluded from Fig. 3.)**

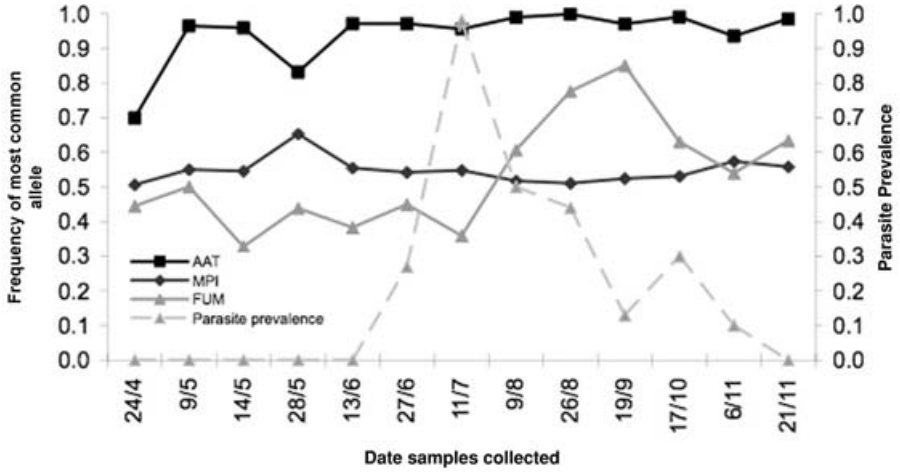

Figure 4. **The frequency of the more common allele at loci aspartate amino transferase (AAT), mannose-6-phosphate isomerase (MPI), and fumerate hydratase (FUM) in the Leitholm *Daphnia* population in relation to a parasite epidemic of the bacterial pathogen *Pasteuria ramosa* in 2003. (Samples collected on the July 27 and December 15 are excluded from Fig. 4.)**

levels when $n > 4$, and rankings are identical, or identical in the reverse). There was no relationship between percentage change in frequency during this period and offspring production ($r = 0.00$, $P = 1.00$) or parasite growth ($r = -0.3$, $P = 0.62$).

Discussion

This study showed that a natural and severe parasite epidemic was associated with genotype frequency (based on allozymes) changes in the host population. A controlled laboratory infection experiment revealed that the degree of decline experienced by particular allozyme genotypes was indeed related to susceptibility. This study therefore offers evidence of parasite-mediated natural selection in the wild.

A previous study on this population (Duncan et al. 2006), also conducted on the 2003 samples, corroborates the finding of parasite-mediated selection in this population. This earlier study simply compared a suite of isolates (which had not been genotyped with any molecular technique) collected before and after the epidemic and showed a decrease in average population susceptibility following the epidemic. Earlier work (Mitchell et al. 2005; Duncan et al. 2006), however, also indicated a mechanism that would limit the effectiveness of selection. In particular, there was a genetic correlation between the tendency to make resting eggs (which, in *D. magna,* are always the product of sexual reproduction) and susceptibility, that is, those genetic backgrounds that tend to engage in sex also tend to be more vulnerable to parasites. This observation (Duncan et al. 2006) implied that genotypes that

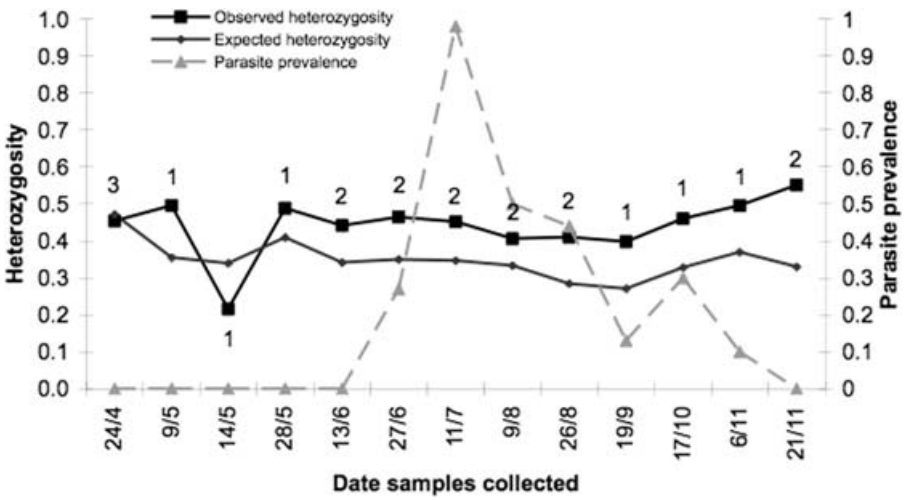

Figure 5. **Comparison of observed heterozygosity to expected heterozygosity (± standard error) over time in the Leitholm *Daphnia* population in 2003. The numbers above the points depicting observed heterozygosity indicate number of loci where deviations from Hardy–Weinberg were detected.**

