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Testing the pluralist approach to sex: the influence of environment on synergistic interactions between mutation load and parasitism in *Daphnia magna*

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Abstract

Both deleterious mutations and parasites have been acknowledged as potential selective forces responsible for the evolutionary maintenance of sexual reproduction. The pluralist approach to sex proposes that these two factors may have to interact synergistically in order to stabilize sex, and one of the simplest ways this could occur is if parasites are capable of causing synergistic epistasis between mutations in their hosts. However, the effects of both deleterious mutations and parasitism are known to be influenced by a range of environmental factors, so the nature of the interaction may depend upon the organisms' environment. Using chemically mutated *Daphnia magna* lines, we examined the effects of mutation and parasitism under a range of temperature and food regimes. We found that although parasites were capable of causing synergistic epistasis between mutations in their hosts, these effects were dependent upon an interaction between parasite genotype and temperature.

Introduction

One of the greatest challenges for evolutionary biology is explaining the evolutionary maintenance of sexual reproduction, and the associated process of genetic recombination (Maynard Smith, 1978; Bell, 1982; Barton & Charlesworth, 1998; West *et al.*, 1999; Burt, 2000; Otto & Lenormand, 2002). The problem is that asexual females can potentially produce twice as many daughters as sexual females, resulting in a 'two-fold' cost of sex. In addition, recombination breaks up favourable gene combinations that have passed the test of natural selection. Yet, despite these costs, sex is widespread throughout eukaryotic taxa.

Although numerous explanations for sex have been suggested, there are currently two leading hypotheses (Kondrashov, 1993; West *et al.*, 1999). First, the mutational deterministic (MD) hypothesis states that sex is advantageous because it allows deleterious mutations to be eliminated more efficiently (Kondrashov, 1982; Charlesworth, 1990). The MD hypothesis requires that each additional mutation leads to a greater decrease in fitness than the last (termed synergistic epistasis), because this leads to negative linkage disequilibrium which slows down the removal of deleterious mutations. Here, sex is advantageous because it destroys linkage disequilibria (Kondrashov, 1982; Charlesworth, 1990). Second, the Red Queen hypothesis states that sex provides an advantage in antagonistic biotic interactions (Bell, 1982). It is usually assumed that selection by coevolving parasites against common host genotypes provides the antagonistic coevolutionary dance that is required to drive the Red Queen (Hamilton *et al.*, 1990; Peters & Lively, 1999).

However, several lines of evidence suggest that a pluralist approach may be required to explain sex, with both (or more) of these mechanisms in operation (West *et al.*, 1999). In particular: (i) empirical estimates suggest that the deleterious mutation rate per genome per generation in a high proportion of sexual species is likely to be too low to fully explain sex (Keightley & Eyre-Walker, 2000); (ii) experimental data suggests that synergistic epistasis is not ubiquitous (Mukai, 1969; Elena & Lenski, 1997; Whitlock & Bourguet, 2000; Peters & Keightley, 2000; Rivero *et al.*, 2003); and (iii) theoretical analyses suggest that the Red Queen hypothesis requires extremely strong selection (Howard & Lively, 1994; Otto & Nuismer, 2004) of a form that is not

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commonly met in natural populations (Little, 2002). Furthermore, empirical work has also shown that genotype-specific infection patterns can be affected by environmental conditions (Blanford *et al*., 2003; Stacey *et al*., 2003; Mitchell *et al*., 2005), which could interfere with Red Queen dynamics.

A pluralist approach can make sex easier to explain in at least three ways (West *et al*., 1999). First, in a purely additive world, multiple mechanisms make it easier to fully balance the two-fold cost of sex (Howard & Lively, 1994,1998). Acting together, each mechanism can play an important role even if it requires extreme, and possibly unreasonable, assumptions in order to fully explain sex on its own. Second, the dynamics of different hypotheses can complement each other (Lively & Howard, 1994). For example, by repeatedly driving asexual genotypes down in frequency (in effect bottlenecking them) Red Queen dynamics can provide a short-term advantage to sex by providing time for and speeding up the accumulation of deleterious mutations (Howard & Lively, 1994,1998; see also Peck, 1994). Third, the fitness consequences of parasites and mutations may interact synergistically (Peters, 1999; West *et al*., 1999). The simplest way this could occur is if parasites lead to increased selection against deleterious mutations or are the factor that drives synergistic epistasis between mutations (West *et al*., 1999; Cooper *et al*., 2005; Buckling *et al*., 2006). However, a further possibility is that there are complex interactions between parasite infection, mutation load and environmental factors, where the shape of the relationship between mutation load and fitness varies depending upon parasite and host genotype, and the prevailing environmental conditions. Understanding these interactions is important, as the general application of the pluralist approach may actually be hindered if it is only relevant under given circumstances.

