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Associations between parasitism and host genotype in natural populations of *Daphnia* (Crustacea: Cladocera)

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Summary

1. Numerous models of parasite–host coevolution have shown that parasitism could play a key role in a wide range of biological phenomena. One critical assumption underlying models of parasite–host coevolution is that natural selection acts on genetic variation for host susceptibility, but this assumption has rarely been tested in the wild.

2. Clonal hosts are particularly suitable for detecting associations between genotypes and parasitism, because multi-locus gene complexes are preserved during reproduction, and following clonal selection, hosts which differ at resistance loci may also show differences at neutral marker loci. In this study, allozymes were used to search for associations between multi-locus host genotypes (clones) and parasitism within 25 cyclically parthenogenetic populations of *Daphnia*.

3. Rates of parasitism were observed ranging from 0% to 48% of adult females, and infection by microparasites was found to significantly reduce host fecundity.

4. Significant differences were detected between the clonal composition of parasitized and healthy *Daphnia* in 12 populations, showing that the proportion of individuals infected varies among clones. Six populations were sampled repeatedly to determine whether this variation was associated with temporal changes in both host clonal frequencies and population genetic characteristics (Hardy–Weinberg equilibrium, genotypic linkage disequilibrium). Three of the six temporally sampled populations showed changes in genetic structure that were consistent with the expectations of parasite-mediated selection, while the other three did not. These latter three populations all had low parasite prevalence (indicating low selection intensities) and/or comprised primarily rare clones, thus limiting the statistical power of the study to detect parasite-mediated effects.

5. The results show the presence of genetic variation for the likelihood of contracting disease within *Daphnia* populations. These associations between infection and host genotype could result from genetic variation for parasite resistance or within-pond spatial arrangement of both host genotypes and parasite prevalences. Either factor would lead to parasite-mediated selection and the modulation of gene frequecies within populations.

Key-words: linkage, microsporidia, parasite, parthenogenesis, Red Queen hypothesis, resistance, selection.

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Introduction

A substantial body of theoretical work suggests that parasitism could play an important role in a variety of ecological and evolutionary phenomena, including sexual selection (Hamilton & Zuk 1982), population densities and dynamics (Anderson & May 1979; May & Anderson 1979; Anderson 1995), host behaviour (Hamilton 1971; Poulin 1994), and the maintenance of sexual reproduction and genetic polymorphism (Levin 1975; Jaenike 1978; Hamilton 1980; Hamilton, Axel-

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rod & Tanese 1990). A principal mechanism determining the impact of parasites on their host population is the differential reproductive success of parasitized vs. healthy hosts (i.e. parasite-mediated natural selection). Natural selection by parasites requires genetic variation for resistance, and indeed the existence of ample resistance-variation is the central assumption underlying models of parasite–host coevolution. However, this assumption has hardly been tested in natural populations (Sorci, Møller & Bouliner 1997).

Present understanding of the extent of genetic variation for disease resistance comes primarily from laboratory studies documenting plant responses to their pathogens (Alexander, Antonovics & Rausher 1984; Burdon & Jarosz 1991; Jarosz & Burdon 1991; reviewed in Thompson & Burdon 1992; Parker 1994), and from studies of humans (Ruwende *et al.* 1995; Singh, Agrawal & Rastogi 1997). Far fewer studies have considered natural systems involving animals, and most of these are local adaptation studies which assayed parasite–host interactions across populations (Lively 1989; Ebert 1994; Mopper 1996). We lack data on genetic variation for resistance *within* natural populations. Those few studies which examined intra-population variation in the wild have typically detected it, indicating the potential for parasite-driven evolutionary change within these populations (Grosholz 1994; Henter & Via 1995). The present study aims to extend our understanding of the significance of parasite-mediated selection in the wild by investigating genetic variation for resistance within multiple populations of freshwater crustaceans in the genus *Daphnia*.

Knowledge of the temporal patterns of resistance-variation is also needed to assess the importance of parasitism as an evolutionary force. However, few studies have sought to demonstrate that genetic variation for resistance results in parasite-mediated natural selection, and these have typically met with mixed results; for example, studying a viral interaction with a lepidopteran host, Fuxa, Mitchell & Richter (1988) recorded an increase in the incidence of resistance (based on laboratory-measured median lethal doses) in just one year of a 3-year study, although they did observe an increase in the heterogeneity of resistance in all three years. Working with an aphid-parasitoid system, Henter & Via (1995) detected no response to selection, despite the presence of genetic variation for susceptibility. From these studies it is apparent that monitoring coevolutionary interactions in the wild will not always be straightforward.

This paper presents the results of a study on cyclically parthenogenetic cladocerans in the genus *Daphnia* and their microparasites. We investigated the utility of using genetic markers to gain insight into the genetic variation associated with infection in a large number of natural populations representing a variety of host and parasite species. Further, we explored whether clonal variation for infection in *Daphnia* is associated with temporal changes in population genotype frequencies (an indication of selection). *Daphnia* and their microparasites combine a number of characteristics which made these aims feasible.

THE *DAPHNIA*-MICROPARASITE SYSTEM

One difficulty associated with detecting genetic variation for resistance within natural populations is that, typically, it is not known what genes are involved. This problem can be circumvented by using parthenogenetic host species such as *Daphnia*. Although most *Daphnia* species do have a sexual phase, throughout the summer in temperate climates, reproduction is largely apomictic. During apomixis, the entire genome is transmitted clonally from parent to offspring, and multi-locus gene complexes are preserved. Such complexes can include non-random associations of neutral loci and loci under selection, and during selection, neutral alleles may 'hitchhike' with selected loci. This is one possible reason why clones identified with allozymes (which are presumably neutral markers) often differ in their response to a variety of selective forces (Carvalho 1987; Carvalho & Crisp 1987; Weider & Hebert 1987). Assuming that *Daphnia* clones which differ at resistance loci, might, in some cases, also differ at allozyme loci, genetic variation for infection was sought by using allozymes to determine whether infected individuals within a population have a different clonal composition from that of healthy individuals. Given a cost of parasitism, it was predicted that clones which are over-represented in the parasitized class will decline in frequency in the population over time, or conversely, that clones which are under-represented in the parasitized class will increase in frequency. Because of the short generation time of *Daphnia*, it is possible to observe gene frequency changes for up to 15 generations per season.

Daphnia are attacked by a wide array of microparasites, with bacterial, fungal and microsporidial parasites being particularly common (Green 1974; Stirnadel & Ebert 1997). Many of these parasites are known to affect the fitness of their victims substantially (Green 1974; Schwartz & Cameron 1993; Ebert 1995; Mangin, Lipsitch & Ebert 1995; Stirnadel & Ebert 1997), with sites of infection including the gut, ovaries, fat cells, the muscles that control the swimming antennae, epidermis, haemolymph and the eggs. Parasite biomass within hosts is often considerable and, because *Daphnia* have a transparent carapace, it is possible to detect many infections without dissection (Ebert & Mangin 1995; Ebert *et al.* 1996; Stirnadel & Ebert 1997; see also http://www.unibas.ch/dib/zoologie/ebert/hostpara/index.html). As individuals had to be separated into parasitized and healthy classes without dissection prior to genetic characterization, analyses in the present

study were centred on externally detectable parasites with apparently severe effects.

Methods

SAMPLING, EGG COUNTS AND ELECTROPHORESIS

During the summer of 1996 and spring 1997, *Daphnia* were collected from 70 ponds in north-west Switzerland (S), north-east France (F), in southern Germany near Munich (SG), and in northern Germany near Plön (NG) by passing an $\approx 300\,\mu$m mesh net through the water near shore. There was one collecting site per pond per date, and successive collections were always made at the same location in the pond. To avoid spreading parasites and hosts among ponds, each pond was sampled with different equipment, which was cleaned and sterilized between sampling excursions. To limit host mortality, collections were immediately cooled and maintained at between 1 and 5 °C until they were analysed in the laboratory, always within 24 h.

Each sample was first screened for the number of parasite species that occurred frequently enough for analysis and which were distinguishable without host dissection under a microscope at 50× to 100× magnification. Parasite prevalences (the proportion of infected individuals in a random population sample) were estimated from a sample of 70–120 adult females. Each parasite species was treated separately and the genetic profile of infected hosts was compared to that of uninfected hosts. The genetic profile of a randomly selected group of individuals was also determined. In total then, from each population sample at least three sub-samples were taken: random, healthy and parasitized (one sub-sample for each parasite species). Using a Folsom plankton divider, the random sub-sample (≈ 70 individuals) was always taken first. To estimate the impact parasites have on their hosts' fitness, the average clutch sizes (number of eggs present in the brood chamber) of infected and uninfected hosts were compared in 14 populations using a Mann–Whitney *U*-test. Comparing clutch sizes is likely to underestimate fitness effects, as it does not consider survivorship.

