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ORIGINAL ARTICLE

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Parasitism and breeding system variation in North American populations of *Daphnia pulex*

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Abstract The Red Queen hypothesis proposes that frequency-dependent selection by parasites may be responsible for the evolutionary maintenance of sexual reproduction. We sought to determine whether parasites could be responsible for variation in the occurrence of sexual reproduction in 21 populations of *Daphnia pulex* (Crustacea; Cladocera) that previous studies have shown to consist of either cyclical parthenogens, obligate parthenogens, or a mixture of both. We measured parasite prevalence over a four-week period (which essentially encompasses an entire season for the temporary snow-melt habitats we sampled) and regressed three different measures of sexuality against mean levels of parasite prevalence. Levels of parasitism were low and we found no relationship between levels of sexuality and mean parasite prevalence. Genetic variation with infection level was detected in 2 of the 21 populations, with several different clones showing signs of overparasitism or underparasitism. Overall, however, our results suggest that parasites are not a major source of selection in these populations and it thus seems unlikely they are responsible for maintaining breeding system variation across the study region.

Keywords Geographical parthenogenesis · Red Queen hypothesis · Evolution of sex · Parthenogenesis · Natural selection · Infection

Introduction

The predominance of sexual reproduction among Eukaryotic taxa has long puzzled evolutionary biologists, because the "twofold cost" associated with male production should put sexual taxa at a major disadvantage when they come into competition with their asexual counterparts (Maynard Smith 1978). This dilemma has received much attention over the past few decades and as a result a large number of genetic and ecological hypotheses have been proposed in an attempt to explain both the long-term and short-term maintenance of sex (Bell 1982; Kondrashov 1993; West et al. 1999).

One of the most prominent ecological explanations for sex, the Parasite Red Queen hypothesis, proposes that frequency-dependent selection by parasites is responsible for preventing the spread of asexuality (Bell 1982). The Parasite Red Queen hypothesis works on the assumption that parasites will be selected to infect the commonest host genotypes (Hamilton et al. 1990), and sex is thought to be advantageous because of the ability of recombination to create novel genotypes, thus limiting parasite adaptation to particular genotypic combinations. Consequently, in the longer term, sexual populations may have higher geometric mean fitness. The hypothesis also makes several predictions of how prevalence may vary between breeding systems, depending on the distribution of parasite abundance. For example, if parasite abundance varies little across populations, then sexuals are expected to bear lower parasite loads than their asexual counterparts. If, on the other hand, the risk of infection varies widely across populations asexuals are predicted to dominate in low-risk areas whereas sexuals are expected to hold precedence in areas where parasites are more abundant (Lively 2001).

Most studies have attempted to test the predictions of the Red Queen by:

1 examining the relationship between host genotype frequency and infection by parasites, a test for frequency-dependent selection (Lively et al. 1990; Kelley 1994; Vernon et al. 1996; Dybdahl and Lively 1998; Little and Ebert 1999);
2 comparing levels of infection in closely related sexual and asexual species (Moritz et al. 1991; Hanley et al.

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1995; Hakoyama et al. 2001; Michiels et al. 2001; Kumpulainen et al. 2004); or
3 seeking a relationship between levels of infection and levels of sex and recombination within species (Lively 1987; Heller and Farstey 1990; Jokela and Lively 1995; Camacho et al. 2002; Lively and Jokela 2002; Ben-Ami and Heller 2005).

Because results from these studies on a range of systems have not been in complete agreement, new studies should ideally attempt to incorporate as many of these approaches as possible (Lively et al. 1990; Jokela et al. 2003).

The objective of this study was to examine patterns of parasitism in cyclically and obligately asexual populations of the Cladoceran crustacean *Daphnia pulex*. Specifically, we wanted to:

1 compare levels of parasitism with levels of sex across populations; and
2 look for evidence of frequency-dependent infection patterns within infected populations.

To put this into context, we will now briefly describe the relevant aspects of *Daphnia* natural history.

Reproduction and parasitism in *Daphnia*

In common with most *Daphnia* species, most populations of *D. pulex* are cyclically parthenogenetic. During favourable periods adult females reproduce by apomixis, although in response to some environmental cues they will instead start to produce pairs of haploid eggs by meiosis. These eggs require fertilisation to develop and, enclosed within a protective ephippium, they constitute the resting stage of the *Daphnia* lifecycle (Hebert 1978).