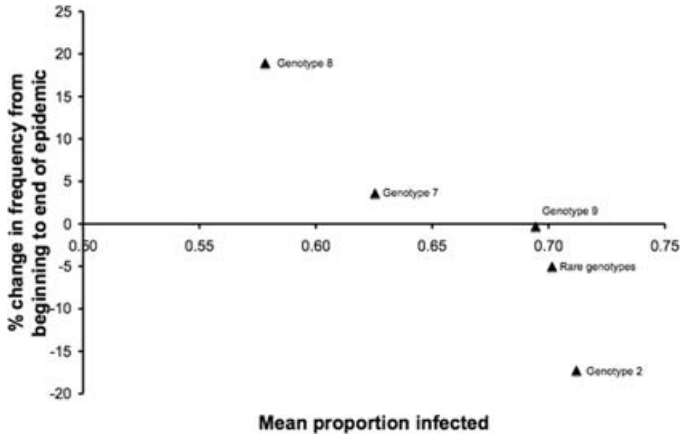

Figure 6. Percent change in frequency for each of the electrophoretic genotypes from the beginning of the parasite epidemic to the end, plotted against their mean levels of infection.

invest in the production of resting eggs will escape the parasite epidemic. The annual recruitment of genotypes from the resting egg bank will contribute relatively susceptible genotypes to the population and thus foster the maintenance of genetic diversity into the host population.

The present study indicates further mechanisms for the maintenance of genetic diversity in this population. Of particular interest is the observation that the genotype that increased most in frequency during the parasite epidemic (and had the lowest levels of infection under controlled conditions; genotype 8, Fig. 6), was prevalent prior to the epidemic at levels lower than the other genotypes and returned to low levels when the epidemic abated. Patterns of clonal dynamics observed in the present study are compatible with a number of hypotheses. For example, it is not inconceivable that this population experiences immigration that influences both allozyme genotype frequencies and mean resistance. Alternatively, genotype 8, the genotype that was most successful during the epidemic, could perform better at the warmer temperatures that coincided with the epidemic. Importantly, such hypotheses are testable in the laboratory using competition experiments.

Deviations from Hardy–Weinberg and multilocus genotypic equilibrium are common in populations of organisms with clonal reproduction and may indicate the occurrence of selection (Hebert 1974). In the present study, however, significant deviations from genetic equilibria did not coincide with parasite associated changes in genotype frequencies. Indeed, disequilibria were detectable even early in the field season. This indicates that this population is possibly not refounded each year solely from the resting egg bank (resting eggs are produced sexually and their hatching tends to shift populations back toward genetic equilibria), but instead may harbor females that survive the winter in a parthenogenetic state. Higher levels of genetic disequilibria are expected in populations that do not experience yearly extinction due to freezing or drying, and indeed we estimate that this <1 m deep pond may remain unfrozen throughout the winter. Neither observed, nor expected heterozygosities changed before, during, or after the parasite epidemic. Figure 4 does however show that observed heterozygosity was higher than expected heterozygosity indicating that deviations from Hardy–Weinberg in this population are due to an excess of heterozygotes.

Nevertheless, the parasite epidemic was associated with genetic change in the host population and laboratory experimentation supported the hypothesis that parasites caused the observed genetic fluctuations. This apparent response to selection is, as far as we are aware, among the clearest examples of direct observation of parasite-driven dynamics. Such observations appear to be rare in natural populations, which could indicate that parasite-mediated dynamics are not as substantial as required by theory on the evolutionary significance of biological interactions (Anderson and May 1982; Howard and Lively 1994; Peters and Lively 1999; Otto and Nuismer 2004). Our capacity to detect selection presently could be due to how parasite-mediated dynamics may interact with environmental factors. We conducted our field work for this study in 2003, which was the year Europe experienced the hottest heat wave on record (Schar et al. 2004). *Pasteuria ramosa* shows greater infectivity and causes higher virulence at higher temperatures in the laboratory (Mitchell et al. 2005). Although epidemics are observed each summer in our study pond (Mitchell et al. 2004), prevalence in 2003 was at least twice as high as in any previous year. The high temperatures of 2003 also caused reduced pond depth, which could have increased the encounter rate of *D. magna* with parasite spores, which lay in the sediment. Thus, our observation of parasite-mediated selection in the wild is probably linked in part to environmental conditions that were conducive to an exceptionally severe epidemic.

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