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Parasite-mediated selection and the role of sex and diapause in *Daphnia*

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Abstract

To gain insight into parasite-mediated natural selection, we studied a natural population of the crustacean *Daphnia magna* during a severe epidemic of the bacterial parasite *Pasteuria ramosa*. We also investigated the relationship between susceptibility and the production of resting eggs, which are only produced during the sexual phase of reproduction. Live host samples were taken before and after this epidemic and resistance to *P. ramosa* was examined in the laboratory. Host clones collected after the epidemic were more resistant to *P. ramosa* than were those collected pre-epidemic, which is consistent with parasite-mediated selection. In our study population, asexually reproducing females were observed across the entire study period, but females carrying resting eggs were observed only prior to the epidemic. For hosts isolated in this pre-epidemic period, we found evidence that those carrying resting eggs (at the time of collection) were more susceptible than those that were reproducing asexually. This was especially apparent for measures of parasite growth, although not all measures of infection success conclusively supported this pattern. Nevertheless, the data suggest that some genotypes invest heavily in diapause at the expense of immunocompetence. Sex could therefore inhibit the evolution of resistance because each spring new genotypes will hatch from resting eggs that are relatively susceptible as they were not exposed to the previous years bout of parasite-mediated selection.

Introduction

Parasites are thought to have extensive effects on host population genetic diversity, and may even be the selective force maintaining sexual reproduction (Haldane, 1949; Jaenike, 1978; Hamilton *et al.*, 1990). This idea has a broad theoretical basis (Bell & Maynard-Smith, 1987; Hamilton *et al.*, 1990; Peters & Lively, 1999) and numerous empirical studies have corroborated the evolutionary significance of parasitism. These include studies showing substantial genetic variation for infection-related traits (Henter & Via, 1995; Kraaijeveld & Godfray, 1999; Little & Ebert, 2000), patterns of genetic variation that are compatible with frequency-dependent coevolutionary dynamics (Ebert, 1994; Dybdahl & Lively, 1995; Lively & Dybdahl, 2000), and a link between breeding system and the distribution of disease prevalence (Lively, 1987; Lively & Jokela, 2002). However, neither theoretical nor empirical support for the notion that parasitism can maintain sex has been universal. For example some models indicate that the selective effects of parasites must be unrealistically severe (May & Anderson, 1983; Howard & Lively, 1994; Otto & Nuismer, 2004), and indeed the expected rapid parasite-mediated dynamics have not been commonly observed in studies of natural systems (Little, 2002).

One important perspective that may require further attention is that sex often serves other functions in organisms that alternate sexual and asexual reproduction. For example sex is often associated with the production of diapausing stages that allow an organism to persist through periods of environmental hostility (Hairston & Kearns, 1996; Grishkan *et al.*, 2003; Slarsarczyk *et al.*, 2005). Parasitism is a ubiquitous source of environmental hostility, so when sex leads to diapause, coevolutionary interactions between hosts and their parasites may be altered. When resting stages hatch,

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they could release a reservoir of genotypes that have escaped the most recent bout of parasite-mediated selection. It may, therefore, be difficult to disentangle the functions of sex in taxa where sex is linked to resting stages.

Daphnia are cyclical parthenogens, reproducing asexually for the majority of the year, with occasional bouts of sexual reproduction. Environmental stimuli such as photoperiod, food shortages, temperature and fish kairomones (Carvalho, 1983; Hobaek, 1990; Kleiven, 1992; Slarsarczyk *et al.*, 2005) are all cues that contribute to the onset of sexual reproduction. *Daphnia* also show genetic differences for levels of sexual and asexual egg production (Hebert, 1974; Deng, 1996). Sexual reproduction in *Daphnia* results in the production of resting eggs encased in an ephippium, which is resistant to freezing and drying and will remain in the sediment for a period of time before hatching. Thus, sexual reproduction in *Daphnia* creates a dormant reservoir of genetic variation that periodically contributes to the population.