The occurrence of obligate parthenogenesis in several *Daphnia* species has been recognised for some time (Hebert 1981; Hebert and Crease 1983), and studies of North American *Daphnia pulex* populations have revealed that asexual (obligately parthenogenetic) and sexual (cyclically parthenogenetic) populations often coexist within the same geographical region (Hebert et al. 1989, 1993; Hebert and Finston 2001). In contrast with cyclically parthenogenetic forms, obligate parthenogens produce ephippial eggs that develop without being fertilised, and genetic studies have revealed that this is mainly because of the action of a sex-linked meiotic-suppressor gene (Innes and Hebert 1988). Allozyme and breeding studies have confirmed that these eggs are diploid and ameiotically produced (Hebert 1981; Innes and Hebert 1988). This aspect of the biology of the species is particularly appealing, because it means the potentially confounding factors of ploidy difference and/or hybridisation, common to other Red Queen study systems (Lively et al. 1990; Moritz et al. 1991; Hanley et al. 1995; Brown et al. 1995; Hakoyama et al. 2001; Michiels et al. 2001) can be avoided.

Daphnia are attacked by a wide array of microparasites (Green 1974; Stirnadel and Ebert 1997), many of which are known to have substantial effects on host fitness (Green 1974; Schwartz and Cameron 1993; Ebert 1995; Mangin et al. 1995; Stirnadel and Ebert 1997; Little 1999). Parasite biomass within hosts is often substantial and, because *Daphnia* have a transparent carapace, it is possible to detect many infections without dissection. This study is one of the first attempts to examine the role of parasitism in the evolution of breeding system in North American *Daphnia pulex*.

Materials and methods

Sampling and examination

Samples were collected from 21 ponds in south-west Ontario (Fig. 1) over a four-week period during the Spring of 2003. This geographic area is known to contain both sexual and obligately asexual populations (Hebert et al. 1988, 1993). Most populations were located in small, temporary snowmelt ponds and as such the length of the sampling period was constrained by the ephemeral nature of these habitats. One sample was taken from each pond per week by use of a fine-meshed aquarium net, and samples were immediately cooled and maintained at between 1 and 5°C to minimise mortality before inspection (always within 24 h). After collection of each sample the sampling equipment was cleaned and sterilised using 70% ethanol, to avoid spreading parasites and hosts between different ponds.

In the laboratory an initial random sub-sample of ~100 to 600 individuals (depending on the density of the whole sample) was examined under a dissecting microscope. These initial sub-samples were used to estimate the sex ratio of the population and the proportion of infected females (on the basis of external examination,

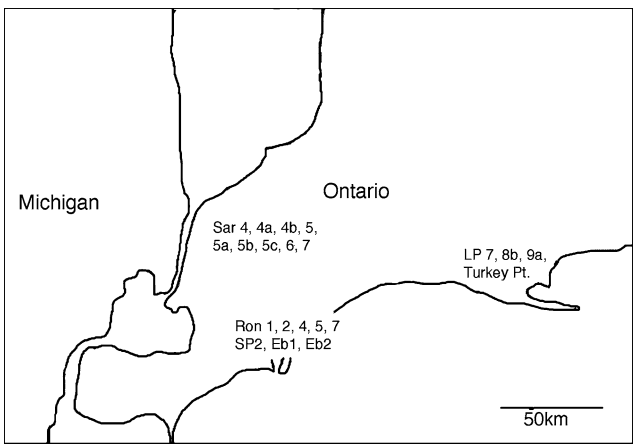

Fig. 1 Map showing the locations of the 21 *Daphnia pulex* populations sampled during the study period

but not dissection, of all size classes, which could result in some infections being overlooked, although the clear carapace of *Daphnia* does make external examination relatively effective). There was no correlation between sample size and parasite prevalence ($r^2 = 0.072$, $F = 1.47$, $P = 0.24$). A second, smaller sub-sample (~50 to 80 individuals) was then frozen at −80°C for allozyme electrophoresis at a later date. This constituted the "random" sample for allozyme analysis. Finally, any infected individuals remaining in the sample were examined to establish the cause of infection and, if enough were available, intact individuals were also frozen at −80°C. These individuals constituted the "infected" sample for allozyme analysis.