Allozyme phenotypes were discriminated using standard methods of cellulose acetate electrophoresis (Hebert & Beaton 1993). Twenty-four individuals from each population were initially screened for variation at from nine to 12 enzyme loci known to be often polymorphic in *Daphnia* and other freshwater crustaceans. These enzymes included aldehyde oxidase (*AO*) (EC 1.2.1), arginine phosphokinase (*APK–1*) (EC 2.7.2), aspartate aminotransferase (*AAT*) (EC 2.6.1.1), fumarate hydratase (*FUM*) (EC 4.2.1.2), glucophosphate isomerase (*GPI*) (EC 5.1.9), isocitrate dehydrogenase (*IDH*) (EC 1.1.1.42), lactate dehydrogenase (*LDH*) (EC 1.1.1.27), malic enzyme (*ME*) (EC 1.1.1.40), malic dehydrogenase (*MDH*) (EC 1.1.1.37), mannose phosphate isomerase (*MPI*) (EC 5.1.8), peptidase-C (*phenyl-proline*) (*PEP*-C), phosphoglucomutase (*PGM*) (EC 2.7.5.1). Following the initial screening, individuals were analysed for variation at only those loci found to be both polymorphic and to have banding patterns that could be reliably interpreted. Occasionally, attempts were also made to detect allozyme bands for parasites by intensely grinding up heavily infected *Daphnia* and electrophoresing them alongside healthy hosts. In a few rare cases additional bands were observed, which presumably originated from the parasites, but these clearly migrated at a different rate and were much less intense than the host allozyme bands.

ANALYSIS

For each population, allozyme electromorphs were labelled alphabetically based on their mobility, with the slowest electromorph being labelled 'a'. Except for populations F4, F5 and F6 (Table 1), banding patterns were not compared among populations. Allozyme bands with unique electrophoretic mobility were assumed to correspond to unique alleles, and distinct multi-locus genotypes were termed clones, but with the following caveat: individuals sharing the same allozyme phenotype may posses amino acid substitutions that do not result in detectable mobility differences, or they might differ at loci not assayed. Thus, an allozymic 'clone' probably represents a clonal group whose members happen to share the same allozyme phenotype. Clonal diversity in each population was estimated using Simpson's (1949) index of diversity modified for finite populations (Pielou 1969).

Significant differences between the clonal composition of the parasitized and healthy classes in each sample were determined with a contingency table and Fisher's Exact Test (SAS Institute Inc. 1989). When a significant difference was detected between the parasitized and healthy class, further Exact Tests were undertaken on particular clones to determine which ones were 'overparasitized' and which were 'underparasitized'. Where possible, when significant associations were found clonal frequencies were followed over time and the standard deviations were calculated (square-root of the sampling variance) around the estimates of these frequencies at each time point. This sampling variance was obtained with the multinomial method used for allele frequency estimates (see Weir 1990).

Using the computer program BIOSYS-1 (Swofford & Selander 1988), with a locus considered polymorphic if the frequency of the common allele did not exceed 0·99, individual heterozygosities, the fixation index (F), and conformance to Hardy–Weinberg (H.W.) equilibrium were determined for the random sample from each population. Measures of heterozygosity

Table 1. Infection data for 25 *Daphnia* populations and their parasites collected from ponds in central Europe. As some host populations were infected with more than one parasite species, these populations yielded 34 samples, each representing one unique parasite species – host species combination. In column one, upper case letters represent the geographical location of the pond (S, north-west Switzerland; F, north-east France; SG, southern Germany near Munich; and NG, northern Germany near Plön) numbers represent single ponds, and lower case letters indicate cases where different parasite species infected the same host population. *P* is the significance of differences in the clonal composition of the parasitized and healthy class of individuals, based on a contingency table analysis. Group A are those samples which showed significant differences, group B are those which showed no difference

Sample	Host	Parasite	Site of infection	Parasite prevalence	*P*
Group A					
F3b	*D. longispina*	*Thelohania* sp. (Microsporidia)	Epidermis	4·1%	<0·001
S2	*D. longispina*	Unknown microsporidium 3	Muscle/ovary/fat cell	<1%	0·006
F7	*D. obtusa*	Unknown microsporidium 5	Ovary/fat cell	7·8%	0·038
F4	*D. pulex*	*Thelohania* sp. (Microsporidia)	Epidermis	40·0%	<0·001
F5	*D. pulex*	*Thelohania* sp. (Microsporidia)	Epidermis	32·0%	<0·001
F6	*D. pulex*	*Thelohania* sp. (Microsporidia)	Epidermis	8·5%	<0·001
F9	*D. pulex*	Unknown microsporidium 1	Ovary/fat cell	10·0%	<0·001
F10a	*D. pulex*	Unknown bacterium 1	Epidermis/haemolymph	4·2%	0·014
F11	*D. pulex*	Unknown microsporidium 2	Ovary/fat cell	4·2%	0·023
S1a	*D. pulex*	Unknown microsporidium 6	Ovary/fat cell	2·3%	0·023
F13	*D. pulicaria*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber/eggs	7·0%	0·014
Ng3	*D. pulicaria*	Unknown microsporidium 4	Muscle/ovary/fat cell	<1%	<0·001
Group B					
F2	*D. ambigua*	Unknown microsporidium 7	Gut	44·0%	NS
F1	*D. longispina*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber/eggs	7·8%	NS
F3a	*D. longispina*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber/eggs	20·0%	NS
F12	*D. longispina*	*Thelohania* sp. (Microsporidia)	Epidermis	<1%	NS
Ng2	*D. longispina*	Unknown microsporidium 4	Ovary/fat cell	2·8%	NS
S4	*D. longispina*	Unknown microsporidium 3	Ovary/fat cell	10·4%	NS
S5	*D. longispina*	Unknown microsporidium 3	Ovary/fat cell	9·7%	NS
Sg2a	*D. longispina*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber	<1%	NS
Sg2b	*D. longispina*	Unknown microsporidium 9	Ovary/fat cell	<1%	NS
Ng1a	*D. magna*	White Bacterial Disease	Ovary/fat cell	<1%	NS
Ng1b	*D. magna*	*Thelohania* sp. (Microsporidia)	Epidermis	3·7%	NS
Sg1	*D. magna*	White Bacterial Disease	Ovary/fat cell	<1%	NS
F8	*D. pulex*	Unknown microsporidium 8	Ovary/fat cell	3·9%	NS
F10b	*D. pulex*	*Gurleya vavrai* (Microsporidia)	Epidermis	4·2%	NS
F10c	*D. pulex*	Unknown microsporidium 2	Ovary/fat cell	4·2%	NS
S1b	*D. pulex*	Unknown bacterium 1	Epidermis/haemolymph	3·4%	NS
S3a	*D. pulex*	Unknown bacterium 1	Epidermis/haemolymph	1·9%	NS
S3b	*D. pulex*	*Thelohania* sp. (Microsporidia)	Epidermis	7·6%	NS
S3c	*D. pulex*	*Caulerya mesnili* (Haplosporidia)	Gut	43·5%	NS
S8a	*D. pulex*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber/eggs	10·4%	NS
S8b	*D. pulex*	White Bacterial Disease	Ovary/fat cell	2·1%	NS
F14	*D. pulicaria*	*Saprolegnia* sp. (Saprolegniaceae)	Brood chamber/eggs	48·0%	NS

were also calculated for both the healthy and parasitized class in each population. Conformance to H.W. equilibrium was determined by considering all alleles except the most common one as a single allele, then calculating exact significance probabilities (Swofford & Selander 1988). Using Fisher's Exact Test, it was also possible to determine whether pairs of genotypes from different loci were found together more often than would be expected by chance alone (i.e. genotypic linkage disequilibrium). Although most analyses of linkage consider haplotypic (gametic) disequilibrium, assessment of genotypic disequilibrium is appropriate for apomictic taxa, where the entire diploid genome represents a single linkage unit upon which selection may act (see also Little & Hebert 1997). When multiple tests for H.W. equilibrium or genotypic disequilibrium were performed within single populations on the same sampling date, significance levels were adjusted with a sequential Bonferroni correction (Rice 1989). The calculations of H.W. equilibrium and genotypic linkage disequilibrium were carried out because populations under selection are expected to show deviations from both these equilibria. Thus, these two population genetic parameters provide an indication of selection independent from observing and correlating gene frequency changes.

Motivated by Haldane's (1949) assertion that the

most successful parasites will be those that can infect common host genotypes, the possibility that common genotypes tend to be 'overparasitized' was investigated. This assumption is made by many models of host–parasite coevolution and the Red Queen hypothesis (see Dybdahl & Lively 1995). To test this idea, we performed a regression of clonal frequencies in the parasitized class on clonal frequencies in the random class in each population. If clonal frequencies in these two classes were, on average, identical, then the slope of the regression line should not differ from one, i.e. 'overparasitized' clones would be found evenly distributed among rare and common clones. A slope above one would indicate that common clones tend to be 'overparasitized', while a slope below one would indicate the opposite.