This study tested for the occurrence of parasite-mediated selection in a natural population of *Daphnia magna* infected with the bacterial pathogen *Pasteuria ramosa*. We further sought to determine whether genetic variation with regards to sexual reproduction in *D. magna* could be subject to natural selection by parasites. Like many *Daphnia* parasites, *P. ramosa* has a cyclical existence within *D. magna* populations, appearing only in summer (Stirnadel & Ebert, 1997). Previously, it was observed that *Daphnia* genotypes that are more susceptible to the parasite tend also to invest more in sexual reproduction (and hence resting eggs) (Mitchell *et al.*, 2004). This situation would arise if genotypes that produce resting eggs in the spring create a reservoir of progeny that escape the peak of parasite-mediated selection in the summer, because once made, resting eggs will often not hatch until the following spring. Thus, the production of resting eggs prior to the summer epidemic could become genetically correlated with higher parasite susceptibility as susceptible genotypes are not removed by selection during the summer.

We monitored a natural population of *D. magna* for bouts of sexual reproduction across a season with a strong parasite epidemic, and brought clones into the laboratory to test their susceptibility. Susceptibility could be indicated by any of three response variables that we measured in the laboratory; parasite growth, the proportion of hosts becoming infected, or parasite-induced fitness losses in hosts. We studied hosts from both before and after the epidemic, and also compared those showing variation in the propensity for sex/resting egg production. Our specific predictions for the infection experiment were as follows:

1. Hosts collected after the epidemic would be less susceptible than hosts collected before, having just experienced that summer's parasite epidemic, i.e. we predicted that the epidemic would select for resistance.
2. Within the pre-epidemic samples, hosts reproducing sexually at time of collection would be more susceptible than hosts reproducing asexually at time of collection being represented by genotypes that do not typically experience parasite-mediated selection during summer epidemics.

Methods

Organisms and collections

Daphnia magna is a planktonic crustacean found in still freshwater bodies and is host to numerous bacterial, microsporidian and fungal parasites (Green, 1974; Stirnadel, 1994; Stirnadel & Ebert, 1997; Little & Ebert, 1999). *Pasteuria ramosa* is a bacterial, spore-forming, obligate endoparasite of *D. magna* that greatly reduces host fecundity. Transmission is horizontal, achieved by the release of spores from the decomposing cadavers of previously infected hosts (Ebert *et al.*, 1996).

Daphnia magna and *P. ramosa* were collected from a farm pond at Leitholm, Scottish Borders (2°20.43′W 55°42.15′N). Samples were taken 1–2 times a month between April and December 2003. Three samples were taken at each collection from different locations around the pond. Variability between samples due to sampling techniques were minimized by always using the same net and sweep length.

Immediately following collection, population composition was estimated. Each sample was sieved and diluted in 250 mL of water. The sample was well mixed, and sub-samples were poured on to a Petri dish. Water was removed, and each sub-sample analysed under a dissecting microscope. Individual *D. magna* were recorded as follows; adult females with asexual eggs, adult females with ephippia (reproducing sexually), barren adult females, juveniles and males. We counted until at least 100 individuals had been recorded. Prevalence of the parasite *P. ramosa* was recorded by eye across all samples. Infected *D. magna* are usually distinct making infection easy to detect by eye.

Each month one live sample of adult females was kept and from these we established isofemale lines. When a relatively large portion of the population was found to be reproducing sexually (this occurred at two sampling dates; 14 May 2003 and 27 June 2003), live samples comprising females with ephippia also were kept. Since we ensure that *D. magna* only reproduces asexually in the lab, once isolated, a female (regardless of whether she was reproducing asexually or sexually at time of collection) and all her subsequent offspring are a genetically identical clone. Thus, each live sample can be considered a representation of clones present in the pond at time of collection.

Infection experiment

An infection experiment was performed on *Daphnia* that had been collected in May and June (before the parasite epidemic reached its peak and consisted of females reproducing sexually or asexually), and November (once the epidemic abated, and was composed entirely of females reproducing asexually at time of collection). Thus there were a total of 96 individual isofemale lines, termed clones, that contributed to three experimental groups; (1) pre-epidemic and reproducing sexually at time of collection, (2) pre-epidemic and reproducing asexually at time of collection and (3) post-epidemic, all of which were reproducing asexually at time of collection. As stated in the introduction, we predicted that group 1 would be more susceptible than group 2, and that group 3 would be least susceptible of all.

To equilibrate maternal effects, three replicates of each clone were kept under experimental conditions for three generations prior to starting the experiment. Replicates contained five females all from the same clutch, in a 200 mL jar of *Daphnia* medium (Klüttgen *et al.*, 1994). All subsequent generations of each replicate, including the experimental generation, were seeded using females from the third, fourth or fifth clutches that were less than 24 h old.