Allozyme electrophoresis

Samples were analysed using the cellulose acetate electrophoresis methods described in Hebert and Beaton (1993). Allozyme phenotypes were discriminated by screening for variation at four enzyme loci known to be polymorphic in *D. pulex* from this region (P. Hebert, personal communication): aldehyde oxidase (AO; EC 1.2.3.1), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), mannose-6-phosphate isomerase (MPI; EC 5.3.1.8), and phosphoglucomutase (PGM; EC 5.4.2.2). For each locus, distinct alleles were labelled according to their mobility, with the fastest labelled "1", the next fastest "2", etc. A single laboratory clone of *D. pulex* was used as a marker clone (allozyme phenotype = AO-"11", GPI-"23", MPI-"33", PGM-"22").

Analysis

Regression analysis was used to study relationships between parasite prevalence and three different continuous measures of sexuality: the genotypic diversity ratio (GDR), the log-transformed probability that the observed genotypic array was in Hardy–Weinberg equilibrium (LOGHW), and the population sex ratio. Pleasingly, we found that the estimates of sexuality from these were highly correlated; we therefore regard our results as robust.

Both the GDR and LOGHW have been used to establish breeding system in previous studies on N. American *D. pulex* populations (Hebert et al. 1988, 1989, 1993; Hebert and Finston 2001). The GDR of each population was calculated by dividing the observed number of multilocus genotypes by the predicted number generated by Monte Carlo simulation. The allozyme data from the first sampling date were always used. The simulation ran for 50,000 repeats and used the observed allele frequencies at each locus to produce the mean number of MLGs predicted for a freely recombining, panmictic population, of sample size N. Obligately asexual populations are expected to have low (usually <0.5) GDR values (Hebert et al. 1993).

LOGHW was calculated by log-transforming the P values obtained from chi-square analyses, which compared the observed genotypic arrays with those expected for populations in Hardy–Weinberg equilibrium. As above, the data from the first sampling date were always used. Loci regarded as monomorphic (those for which the frequency of the most common allele was ≥0.9) were excluded from the analysis. If a population was polymorphic at more than one locus the mean log P value was determined. Log probability values of approximately −2.00 or less are normally indicative of obligate parthenogenesis (Hebert et al. 1993).

The final measure of sexuality, the sex ratio of each population, was estimated by calculating the mean proportion of males in the population relative to the mean proportion of ephippial females. Although male frequency alone has been used as an indicator of sexuality in previous Red Queen studies (Lively and Jokela 2002; Ben-Ami and Heller 2005), the nature of the reproductive cycle of *D. pulex* makes it potentially unreliable here; in sexual populations, males are normally only produced during periods where there is also ephippia production, so lack of males may simply indicate that the cues responsible for initiating the sexual phase of the life-cycle have not yet occurred. Males would be expected in a sexual population containing ephippial females, however. As such, any populations in which ephippial females were not present were excluded from the regression analyses. We found that this did not quantitatively affect our results.

To test for evidence of frequency-dependent patterns of parasitism, we sought to determine whether common host genotypes tended to be significantly over-parasitized. The genotypic compositions of the random and infected samples were compared using contingency table analysis. Genotypes with a frequency of 5% or less were always pooled in a single "rare" clonal class. If a significant difference was found between the infected and random samples, Fisher's exact tests were performed on individual clones to establish which were "overparasitized" and which were "underparasitized". In all cases, sequential Dunn–Sidak tests were used to adjust for multiple comparisons (Sokal and Rohlf 1995).