Results

Parasite prevalences were sufficiently high for analysis in 25 of 70 *Daphnia* populations examined. Five of these 25 populations were infected with two parasite species, while two populations were infected with three parasite species, giving a total of 34 samples, each representing one unique parasite species–host species combination (Tables 1 and 2). Parasite prevalences ranged from less than 1% to 48%, averaging 10% (Table 1). In total 15 different parasite species were found which included 11 microsporidians, two bacteria, one fungus (Saprolegniaceae), and one haplosporidium. Many of the parasite species encountered had not previously been described, and full descriptions of some of these will be presented elsewhere (R. Larsson and D. Ebert, unpublished data). Multiple infections of single individuals were rare, the only notable exception being population S3 (Table 1), where many individuals infected with the gut parasite *Caulerya mesnili* also harboured one of the other parasites present in this population. In this case, no evidence was found that infection by one parasite species tended to increase susceptibility to other parasites.

The clutch sizes of infected and healthy females were compared in 14 samples. Infected females carried significantly fewer eggs than healthy ones in nine of these (Table 3), an observation compatible with previous studies of parasite-induced fecundity reduction in *Daphnia* (Mangin *et al.* 1995; Ebert *et al.* 1996; Stirnadel & Ebert 1997).

Clonal diversity in host populations, as estimated by Simpson's diversity index, ranged from 0·14 to 0·97 and averaged 0·68. In 12 of the 34 samples the clonal composition of the healthy class of hosts differed significantly from that of the parasitized hosts. In Tables 1 and 2, therefore, the data from these 34 samples were split into two groups. Group A contains the 12 samples that showed significant differences between the parasitized and healthy class, while group B includes the 22 samples for which no such difference was detected. All of the significant associations were evident the first time the populations were sampled, except for population F10 in which the significant association was detected the second time the relevant parasite was present. The values of zero entered for clonal diversity, heterozygosity and the fixation index for sample NG3 (Table 2) are a result of the fact that no polymorphism was detected in the random class, although the parasitized class was polymorphic.

To compare the population genetic characteristics (determined for the random sub-sample) of groups A and B, duplicate samples from the same date were discarded (NG1b, SG2b, S1b, S3b and S3c; see Table 2), and thus $n = 12$ for group A, and $n = 17$ for group B. Populations from Group A had lower clonal diversity than those from group B (Mann–Whitney U-test: $U = 53{\cdot}3$, $z = 2{\cdot}15$, $P = 0{\cdot}032$). Group A populations also seemed to show a greater incidence of both linkage disequilibrium (18 of 41 tests significant in group A vs. 5 of 67 in Group B) and H.W. deviations (7 of 32 tests significant in group A vs. 5 of 51 in Group B). Accordingly, the fixation index in Group A samples (average $F = -0{\cdot}141$) was larger and more negative, than in the group B populations (average $F = +0{\cdot}033$) (Mann–Whitney U-test: $U = 50$, $z = -2{\cdot}3$, $P = 0{\cdot}02$). This difference in the fixation index suggests that H.W. disturbances towards heterozygote excess were more severe in those populations where significant clonal variation for infection was evident. In an effort to verify that deviations from H.W. equilibria were more prevalent in group A populations, populations were coded as either having a deviation (1) or not (0). Contingency tables were then constructed, and using a χ^2 analysis, the total number of deviations in group A were compared with the total number in group B. A similar comparison of the incidence of genotypic linkage disequilibria was performed, but neither of these (conservative) comparisons revealed a significant difference between the two groups, although the trend towards more disequilibria in group A was evident.

A paired t-test, comparing healthy and parasitized *Daphnia* in all 34 samples, indicated that neither expected nor observed heterozygosity differed between the parasitized and the healthy classes (Table 2).

Table 4 contains detailed information on the clonal composition of group A samples, as well as the results of tests to determine whether particular clones (as opposed to comparing the healthy vs. the parasitized class of hosts) were under- or overparasitized. Although Table 4 primarily includes data from only one sample per population, a second sample from the same parasite–host combination was included in population S2. This second sample (S2-$t2$), taken 6 days after the first sample, showed a significant clonal association with parasite infection, but this association now largely involved a clone (clone 2) which had not contributed to the significant results of the previous week. For population F3, clone 4 is the only

Table 2. Population genetic data for 25 populations of *Daphnia* representing 34 unique parasite species–host species combinations (see Table 1). Individual heterozygosities were calculated separately for the random, healthy and parasitized class in each population, while other parameters considered only the random population sample. Clonal diversity is based on the Simpson diversity index. F is the fixation index. H.W. denotes a Hardy–Weinberg disturbance ($P < 0.05$) at X of Y polymorphic loci, while L.D. denotes significant ($P < 0.05$) linkage between X of Y possible two-loci genotype combinations. Para = parasitized. Group A represents those samples which showed a significant difference between the clonal composition of healthy and parasitized hosts (see Table 4 for details), group B shows those which demonstrated no difference. Averages do not include duplicate samples from the same date (NG1b, SG2b, S1b, S3b, S3c)

			Observed heterozygosity			Expected heterozygosity					
Sample	Date	Clonal Diversity	Random	Healthy	Para	Random	Healthy	Para	F	H.W.	L.D.
Group A											
F3b	17/05/96	0·76	0·111	0·111	0·111	0·121	0·121	0·056	0·062	1 of 3	2 of 3
S2	10/07/96	0·85	0·184	0·184	0·228	0·137	0·137	0·165	−0·415	2 of 4	4 of 6
F7	05/05/96	0·87	0·099	0·100	0·109	0·097	0·098	0·094	−0·045	0 of 3	1 of 3
F4	11/05/96	0·48	0·034	0·048	0·013	0·032	0·044	0·012	−0·084	0 of 2	0 of 1
F5	11/05/96	0·40	0·026	0·038	0·011	0·024	0·034	0·011	−0·064	0 of 2	0 of 1
F6	11/05/96	0·50	0·042	0·046	0·000	0·036	0·038	0·000	−0·113	0 of 2	0 of 1
F9	27/04/96	0·39	0·110	0·115	0·085	0·107	0·113	0·059	0·065	2 of 5	9 of 10
F10a	14/06/96	0·95	0·160	0·160	0·141	0·141	0·141	0·142	−0·122	0 of 4	1 of 6
F11	14/06/96	0·97	0·143	0·140	0·141	0·154	0·153	0·136	0·046	0 of 4	0 of 8
S1a	19/07/96	0·14	0·293	0·293	0·280	0·151	0·151	0·149	−0·957	2 of 2	0 of 1
F13	22/05/96	0·21	0·007	0·006	0·016	0·006	0·006	0·015	−0·063	0 of 1	1 of 1
NG3	15/05/97	0·00	0·000	0·000	0·058	0·000	0·000	0·039	0·000	0 of 0	0 of 0
Mean (A)		0·54	0·101	0·103	0·099	0·084	0·086	0·073	−0·141	7 of 32	18 of 41
Group B											
F2	31/05/96	0·42	0·048	0·066	0·035	0·081	0·107	0·059	0·668	1 of 2	1 of 2
F1	14/05/96	0·73	0·118	0·118	0·116	0·126	0·127	0·114	0·008	1 of 3	1 of 3
F3a	12/05/96	0·86	0·116	0·113	0·111	0·118	0·122	0·116	0·005	1 of 3	1 of 3
F12	28/05/96	0·94	0·205	0·190	0·171	0·208	0·199	0·187	0·077	0 of 3	0 of 3
NG2	10/04/96	0·94	0·158	0·154	0·184	0·155	0·152	0·179	0·098	1 of 1	1 of 10
S4	08/02/96	0·77	0·075	0·073	0·077	0·069	0·070	0·061	−0·080	0 of 2	0 of 1
S5	08/02/96	0·61	0·034	0·035	0·021	0·041	0·040	0·021	0·052	0 of 3	0 of 3
SG2a	24/08/96	0·93	0·141	0·141	0·131	0·125	0·125	0·124	−0·126	1 of 3	0 of 3
SG2b	24/08/96	0·93	0·141	0·141	0·154	0·125	0·125	0·131	−0·126	1 of 3	0 of 3
NG1a	10/04/96	0·95	0·132	0·132	0·142	0·135	0·135	0·140	0·019	0 of 3	0 of 3
NG1b	10/04/96	0·95	0·132	0·132	0·141	0·135	0·135	0·135	0·019	0 of 3	0 of 3
SG1	24/08/96	0·85	0·107	0·107	0·097	0·105	0·105	0·098	−0·045	0 of 4	1 of 6
F8	05/10/96	0·81	0·068	0·068	0·064	0·075	0·074	0·075	0·104	0 of 2	0 of 1
F10b	06/06/96	0·96	0·154	0·156	0·147	0·147	0·149	0·148	−0·052	0 of 4	0 of 6
F10c	24/05/96	0·97	0·168	0·154	0·142	0·168	0·154	0·154	−0·063	0 of 4	0 of 6
S1b	19/07/96	0·14	0·293	0·293	0·286	0·151	0·151	0·151	−0·957	2 of 4	2 of 4
S3a	09/04/96	0·79	0·079	0·074	0·063	0·071	0·070	0·070	−0·112	0 of 2	0 of 1
S3b	09/04/96	0·79	0·079	0·074	0·072	0·071	0·070	0·069	−0·112	0 of 2	0 of 1
S3c	09/04/96	0·79	0·079	0·074	0·077	0·071	0·070	0·069	−0·112	0 of 2	0 of 1
S8a	17/08/96	0·94	0·133	0·124	0·168	0·145	0·144	0·165	0·064	0 of 5	0 of 10
S8b	21/08/96	0·92	0·171	0·172	0·138	0·168	0·168	0·146	−0·026	0 of 5	0 of 5
F14	31/05/96	0·68	0·063	0·054	0·074	0·064	0·060	0·066	−0·024	0 of 2	0 of 1
Mean (B)		0·83	0·116	0·114	0·111	0·118	0·118	0·113	0·033	5 of 51	5 of 67
Mean (A & B)		0·71	0·109	0·109	0·106	0·104	0·105	0·096	−0·039	12 of 83	23 of 108