A solution of *P. ramosa* transmission spores that had been frozen at −20°C was used for the infection experiment. The spores in solution originated from a large mixture of *D. magna* infected with *P. ramosa* collected from the same pond in 2000. Creating the solution involved infecting a mixture of *Daphnia* individuals (from fifteen 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.

The infection experiment comprised three replicate jars containing five females of each of the 96 clones set up over 4 days. Five female offspring less than 24 h old were placed in a jar containing 50 mL of *Daphnia* medium, with purified sand at the bottom. Sand in jars during infection periods reduces variation in infection levels and increases the incidence of infection (Mitchell *et al.*, 2004). To each jar, 1×10^5 *P. ramosa* transmission spores were added. Everyday, until day 8, each jar was stirred to increase chances of contact with parasite spores. During the infection period *Daphnia* were fed 1×10^7 algae cells 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 hours.

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, and the offspring in the clutch counted. In the absence of any clutches *Daphnia* were transferred to a new jar with fresh medium every 3 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 *P. ramosa* transmission stages (an estimate of parasite fitness).

Data analysis

We used general linear models as implemented in JMP 5.1 to investigate how parasite transmission spore production, the proportion of hosts infected, and host offspring production were affected by 'field history'. Field history is a fixed factor with three levels: (1) pre-epidemic hosts, reproducing sexually, (2) pre-epidemic hosts, reproducing asexually and (3) post-epidemic hosts (which all happened to be reproducing asexually at the time of collection). Host clone was included in each model as a random effect, nested within 'field history'. The experiment was set up over 4 days and thus 'set up day' was also included as a random effect. Proportion data were arcsine-square root transformed, offspring counts were square-root transformed, and transmission spore counts were log transformed.

We next addressed our two hypotheses separately. First, we compared all pre-epidemic hosts with post-epidemic hosts to test for parasite-mediated selection. We then looked solely at hosts collected before the parasite epidemic, and compared those that were reproducing sexually at time of collection with those that were reproducing asexually to test for susceptibility differences associated with life-history.

Results

Population composition

Pasteuria ramosa was not present in samples collected early or late in the year, however an epidemic occurred during the summer reaching 100% prevalence in July (Fig. 1). Peak parasite prevalence corresponded with a dramatic drop in *Daphnia* abundance (Fig. 1). The incidence of sexual reproduction (measured as the occurrence of both males and females carrying ephippia) was highest in May and June (Fig. 2). Barren and juvenile females consistently composed between 70 and 100% of the population and these are omitted from Fig. 2 as they obscure the dynamics of the reproducing portion of the population. It should be noted that these population

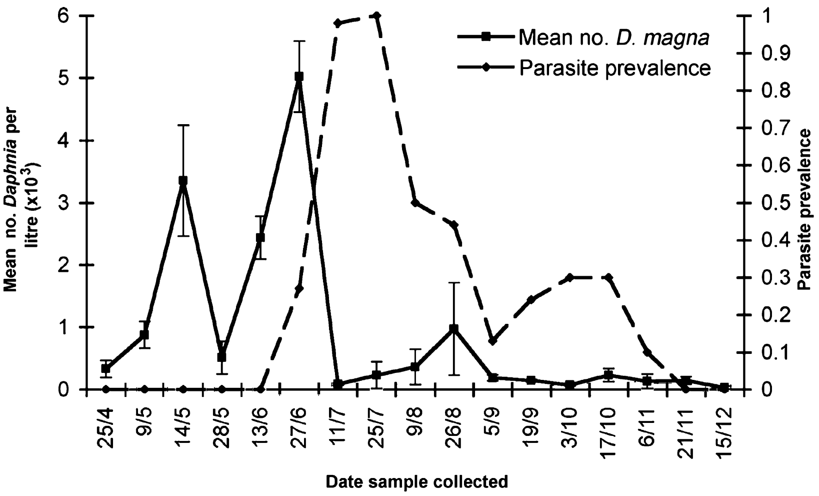

Fig. 1 Mean number of *Daphnia* per litre collected from the Leitholm population in 2003 and proportion of population infected with *Pasteuria ramosa*. (±standard error).