Results

Data for each population are summarised in Table 1. Mean parasite prevalence ranged from 0 to 3.73%, averaging 0.73% overall. Three different parasite species were found, two microsporidians and one bacterium, although only one parasite species at a time was ever present in a single population. All three species infected the swimming muscles, fat cells, and ovaries, although the bacterium was also found to infect the haemolymph in the later stages of infection. Attempts were made to identify each species using Green (1974); these attempts were unsuccessful, however, and we are currently pursuing further taxonomic identification. Because of their

Table 1 Mean levels of parasite prevalence and measures of sexuality for 21 population of *Daphnia pulex* from south-west Ontario

Population	Region	Observed MLG (*N*)	Predicted MLG	GDR	LOGHW	Sex ratio (proportion male)	Mean prevalence (*total N*)
Sar4	Sarnia	14 (57)	16.12	0.86	−1.101	0.195	0.01 (1446)
Sar4a	Sarnia	25 (44)	21.44	1.17	−1.244	0.362	0.003 (1684)
Sar4b	Sarnia	19 (58)	18.51	1.03	−0.127	0.204	0.034 (1999)
Sar5	Sarnia	2 (40)	5.67	0.35	−4	0.033	0.001 (1067)
Sar5a	Sarnia	11 (57)	9.86	1.12	−0.116	0.37	0.006 (1573)
Sar5b	Sarnia	21 (43)	21.17	0.99	−0.652	0.235	0 (1058)
Sar5c	Sarnia	26 (48)	28.21	0.92	−0.694	0.218	0.015 (1237)
Sar6	Sarnia	12 (40)	10.45	1.15	−0.246	0.123	0.001 (1308)
Sar7	Sarnia	1 (40)	3	0.33	−4	0.068	0.037 (786)
Turkey Pt.	Long Point	3 (40)	8.09	0.37	−2.112	0.5	0 (1097)
LP7	Long Point	19 (44)	17.85	1.06	−1.016	0.5	0 (707)
LP8b	Long Point	15 (40)	17.28	0.87	−0.903	0.5	0.003 (1940)
LP9a	Long Point	21 (55)	17.33	1.21	−0.793	0.5	0 (2525)
Ron1	Rondeau	8 (60)	26.83	0.3	−2.751	–	0 (177)
Ron2	Rondeau	11 (50)	32.09	0.34	−1.974	0.034	0.002 (1301)
Ron4	Rondeau	4 (80)	18.82	0.21	−4	0.013	0 (1190)
Ron5	Rondeau	4 (80)	16.12	0.25	−4	0.024	0.031 (1935)
Ron7	Rondeau	10 (59)	44.61	0.22	−2.71	0.012	0 (1350)
SP2	Rondeau	15 (43)	29.81	0.5	−3.385	0.006	0 (1129)
Eb1	Rondeau	17 (60)	29.37	0.58	−1.375	0.049	0.01 (2017)
Eb2	Rondeau	21 (60)	33.96	0.35	−1.958	0.027	0.003 (2334)

Values in parentheses in the "Observed MLG" column indicate the number of individuals analysed by allozyme electrophoresis to estimate MLG values, GDR, and LOGHW. Values in parentheses in the "Mean prevalence" column indicate the total number of individuals sampled for each population over the whole study period to estimate sex ratio and mean prevalence

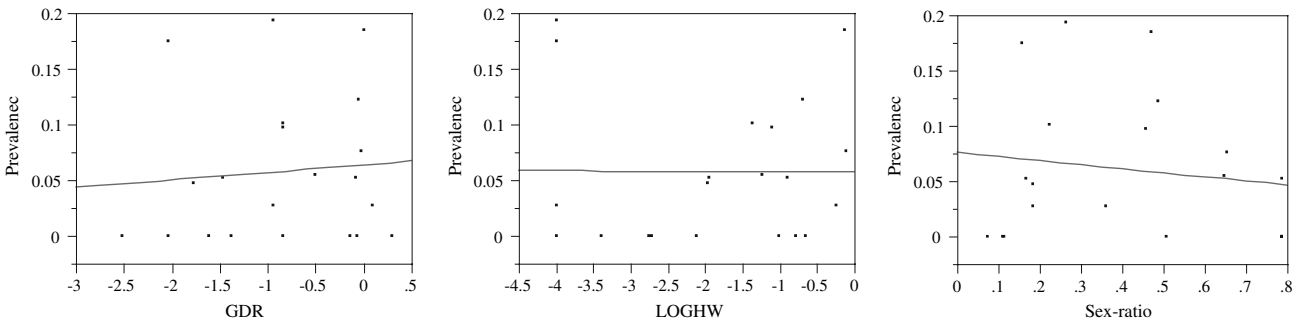

Fig. 2 Bivariate plots of mean parasite prevalence against GDR, LOGHW, and sex ratio. GDR and LOGHW data have been log-transformed; prevalence and sex-ratio data were arcsine square-root transformed