clone shown to have *PGM* allele 'b' (which might suggest that it is a different species or a hybrid), but this allele was also present among the rarer clones whose phenotypes are not listed in Table 4. Moreover, when all clones are considered, *PGM* allele 'b' was not consistently associated with any particular clone, but was, instead, found in a range of different multilocus phenotypes, and thus the distribution of this allele provides no indication that a second species was present in the sample.

A regression of [log (x + 1 transformed] clonal frequencies in the parasitized class vs. clonal frequencies in the random class, to test the notion that common clones tend to be disproportionately infected, produced a line ($y = 0.999x - 0.0005$) with a slope not significantly different from one ($z = 1.13$, $n = 626$, $P = 0.26$) (Fig. 1a). When only the [log(x + 1) transformed] frequencies of the significantly disproportionately infected clones listed in Table 4 were considered, the slope of the resulting line

Table 3. The average number of eggs found in the brood chambers of parasitized and healthy adult *Daphnia* females. *P* is the significance of differences in the averages, based on a Mann–Whitney *U*-test

Sample	Date	Host	Parasite	Average number of eggs (SE)		Sample size		
				Healthy	Parasitized	Healthy	Parasitized	*P*
F2	31/05/96	*D. ambigua*	Unknown microsporidium 7	3·7 (0·37)	3·4 (0·33)	33	30	NS
F3a	17/05/96	*D. longispina*	*Saprolegnia* sp. (Saprolegniaceae)	1·2 (0·16)	0·0 (0·00)	54	44	0·001
F12	28/05/96	*D. longispina*	*Thelohania* sp. (Microsporidia)	0·9 (0·36)	0·0 (0·00)	72	48	0·001
S2	19/07/96	*D. longispina*	Unknown microsporidium 3	0·4 (0·07)	0·06 (0·03)	91	79	0·001
S4	08/02/96	*D. longispina*	Unknown microsporidium 3	0·5 (0·09)	0·1 (0·06)	82	70	0·003
NG1a	10/04/96	*D. magna*	White Bacterial Disease	1·1 (0·25)	0·8 (0·17)	78	63	NS
F7	05/05/96	*D. obtusa*	Unknown microsporidium 5	10·6 (1·05)	7·9 (1·40)	18	9	NS
F7	14/05/96	*D. obtusa*	Unknown microsporidium 5	7·1 (0·51)	6·9 (0·73)	37	36	NS
F7	21/05/96	*D. obtusa*	Unknown microsporidium 5	2·3 (0·39)	3·6 (0·40)	44	46	NS
F10a	14/06/96	*D. pulex*	Unknown bacterium 1	3·5 (0·36)	0·0 (0·00)	36	24	0·001
F10b	14/06/96	*D. pulex*	*Gurleya vavrai* (Microsporidia)	3·5 (0·36)	0·0 (0·00)	36	24	0·001
F10c	24/05/96	*D. pulex*	Unknown microsporidium 2	0·7 (0·21)	0·2 (0·11)	34	39	0·04
S3a	09/04/96	*D. pulex*	*Caulerya mesnili* (Haplosporidia)	1·6 (0·33)	0·4 (0·14)	51	33	0·03
S8a	17/08/96	*D. pulex*	*Saprolegnia* sp. (Saprolegniaceae)	0·8 (0·14)	0·2 (0·08)	65	52	0·005

($y = 0{\cdot}969x + 0{\cdot}01$) also did not differ from one ($z = 0{\cdot}67$, $n = 19$, $P = 0{\cdot}50$) (Fig. 1b).

TEMPORAL PATTERNS

Six populations (F4, F5, F6, F7, F10, and S2) which showed significant parasite–host clone associations were sampled repeatedly to test for parasite-associated changes in clone frequencies in a random population sample (Figs 2, 3, 4, 5). Populations F4, F5 and F6 contained the same host and parasite species (Table 1), were all roughly the same size ($\approx 100\ m^2$, 2–3 m depth), were located within 20 m of each other, and were each sampled on the same three dates. Thus, changes in clonal frequencies, parasite prevalences and population genetic characters are presented together for F4, F5 and F6 in Fig. 2. These three ponds each harboured the same 'overparasitized' clone (clone 1), which was significantly 'overparasitized' on 14 and 18 May in population F5, and on all three time points in both F4 and F6. There were, however, differences among these ponds with regard to which clones were significantly 'underparasitized' (Table 4). In populations F4 and F5, 'underparasitized' clones increased in frequency over time while the 'overparasitized' clone decreased in frequency. Population F6 showed the reverse pattern. Construction work at this field site prevented continued sampling of these three ponds.

Figure 3 presents the temporal data for population F7, which showed a significant parasitism–genotype association on 5 May, but not on later sampling dates. No single clone in this population was significantly 'under-' or 'overparasitized' at any time during the sampling period.

Figure 4 presents temporal data for population F10, a population composed entirely of rare clones (none with a frequency greater than 0·17). Three parasite species were present in this population (parasites a, b and c in Fig. 4, corresponding to F10a, F10b, F10c in Tables 1 and 2). The significant association between parasite 'a' and host genotype was evident on 14 June but not on either the previous sampling date or on later sampling dates. On 14 June it was possible to identify clone 2 (Table 4, Fig. 4) as being 'underparasitized'.

Figure 5 depicts allele frequencies, and the frequencies of the five dominant clones in population S2, which was sampled about once per week for 11 weeks. The strongly 'overparasitized' clone 5 (Table 4) was omitted from Fig. 4 because it was detected exclusively in the parasitized class on 10 July ($n = 7$ of 46 individuals), and was not found again in later samples. Population S2 had a number of 'overparasitized' clones on 10 and 16 July, primarily involving clones heterozygous at *GPI* and *PGM* (see Table 4). These associations were absent by 23 July, concordant with a decline in the frequency of the *GPI* and *PGM* heterozygotes. From 16 July onwards, significant associations were primarily attributable to the two most common clones, clone 2 ('underparasitized') and clone 3 ('overparasitized')(Fig. 5).

Some populations that showed no significant clonal associations with parasitism when first sampled were genetically characterized over time to determine whether associations might develop. These populations, the number of additional samples taken, and over what time period, were: S5, one additional sample, 1 week later; S4, six times in 9 weeks; F3a (*D. longispina–Saprolegnia* fungus combination), three times over 3 weeks; F14, twice in 1 week; F2, twice in a 2-week period; and S8, three times over 3 weeks. No associations between host clones and parasitism were detected in these samples.