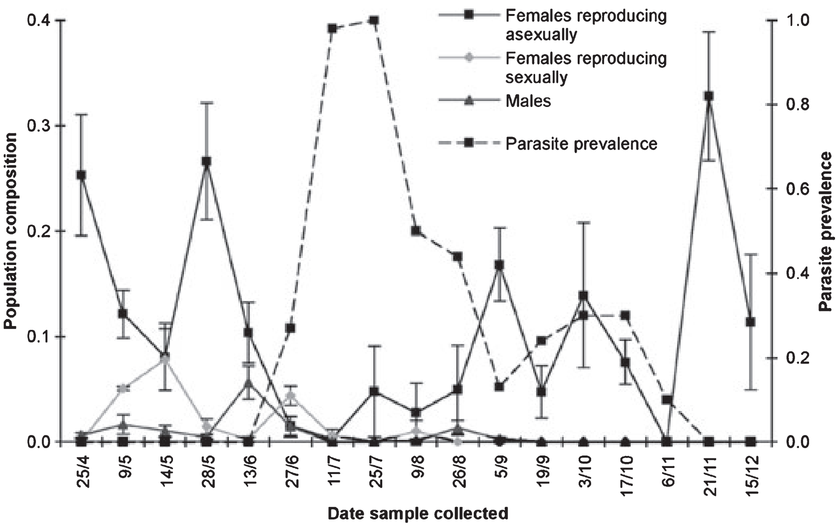

Fig. 2 Proportion of sample composed of reproducing females and males in the Leitholm *Daphnia* population (estimated from three live samples collected on each date) and proportion of population infected with *Pasteuria ramosa* (±standard error).

density dynamics would have been influenced by a variety of factors such as competition for food.

Infection experiments

Parasite growth, measured as mean number of transmission spores per host, was significantly affected by field history in the predicted direction (Fig. 3; $F_{2,87} = 9.79$, $P < 0.0001$). Field history also significantly impacted levels of infection among the three host groups in the predicted direction (Fig. 4; $F_{2,93} = 9.14$, $P < 0.0002$). Reflecting these differences in infectivity and parasite growth, field history significantly impacted offspring production among the three host groups (Fig. 5; $F_{2,93} = 3.37$, $P = 0.039$). It should be noted that the overall differences in offspring production when exposed to parasites is not due to intrinsic differences in the clones in the absence of infection. In a separate study using identical methods, offspring production in the absence of parasites did not differ among the three groups (mean number of offspring from pre-epidemic sexual females = 42.21, mean number of offspring from pre-epidemic asexual females = 41.17, mean number of offspring from post-epidemic females = 40.16, $F_{2,77} = 0.16$, $P = 0.85$).

Regarding the prediction that the epidemic will have selected more resistant hosts, parasite growth was higher on host clones collected before the parasite epidemic than those collected after (Fig. 3; $F_{1,88} = 17.32$, $P < 0.0001$). Infection levels were also higher in hosts collected before the parasite epidemic (Fig. 4; $F_{1,94} = 14.85$, $P < 0.0002$). Although not significant there was a trend for hosts collected after the epidemic to have more offspring in the face of parasitism (Fig. 5; $F_{1,94} = 3.02$, $P = 0.085$).

Examining only the pre-epidemic samples, parasite growth was higher on hosts that were reproducing sexually in the field than those reproducing asexually

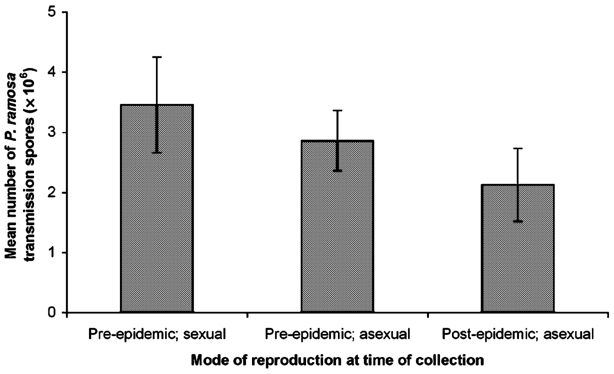

Fig. 3 Parasite fitness measured as mean number of transmission spores per host produced across those reproducing sexually and asexually before the epidemic, and asexually after the epidemic (±standard error). This figure shows transformed data.