low prevalence and infrequent distribution, the data for all three-parasite species were pooled for analysis. Parasite prevalence was not related to sex ratio ($r^2 = 0.021$, $P = 0.543$), GDR ($r^2 = 0.007$, $P = 0.711$), or LOGHW ($r^2 < 0.0001$, $P = 0.985$) (Fig. 2). Sex ratio was significantly related to both GDR ($r^2 = 0.6$, $P < 0.0001$) and LOGHW ($r^2 = 0.44$, $P = 0.001$), and GDR and LOGHW were also highly correlated ($r^2 = 0.599$, $P < 0.0001$).

Contingency table analysis found significant differences between the clonal frequencies of infected and random samples in two of the sixteen infected populations—Sar5c and Eb2. In both populations, when corrections for multiple tests were made, further analysis could not detect the presence of overparasitized or underparasitized clones.

Discussion

The level of parasitism and patterns of parasite prevalence observed in this study do not support the hypothesis that parasite-mediated selection has been responsible for the maintenance of sexual reproduction across this part of the range of *D. pulex*. Infection levels were variable, but always low (0–4%). This is in contrast with results from field studies on parasitism in European *Daphnia* populations (Little and Ebert 1999) but does indicate the possibility that parasitism is too weak a force to affect *D. pulex* breeding systems. Highly virulent parasites may be rare, however, because they kill their hosts quickly. It is, moreover, unclear whether low parasite prevalence is consistent across years, and,

indeed, parasite prevalence in other *Daphnia* species has been shown to vary widely both within and between seasons (Little and Ebert 1999; Duncan et al. 2006). The reason(s) for this variability are not clear, although climate has been linked to fluctuations in parasite prevalence in *D. magna* (Duncan et al. 2006). If levels of infection of *D. pulex* are prone to climate-mediated fluctuations the Red Queen hypothesis is not implausible, because substantial parasite effects are, perhaps, apparent in some years. Our correlations between parasite prevalence and sexuality could still test the Red Queen hypothesis if the between-pond variation we observed reflects that seen in years of greater parasitism. We found no significant relationship between parasite prevalence and three different measures of sexuality, however.

The observed positive relationship between sex ratio and genetic estimates of sexuality (GDR and LOGHW) suggests that all three provide reliable estimates of the amount of sexual reproduction in a population. This is consistent with previous studies showing that male production is much reduced in obligately asexual *D. pulex* populations (Hebert et al. 1989; Innes et al. 2000). Genetic variation for male production has been shown to exist among clones of *D. pulex*, and Innes et al. (2000) have suggested this may lead to selection against male production in obligate parthenogens. Earlier work has shown that clones producing few or no males tend to dominate populations in the areas in which they are found (Hebert et al. 1989) and this is thought to be because of their ability to avoid the "cost of males" (Innes et al. 2000).

For two populations there were significant differences between the clonal composition of infected and uninfected samples, which suggests that some parasite-mediated selection may be occurring, despite the low levels of infection. As with other *Daphnia* studies, both those on parasites (Little and Ebert 1999) and those that do not consider parasites (e.g. Hebert 1974), we observed some dramatic genotype frequency fluctuations through time (data not shown). The general cause of these dynamics remains unknown, but at least one study on a different *Daphnia* species (*D. magna*) was able to successfully link allozyme frequency changes to parasitism (Duncan and Little 2007).

If parasites are not responsible for maintaining breeding system variation in *D. pulex*, it is not immediately obvious what is responsible. The populations surveyed all occupy very similar habitats (temporary meltwater ponds in deciduous forest), although the possibility of substantial ecological variation between ponds cannot be discounted. The Great Lakes region is dominated by obligate parthenogens (Hebert et al. 1988, 1989), yet sexual populations persist, even coexist within the same pond, despite evidence they have repeatedly been exposed to "contagiously" asexual clones (Crease et al. 1989; Hebert et al. 1989). Perhaps given sufficient time and a consistently low level of Infection risk, asexual domination will become complete.

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