Table 4. Clonal frequencies and allozyme phenotypes in 12 populations (group A from Tables 1 and 2) in which the clonal composition differed significantly between the healthy and parasitized classes based on a contingency table analysis and Fisher's Exact Test. *P*-clone is the significance of Exact Tests applied to individual clones. Note that data from two sampling dates are shown for population S2 (*t*1 and *t*2)

				Frequency		
Pond	Host	Clone	ENZYME/phenotype	Healthy	Parasitized	*P*-clone
F3b	*D. longispina*	1	PGM/ac AAT/ab	0·07	0·00	NS
		2	PGM/cc AAT/ab	0·20	0·00	NS
		3	PGM/cc AAT/aa	0·07	0·00	NS
		4	PGM/ab AAT/bb	0·43	1·00	<0·001
		5	PGM/cc AAT/bb	0·07	0·00	NS
		6	PGM/ac AAT/bb	0·05	0·00	NS
		6 rare		0·11	0·00	
				$n = 74$	$n = 45$	
F4	*D. pulex*	1	MPI/aa AO/aa	0·58	0·92	<0·001
		2	MPI/aa AO/ab	0·23	0·08	0·043
		3	MPI/ab AO/aa	0·19	0·00	0·007
				$n = 75$	$n = 52$	
F5	*D. pulex*	1	MPI/aa AO/aa	0·70	0·90	NS
		2	MPI/aa AO/ab	0·00	0·10	NS
		3	MPI/ab AO/aa	0·30	0·00	<0·001
				$n = 72$	$n = 31$	
F6	*D. pulex*	1	MPI/aa AO/aa	0·58	1·00	<0·001
		2	MPI/aa AO/ab	0·03	0·00	NS
		3	MPI/ab AO/aa	0·39	0·00	<0·001
				$n = 66$	$n = 30$	
F7	*D. obtusa*	1	GPI/aa PGM/aa MDH/aa	0·28	0·24	NS
		2	GPI/aa PGM/ab MDH/aa	0·05	0·17	NS
		3	GPI/aa PGM/ab MDH/ab	0·09	0·24	NS
		4	GPI/ab PGM/ab MDH/aa	0·07	0·00	NS
		5	GPI/aa PGM/aa MDH/ab	0·20	0·15	NS
		11 rare		0·31	0·20	
				$n = 71$	$n = 48$	
F9	*D. pulex*	1	GPI/aa PEP/aa MDH/aa	0·33	0·11	0·027
		2	GPI/aa PEP/ab MDH/aa	0·33	0·77	<0·001
		3	GPI/aa PEP/aa MDH/ab	0·13	0·05	NS
		7 rare		0·21	0·07	
F10a	*D. pulex*	1	GPI/cc PGM/aa GOT/bb MDH/aa	0·03	0·08	NS
		2	GPI/cc PGM/aa GOT/ab MDH/aa	0·17	0·03	0·010
		3	GPI/bc PGM/aa GOT/ab MDH/aa	0·07	0·08	NS
		4	GPI/ac PGM/ab GOT/ab MDH/aa	0·06	0·05	NS
		5	GPI/bc PGM/aa GOT/bb MDH/aa	0·06	0·00	NS
		6	GPI/cc PGM/ab GOT/ab MDH/aa	0·08	0·05	NS
		7	GPI/bc PGM/aa GOT/bb MDH/aa	0·07	0·18	NS
		8	GPI/ac PGM/aa GOT/ab MDH/aa	0·08	0·00	NS
		19 rare		0·39	0·54	
				$n = 72$	$n = 40$	
F11	*D. pulex*	1	GPI/cc PGM/bb GOT/ab MDH/aa	0·06	0·02	NS
		2	GPI/cc PGM/ab GOT/ab MDH/aa	0·10	0·02	NS
		3	GPI/cc PGM/ab GOT/aa MDH/aa	0·06	0·02	NS
		4	GPI/cc PGM/aa GOT/ab MDH/aa	0·04	0·17	0·025
		5	GPI/cc PGM/aa GOT/aa MDH/aa	0·07	0·11	NS
		6	GPI/bc PGM/ab GOT/bb MDH/aa	0·01	0·13	0·014
		7	GPI/bc PGM/ab GOT/ab MDH/aa	0·06	0·11	NS
		8	GPI/bc PGM/aa GOT/bb MDH/aa	0·06	0·07	NS
		9	GPI/bc PGM/aa GOT/ab MDH/aa	0·06	0·04	NS
		10	GPI/bb PGM/aa GOT/ab MDH/aa	0·06	0·02	NS
		24 rare		0·43	0·28	
				$n = 70$	$n = 46$	

Table 4—*continued.*

Pond	Host	Clone	ENZYME/phenotype	Frequency		
				Healthy	Parasitized	*P*-clone
F13	*D. pulex*	1	PGM/ab	0·10	0·21	NS
		2	PGM/bb	0·90	0·70	0·011
		2 rare		0·00	0·09	
				$n = 62$	$n = 47$	
S1	*D. pulex*	1	PGM/ab MPI/ab AO/ab	0·93	0·80	0·023
		2	PGM/ab MPI/bb AO/ab	0·07	0·20	0·023
				$n = 84$	$n = 59$	
S2-*t*1 10/06/97	*D. longispina*	1	GPI/bb PGM/ab MDH/ab AAT/ab	0·10	0·15	NS
		2	GPI/bb PGM/aa MDH/bb AAT/ab	0·06	0·07	NS
		3	GPI/bb PGM/aa MDH/ab AAT/ab	0·31	0·17	NS
		4	GPI/ab PGM/ab MDH/ab AAT/bb	0·19	0·28	NS
		5	GPI/ab PGM/ab MDH/aa AAT/ab	0·00	0·15	0·005
		13 rare		0·34	0·18	
				$n = 48$	$n = 46$	
S2-*t*2 16/06/97	*D. longispina*	1	GPI/bb PGM/ab MDH/ab AAT/ab	0·01	0·10	0·009
		2	GPI/bb PGM/aa MDH/bb AAT/ab	0·46	0·30	0·036
		3	GPI/bb PGM/aa MDH/ab AAT/ab	0·36	0·35	NS
		4	GPI/ab PGM/ab MDH/ab AAT/bb	0·03	0·12	0·009
		5	GPI/ab PGM/ab MDH/aa AAT/ab	0·00	0·00	
		6	GPI/bb PGM/aa MDH/bb AAT/aa	0·01	0·06	NS
		5 rare		0·13	0·07	
				$n = 112$	$n = 81$	
NG3	*D. pulicaria*	1	GPI/aa PGM/aa	1·00	0·58	<0·001
		2	GPI/ab PGM/aa	0·00	0·12	0·027
		3	GPI/aa PGM/ab	0·00	0·19	0·002
		4	GPI/aa PGM/bb	0·00	0·09	0·046
		5	GPI/ab PGM/ab	0·00	0·02	NS
				$n = 48$	$n = 43$	

Discussion

The central assumption underlying models of parasite–host coevolution is that natural selection acts on genetic variation for host susceptibility. In this study, differences were detected, based on allozyme variation, between the clonal composition of parasitized and healthy *Daphnia* within 12 of 34 (35%) samples taken from 25 populations. The use of allozyme electrophoresis to investigate genetic variation for resistance in clonal taxa is analogous to studies of *Daphnia* that have detected clonal differences for tolerance to abiotic factors (Carvalho 1987; Weider & Hebert 1987). The clonal variation for infection reported here was often extremely pronounced, the strongest example being population F3, where only one clone of 12 was infected with *Thelohania* sp. (Table 4). If this strong clonal variation reflects a genetic basis for disease resistance, natural selection against susceptible clones is likely to be a prominent evolutionary force in *Daphnia* populations. This possibility is supported by the higher incidence of deviations from genetic equilibria in those populations showing clonal variation for susceptibility, as selection acting on resistance-variation should result in shifts away from equilibria.

In ameiotic organisms, nonrandom associations among genotypes are expected to be common as a result of clonal selection (Hebert 1974; Hebert & Ward 1976; Lynch 1984). Associations between parasitism and allozymes presumably reflect linkage disequilibrium between resistance genes and neutral markers. Such associations could become evident through the hitchhiking of allozymes with resistance genes that are under selection (the amplification of pre-existing linkage disequilibrium), or if selection acts on both the allozymes and the resistance genes (epistatic associations). However, non-random associations among loci can also arise for a variety of ecological reasons such as the recent admixture of different populations or spatial structuring of genotypes within a single habitat (see Crow & Kimura 1970). Spatial structuring of genotypes and occasional bouts of admixture (e.g. wind mixing) have been observed in *Daphnia* (Hebert & Ward 1976; Weider 1985), and if such phenomena were coupled with within-pond