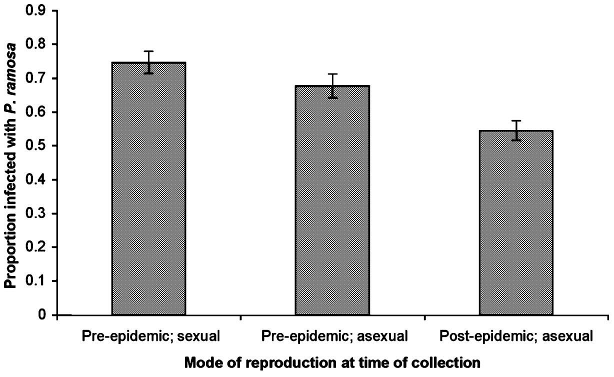

Fig. 4 Resistance to *Pasteuria ramosa* among *Daphnia* collected before the epidemic reproducing sexually and asexually before the epidemic, and asexually after the epidemic (±standard error). Infection inferred through direct observation of *Pasteuria ramosa* spores for 194 of the replicates. This figure shows original untransformed data.

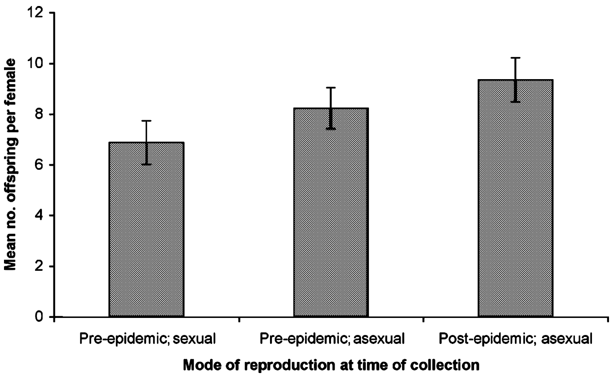

Fig. 5 Comparisons in host fitness between *Daphnia* that were reproducing sexually and asexually before the epidemic, and asexually after the epidemic in the presence of the parasite; measured as mean number of offspring per female (±standard error). This figure shows original untransformed data.

at the same time (Fig. 3; $F_{1,55} = 4.23$, $P = 0.044$). Despite this, intrinsic infection levels were not found to differ between females reproducing sexually at time of collection and those reproducing asexually (Fig. 4; $F_{1,58} = 2.02$, $P = 0.16$), however the difference is again in the predicted direction. There was not found to be a significant difference in offspring production between these two groups (Fig. 5; $F_{1,58} = 2.93$, $P = 0.092$).

Discussion

We observed a severe summer epidemic of the bacterium *P. ramosa*, with infection prevalence reaching 100% in the *Daphnia magna* host population. To test if this epidemic was a source of natural selection, we collected hosts from before and after this epidemic and found those collected before the epidemic were more susceptible to *P. ramosa* than post epidemic hosts. This pattern of susceptibility was evident as higher parasite growth and a greater proportion of hosts becoming infected in the pre-epidemic set of isolates. Thus the parasite epidemic appears to have pruned the more susceptible genotypes from the population.

This study therefore demonstrates parasite-mediated selection in a naturally interacting host-parasite system. Previous *D. magna–P. ramosa* studies have had variable success at finding such evidence for parasite mediated selection in the wild (Little, 2002; Little & Ebert, 1999,2001; Mitchell *et al.*, 2004; but see Haag & Ebert (2004) for an example with a different parasite in an semi-natural population). Indeed, field work on our study population at Leitholm in the year 2000 (i.e. three years prior to the present study, see Mitchell *et al.*, 2004) was unable to demonstrate parasite-mediated selection in a experimental design similar to the present one.

It is notable that parasite prevalence in 2000 reached only 30%, while in 2003, the year sampled for the present study, it reached 100%. Thus the 2003 host population almost certainly experienced stronger parasite-mediated selection. This large difference in parasite prevalence and selection pressure between years could well be due to temperature. The summer of 2003 was one of the hottest on record in Europe (Schar *et al.*, 2004), and *P. ramosa* shows greater infectivity and causes higher virulence at higher temperatures (Ellner *et al.*, 1999; Mitchell *et al.*, 2005). 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.