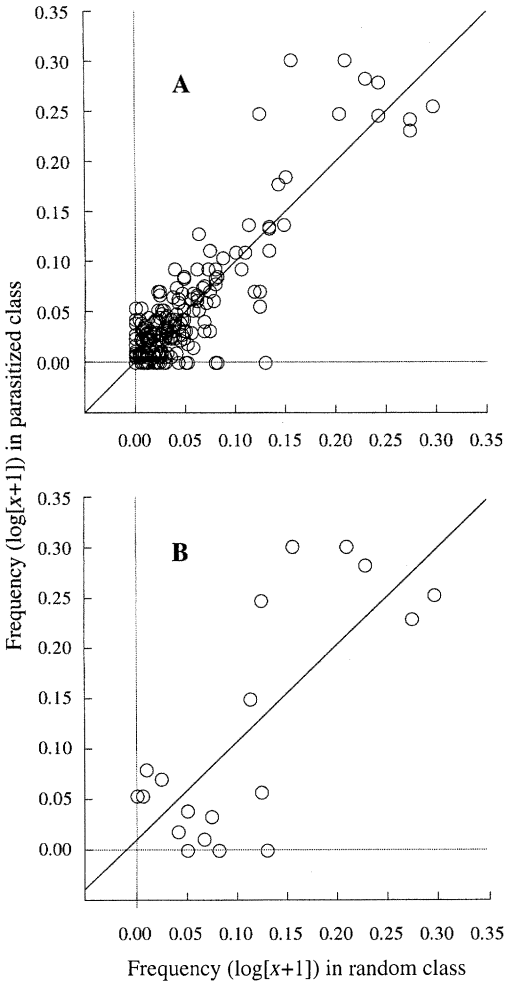


Fig. 1. Regressions of clonal frequencies [log $(x + 1)$ transformed] in the parasitized class of individuals vs. those in a random population sample. Graph A is a regression ($y = 0{\cdot}99x - 0{\cdot}0005$; $R^2 = 0{\cdot}772$; $n = 626$) including all clones from 34 parasite–host combinations involving *Daphnia* as host. Graph B ($y = 969x + 0{\cdot}01$; $R^2 = 0{\cdot}576$; $n = 19$) includes only those clones found to be significantly under- or overparasitized (see Table 4).

spatial differences in parasite prevalence, patterns similar to those observed in the present study could arise. In this case, clonal differences for infection would not necessarily indicate a genetic basis for disease resistance. Nevertheless, whether associations between parasitism and allozymes are a result of direct genetic differences for resistance or because of genetic differences which lead to differential exposure to parasitism, both would lead to selection against overparasitized clones. In the longer term, however, spatial refuges for hosts could significantly impact the coevolutionary dynamics within single ponds. To distinguish between genetic variation for resistance and genetic variation associated with exposure to spatially heterogeneous parasites, it is necessary to compare hosts and parasites under controlled laboratory conditions.

Some parasites of *Daphnia* are known to be both virulent and transmitted vertically through parthenogenetic eggs (Larsson *et al.* 1998; Mangin *et al.* 1995). These parasites are likely to have some degree of horizontal transmission as well, since virulent parasites cannot persist with vertical transmission alone (Fine 1975; Lipsitch *et al.* 1995). During phases of exclusively vertical transmission (e.g. when the vector for horizontal transmission is absent), an existing association between parasitism and host genotype (as found in this study) might be maintained for several generations, leading to the erroneous conclusion that some clones are always more susceptible. However, such associations are likely to have originally arisen through a non-random horizontal transmission event. The conclusions presented here do not need, therefore, to be modified to account for virulent vertically transmitted parasites, although for these parasites the probability of finding an association between host genotype and infection might be greater than in the case of exclusively horizontally transmitted parasites, because associations are maintained for longer.

In four populations, subsequent sampling confirmed the original observation of clonal variation for infection, suggesting at least that the original result was not a statistical error. However, in two other populations (F7 and F10, Figs 3 and 4) significant clonal variation for infection could not be confirmed on later dates. While this loss of significance could certainly indicate that the original observation was an artefact of some other factor, this may not necessarily be so, as theory predicts dynamic patterns of susceptibility. Specifically, coevolutionary models predict time-lagged, frequency-dependent selection, where single clones oscillate between high and low frequency, and also between being under- and overparasitized (Haldane 1949; Hamilton *et al.* 1990). As a clone moves towards an intermediate frequency, it becomes only very slightly over- (or under-) parasitized, and consequently, associations are expected to change from strong to weak to nonexistent (see Dybdahl & Lively 1995). These same clonal oscillations suggest that sampling populations at a single time point will tend to underestimate clonal variation for resistance, as some of the populations lacking detectable associations may be at a stage of the cycle where detection is simply not possible. The complete disappearance of the heavily parasitized clone 5 from population S2, indicates just how strong the dynamic may be.

There are two further reasons why the estimate presented here of the prevalence of clonal variation for resistance is likely to be conservative. First, infected individuals were identified without dissection, and other studies (Bengtsson & Ebert 1998) have shown that as many as 20% of infections, early stage infections in particular, are overlooked by only external examination of hosts. Thus, the genetic profile of the healthy class of individuals was probably biased by the presence of undetected infections. Second, the method used in the present study for detecting resistance variation is

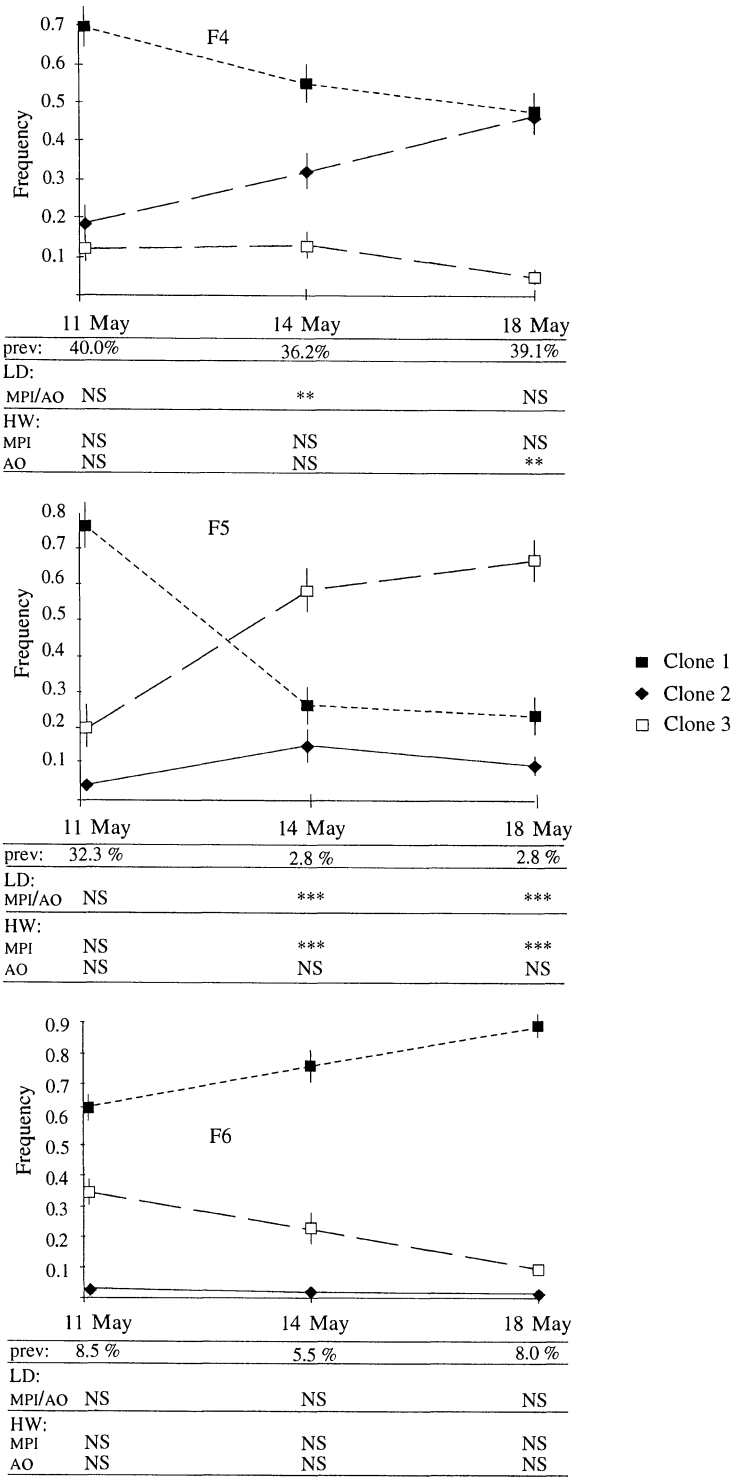
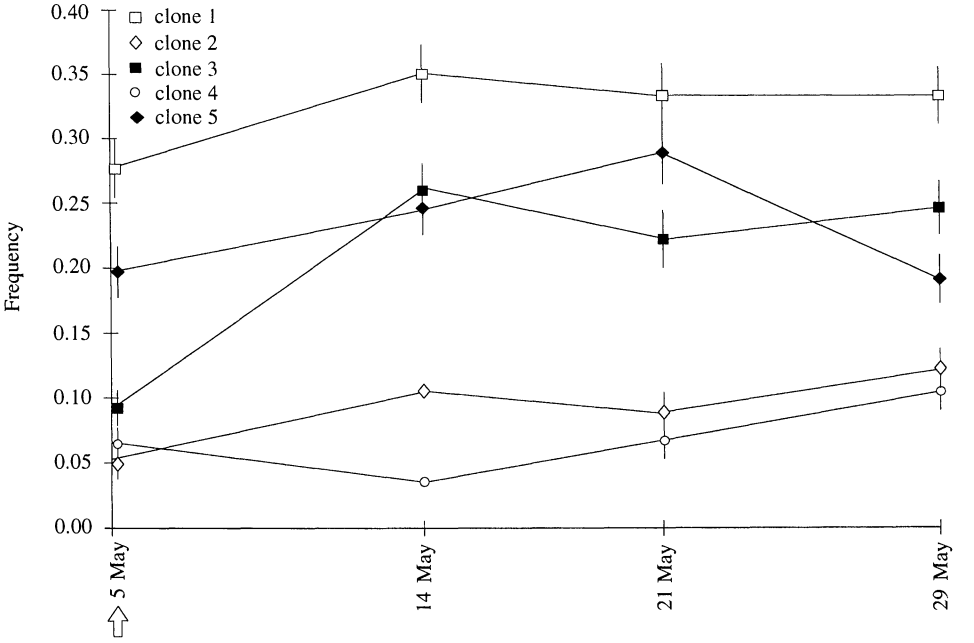


Fig. 2. Clonal frequencies (± SD), parasite prevalences and population genetic characters for three *Daphnia pulex* populations (F4, F5 and F6) infected with the microsporidian endoparasite *Thelohania* sp. Throughout the study period, the parasitized class of individuals had a significantly different clonal composition from that of the healthy class in each population. There were two polymorphic loci (*MPI*, *AO*) and three clones in each population (see Table 4). Clone 1, depicted by the small dashes, was consistently 'overparasitized', while clones depicted by long dashes were consistently 'underparasitized'. LD indicates deviations from genotypic linkage equilibrium between pairs of loci, while HW depicts deviations from Hardy–Weinberg equilibrium (**$P < 0{\cdot}01$, ***$P < 0{\cdot}001$). The significance levels for these tests were Bonferroni corrected when multiple tests were performed on samples from the same date.

indirect, relying on linkage between loci. An allozymic clone (=clonal group that share the same allozyme phenotype) probably consists of a mixture of resistant and susceptible clones, and the ability of the present study to identify a clonal group as being, for example, 'overparasitized', will vary with the strength of the link-



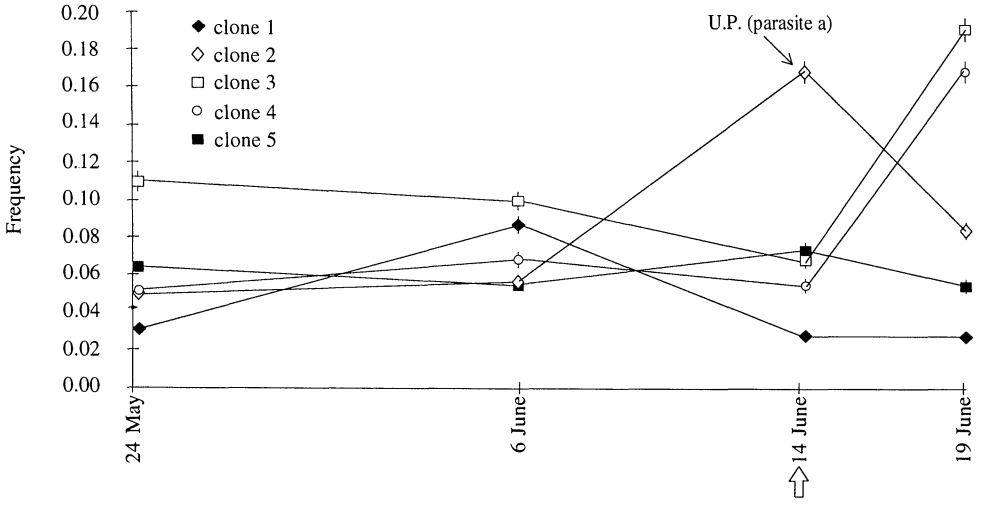
Prevalence: 7.8 %		5.5 %	6.7 %	1.4 %
LD:				
PGI/PGM	NS	NS	NS	NS
PGI/MDH	NS	NS	NS	NS
PGM/MDH	NS	NS	NS	NS
HW:				
PGI	NS	NS	NS	NS
PGM	NS	NS	NS	NS
MDH	NS	NS	NS	NS

Fig. 3. Clonal frequencies (± SD), parasite prevalences and population genetic characters for a population of *Daphnia obtusa* (population F7, see Tables 1, 2 and 4) infected with a microsporidian endoparasite. Clones with frequency less than 0·05 are not shown. The large arrow at the *x*-axis indicates the time point when the parasitized class had a significantly different clonal composition than that of the healthy class. LD indicates deviations from genotypic linkage equilibrium between pairs of loci, while HW depicts deviations from Hardy–Weinberg equilibrium at each of three polymorphic loci. The significance levels for these tests were Bonferroni corrected when multiple tests were performed on samples from the same date.

age between the allozymes and the selected loci, as well on the duration and intensity of past selection. Furthermore, when parasite prevalence is low, susceptible members of a clonal group my be infected in such small numbers that comparisons among clonal groups are uninformative without extremely large sample sizes. If any of the above factors are weak, the method used here will be conservative.

Oscillations of clonal frequencies also make it difficult, perhaps impossible, to determine whether 'overinfected' clones tend to be common ones (Haldane 1949). Frank (1991) predicted that such temporal dynamics should generate a spatial pattern where each population is in a different part of the cycle by chance alone. Thus, even in the presence of substantial clonal variation for infection, averaged over many populations, the clonal composition of the parasitized class should not differ from that of the random class. In agreement, this study found significantly 'overparasitized' clones to span a full range of frequencies (see Fig. 1b), but the slope of the line of the regression of clonal frequencies in these two classes did not differ significantly from one.

Temporal sampling of six populations with significant parasite–host genotype associations allowed exploration of whether clone frequency changes are associated with parasite susceptibility. Past work (Hebert 1974; Hebert & Ward 1976; Young 1979; Lynch 1984, 1987; Korpelainen 1986) has shown *Daphnia* genotype frequencies to vary over time. As *Daphnia* populations are usually extremely large, genetic drift cannot account for these fluctuations, and selection is thought to be the primary determinant of short-term changes. In all six temporally sampled populations changes were observed in clone frequencies, but an association with parasitism was not always evident. However, in populations with high clonal diversity, and hence a low sample size per clone (e.g. F10), the significance of clone frequency changes are difficult to assess, and could be influenced by sampling error. In addition, with low parasite virulence or prevalence (low selection intensity), or when the linkage between allozyme loci and resistance loci is weak, parasite-mediated effects could be easily obscured by other factors. This could be the case in population F7, where the parasitism–genotype association was