Despite the occurrence of parasite-mediated selection within a season, there may be limits to the evolution of host resistance in the longer term. Most obviously, a subsequent evolutionary response in the parasite population would erode any gains made by the host population. In addition, this study provides support for a novel hypothesis on the limits of evolution imposed by the impact of recruitment from the 'seed bank' of resting eggs. In an earlier study (Mitchell *et al.*, 2004), resting egg production was induced in the laboratory and it was observed that those genotypes that tended to produce more resting eggs (in the absence of parasites) also tended to be more susceptible when exposed to parasites. In the present study, we corroborated this by showing that one of our measures of susceptibility, parasite growth, was higher on those hosts that were carrying resting eggs than on those that were reproducing asexually at the time of collection. This corroboration, however, was not complete as two additional measures of susceptibility, infection levels and host reproduction, did not fit this pattern, although the trend was in the correct direction. However, we consider our statistical tests to be conservative given that they do not incorporate the directional nature of our predictions.

Thus, a life history strategy that employs sexual reproduction prior to a parasite epidemic appears to be genetically associated with lower parasite resistance. This association will arise because one set of genotypes invests in resting eggs (that lie dormant until the following spring) prior to the summer parasite epidemic. Consequently these genotypes escape the peak of parasite mediated selection pressure for higher resistance. Simultaneously, another set of genotypes that invest less in the production of resting eggs do not escape the epidemic, and will potentially evolve higher resistance. Subsequently, the spring emergence from the resting egg bank of more susceptible genotypes will reduce the population mean resistance to parasites that was gained in response to the previous season's parasite epidemic.

This potential link between susceptibility and resting egg production is, at least in part, genetic, i.e. a negative genetic association between resistance to parasites and sex/resting egg production. Nevertheless, we do not rule out the possibility that parasites can also directly induce sex in *Daphnia*. Slarsarczyk *et al.* (2005), for example, found *Daphnia* to have increased ephippia production in response to the threat of fish predation. Our field observations showed two bouts of ephippia production prior to the parasite epidemic, the second occurring just as the parasite appeared in the population. It may not be a coincidence that the second bout of sex occurred at this time, and we are currently investigating how ephippia production in our sexual clones could be directly induced by parasite presence.

Furthermore, a direct physiological trade-off between the production of ephippia and immune function is conceivable. Ephippia are composed largely of melanin, and melanin, being the end product of the phenoloxidase cascade, is also an important component of the arthropod immune system (Soderhall & Cerenius, 1998). Those genotypes that reproduce sexually early in the season may be predetermined to invest their melanin in ephippia, whereas other genotypes have melanin available for investment in immune defence. This hypothesis might be testable through environmental induction of melanin production, for example by exploiting the natural variation in degree of carapace melanisation in some *Daphnia* populations which is associated with UV protection (Hebert & Emery, 1990).

The genetic association we hypothesise is similar to one generated from a study of predation that compared behavioural traits of copepods that hatched from resting eggs collected from different depths of pond sediment. Copepods from greater depths tended to hatch later, and switch to production of resting eggs at a later date (Hairston & Kearns, 1996). Hairston & Kearns (1996) postulated that genotypes within the population have adopted one of two life history strategies regarding these traits, each having different fitness values between years depending on onset of fish predation. In years when onset of fish predation is late, those genotypes that both hatch later and switch to production of resting eggs later will enjoy greater fitness advantages. However, in years when onset of fish predation is early, these later hatching genotypes will experience fitness losses due to the reduced security of offspring survival through resting eggs. In years when the onset of fish predation is early, early hatching copepods that also switch to resting egg production earlier have higher fitness.

In summary, the present study provides evidence for parasite-mediated selection in the wild. This observation may have only been possible due to the exceptionally high temperatures and levels of parasitism that occurred in the year of sampling. We also found support for the hypothesis that sexual reproduction (and hence resting egg production) prior to a parasite epidemic might be associated with susceptibility. Those genotypes that tend to make more resting stages secure survival of their offspring by avoiding summer epidemics, but their immune systems are subject to less parasite-mediated selection, the result being immune systems that permit greater parasite growth. We expect the annual emergence from the resting egg bank of these more susceptible individuals to diminish gains in mean population fitness that were caused by the previous season's parasite epidemic. Sexual reproduction is typically associated with the production of fitter offspring due to the purging of deleterious mutations, or the creation of novel highly adaptive genotypes (Hamilton *et al.*, 1990; Burt, 2000). Our results suggest, however, that sex and resting egg production may impart a type of genetic slippage (Lynch & Deng, 1994) upon a population, such that sex directly reduces population mean fitness.

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