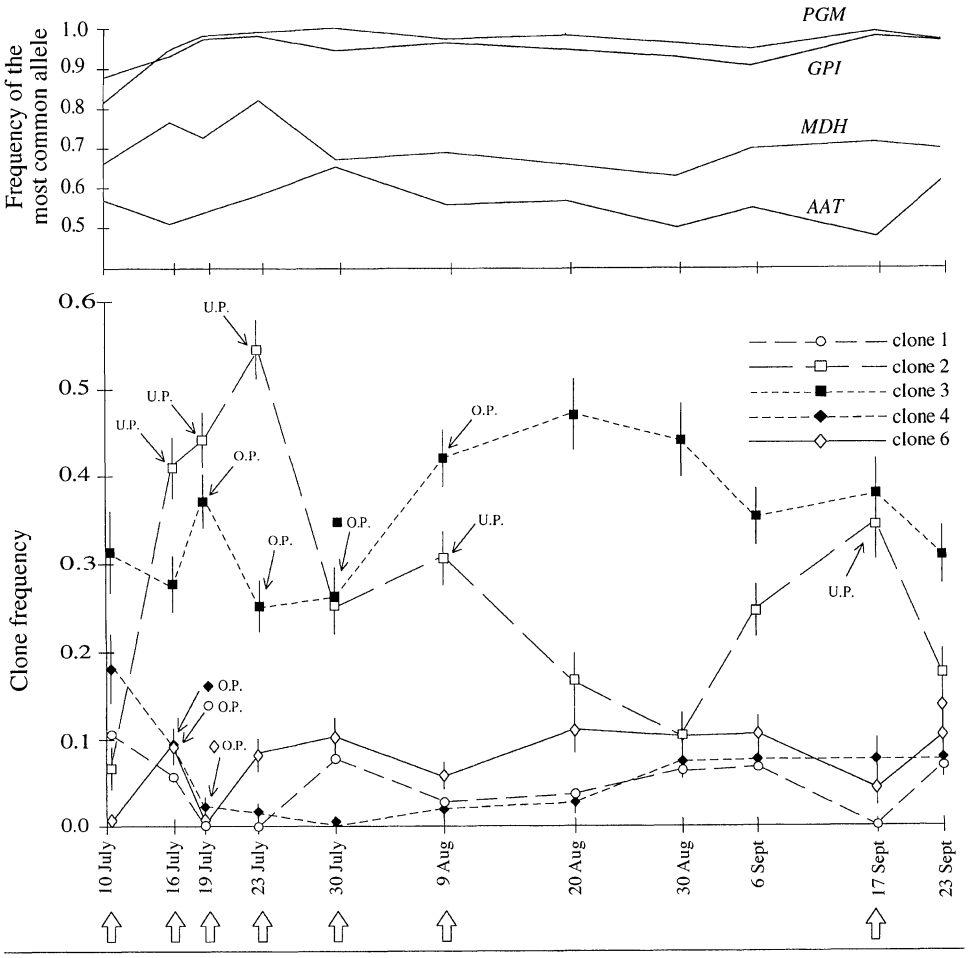
Prevalence(a):	-	10 %	4.2 %	< 1 %
Prevalence(b):	-	4.2 %	< 1 %	< 1 %
Prevalence(c):	4.2 %	< 1 %	< 1 %	< 1 %
LD:				
PGI/PGM	NS	NS	NS	***
PGI/MDH	NS	NS	NS	NS
PGI/GOT	NS	NS	NS	NS
PGM/MDH	NS	NS	NS	NS
PGM/GOT	NS	NS	NS	NS
MDH/GOT	NS	NS	NS	NS
HW:				
PGI	NS	NS	NS	NS
PGM	NS	NS	NS	NS
GOT	NS	NS	NS	NS
MDH	NS	NS	NS	NS

Fig. 4. Clonal frequencies (±SD), parasite prevalences and population genetic characters for a population of *Daphnia pulex* (population F10, see Tables 1, 2 and 4) infected with three endoparasites: an undescribed bacteria infecting the epidermis (parasite a), *Gurleya vavrai* (parasite b) and an undescribed microsporidian infecting the ovaries and fat cells (parasite c). Parasites a and b were not detected on May 24. Clones with frequency <0·05 are not shown. The large arrow at the *x*-axis indicates the time point when the parasitized class (parasite a only) had a significantly different clonal composition than that of the healthy class. The small arrow in the graph indicates when clone 2 was significantly 'underparasitized'(U.P.) with parasite a. LD indicates deviations from genotypic linkage equilibrium between pairs of loci, while HW depicts deviations from Hardy–Weinberg equilibrium at each of four polymorphic loci (***$P < 0{\cdot}001$). The significance levels for these tests were Bonferroni corrected when multiple tests were performed on samples from the same date.

comparatively weak, and clutch size comparisons indicated that the parasite may have low virulence.

In three of six study populations, parasite-associated changes in clonal frequencies were documented that were compatible with predictions based on parasite-mediated selection, and in each of these three, the initial observations of clonal variation for resistance were confirmed on later sampling dates. Populations F4 and F5 are clear examples where an 'overparasitized' clone dropped dramatically in frequency, while a significantly 'underparasitized' clone rose to prominence (Fig. 2). These changes occurred within just 7 days and involved differences in survivorship among clones, rather than differences in fecundity. Unfortunately it is not possible to assess survivorship in the field, but laboratory experiments have shown that most *Daphnia* microparasites shorten lifespan drastically (Ebert 1994; Mangin *et al.* 1995).

Adjacent to ponds F4 and F5, was population F6, which contained the same parasite and host species, but showed the reverse pattern (Fig. 2). However, parasite prevalence in this pond was comparatively low (never more than 9%, compared to up to 32% in F5 and 40% in F4). Low prevalence does not necessarily imply low selection intensity by parasites, as a highly transmissible parasite that quickly kills its host could exert strong selection despite being found at a low prevalence at any single time point (Anderson & May 1991). However, it should be emphasized that prevalence in F6 is low relative to other populations (F4 and F5) with the same parasite–host combination. In addition, clonal frequencies in F4 and F5 show an interesting concordance with parasite prevalences. Population F4 has consistently high prevalence throughout the sampling period, and the decline of the 'overparasitized' clone is constant. Population F5, however, shows a decline of the 'overparasitized' clone concordant with an initially high parasite prevalence, but the gene frequencies seem to stabilize when parasite prevalence drops to 2·8%. Finally, Hardy–Wein-



Prevalence:	<1%	9.4%	6.7%	8.3%	2.3%	1%	<<1%	<1%	2.8%	6.9%	2.3%
LD:											
PGI/PGM	**	***	***	**		***	**	***	***	NS	NS
PGI/MDH	NS	***	NS	NS		NS	NS	NS	***	NS	***
PGI/GOT	**	***	***	***		***	**	**	***	NS	NS
PGM/MDH	NS	***	NS	NS	NS	NS	NS	NS	**	NS	**
PGM/GOT	NS	***	***	***	NS	***	NS	NS	***	NS	***
MDH/GOT	NS	NS	NS	NS	NS	NS	NS	***	**	NS	**
HW:											
PGI	NS	NS	NS			NS	NS	NS	NS		NS
PGM	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
GOT	**	***	***	**	**	***	**	**	**	**	**
MDH	NS	**	**	NS	*	***	**	NS	***	NS	NS

Fig. 5. Allele frequencies, clonal frequencies (±SD), parasite prevalences and population genetic characters for a population of *Daphnia longispina* (population S2, see Tables 1, 2 and 4) infected with an undescribed microsporidian infecting the ovaries and fat cells. Large arrows at the *x*-axis indicate time points when the parasitized class had a significantly different clonal composition than that of the healthy class. Small arrows in the graph indicate when particular clones were significantly 'over-' (O.P.) or 'underparasitized' (U.P.). LD indicates deviations from genotypic linkage equilibrium between pairs of loci (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$), while HW depicts deviations from Hardy–Weinberg equilibrium. Blank entries for these population genetic characters indicate that the test could not be done because of insufficient polymorphism. The significance levels for these tests were Bonferroni corrected when multiple tests were performed on samples from the same date.

berg deviations and linkage disequilibrium, which indicate the presence of selection, were evident in both F4 and F5, but not in F6. Indeed, these departures from equilibria developed over the course of the sampling period (Fig. 2). If the evolutionary changes in F4 and F5 were due to sexual recruitment from the ephippial bank, these populations would not have strayed from genetic equilibria.

Genetic disequilibria were ubiquitous in the longer-term study population, S2, for which a variety of marked clonal frequency changes were evident. The relatively rare and 'overparasitized' *PGM/GPI* heterozygote clones detected on the early sampling dates, declined in frequency as expected with parasite-mediated selection. The significant associations involving the two common clones, clone 2 ('underparasitized') and clone 3 ('overparasitized'), coupled with observations of changes in parasite prevalences,

also indicate the presence of parasite-mediated effects. When parasite prevalence was relatively low, clone 3 is more common than clone 2, but the reverse is true during periods of higher parasite prevalence (see Fig. 5). Such a pattern could arise if resistance were both genetically determined and costly. During periods of low parasite prevalence, a resistant clone would pay this cost of resistance, but receive no benefit, and as a consequence, be competitively inferior. Although these data are compatible with a cost of resistance, experimental verification is needed. In general, costs of resistance have proven difficult to detect (but see Fineblum & Rausher 1995; Kraaijeveld & Godfray 1997).

CONCLUSIONS AND FUTURE DIRECTIONS

This study has established that within single populations, allozymic clones of *Daphnia* may differ in their parasite load. Three of the populations (F6, F7, F10) that were sampled over time showed clone frequency changes that did not indicate an effect of parasites, while the other temporally sampled populations (F4, F5, S2) showed patterns suggestive of parasite-associated changes. These observations may indicate the presence of parasite-mediated clonal selection, but may also be influenced by ecological complexities, such as spatial structuring of clones and parasites within ponds. With a study designed to achieve a broad impression of both the extent of genetic variation for parasite resistance in the wild, and the utility of using allozymes as a marker for this variation, it is not possible to assess to what extent susceptibility is dependent on other selective forces and ecological complexity. Experimental work is necessary to control for multiple confounding factors. Fortunately, a number of parasite–host combinations involving *Daphnia* can be manipulated in the laboratory (e.g. Ebert 1994). Future work of this study group will focus on one or a few populations, and will involve measuring clonal variation for resistance under controlled conditions in an effort to establish a stronger causal link to the field data reported here.

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