Here, we carry out the first empirical test of whether the fitness consequences of deleterious mutations and parasites interact, and vary with both parasite genotype and environmental conditions. We performed experiments on *Daphnia magna*, where there is already evidence that the fitness consequences of parasite infection depends upon an interaction between host and parasite genotype, as required by the Red Queen hypothesis (Carius *et al*., 2001; Mitchell *et al*., 2005). We experimentally manipulated mutation load with an artificial mutagen, as has proved a useful method in studies on other organisms (Peters & Keightley, 2000; Rivero *et al*., 2003). As the MDH relies upon mildly deleterious mutations in the heterozygous state (Rivero *et al*., 2003), this also allowed us to avoid the confounding effects of inbreeding encountered in similar studies on *D. magna* (Haag *et al*., 2003; Salathé & Ebert, 2003). Our specific aim was to determine the relationship between fitness and the number of deleterious mutations, and how this varies with: (i) parasite infection; (ii) parasite genotype; (iii) environmental conditions (temperature and food availability).

Materials and methods

Study system

Daphnia magna is a cyclically parthenogenetic planktonic crustacean commonly found in freshwater ponds. Gender is environmentally determined, and *D. magna* will reproduce sexually given the right environmental cues. However, it is possible to maintain clonal lineages indefinitely in the laboratory by maintaining them under the appropriate conditions.

The *D. magna* clone used in this study is a long-term laboratory clone that originated from a pond near Gaarzefeld, northern Germany. The use of a single genotypic 'clone' allows us to control the effects of host genetic background in a more precise way than is normally possible in studies on other sexual species. *Daphnia* were kept in jars containing 200 ml of *Daphnia* medium [Aachener Daphnien Medium, as described by Klüttgen *et al.* (1994)] at 20 °C in a 12 L: 12D cycle, with six *Daphnia* per jar. The animals were fed 5×10^6 algal cells (*Scenedesmus* sp.) per *Daphnia* per day and the medium was changed every other day. New generations were started using six second-clutch neonates from each jar.

Pasteuria ramosa is a horizontally transmitted bacterial endoparasite of *D. magna*. Infection occurs when water-borne spores, released from dead hosts, are ingested by the animals as they filter-feed. Development of infection is associated with a severe reduction in host fecundity and in many cases the host is permanently sterilized. The three *P. ramosa* isolates used in this study (designated Sp1, Sp7 and Sp13) originated from the same location as the host clone, and were isolated during a previous study examining strain-specific patterns of infectivity (Carius *et al*., 2001). These strains are known to vary substantially in their infectivity when exposed to this particular host clone – strain Sp13 is relatively avirulent (0–2% infection), Sp1 is highly virulent (80–100% infection) and Sp7 is of intermediate virulence (20–60% infection) (Fig. 1). A short time prior to the experiments, solutions containing 50 000 spores ml^{-1} were made up for each parasite strain. These were frozen at −20 °C until required, after which they were stored at 4 °C.

Mutagen treatment

Mutations were induced using *N*-ethyl-*N*-nitrosourea (ENU), a chemical mutagen known to be highly efficient at inducing point mutations (Solnica-Krezel *et al*., 1994; Nolan *et al*., 2000). The mutagen was prepared by injecting 85 ml of *Daphnia* medium (buffered to pH <6 using 5 mM MES) into a 1 g ENU Isopac® (Sigma no. 3385, St Louis, MO, USA), producing approximately 100 mM solution.

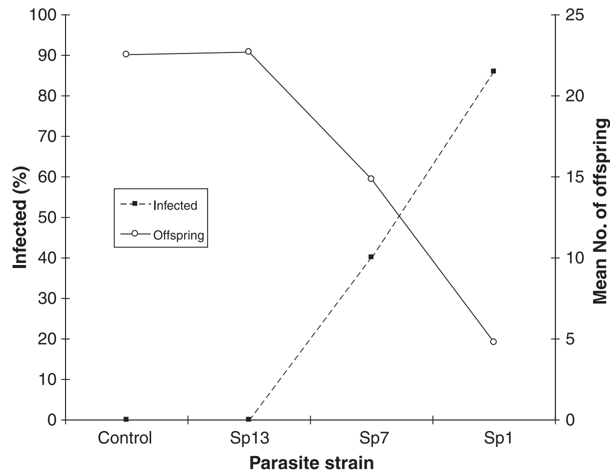

Fig. 1 Infectivity, and its consequences on offspring production, of the three *Pasteuria ramosa* strains used in this study, compared with uninfected controls. (S.C. Killick, unpublished data).

We exposed forty-eight 4-day old females to the mutagen by placing them individually into tubes containing 10 ml of 3 mM ENU solution. Exposure lasted for 1 h, after which the animals were moved to a small tank containing fresh *Daphnia* medium to recover and rinse off the mutagen. This 'rinse and recover' step was repeated three times in order to remove as much of the mutagen as possible. Individual females were then moved to 200 ml jars and the lines were maintained as described above.

One generation later, 24 lines were selected at random and one female from each of these lines was selected to go through another round of mutagenesis. These double-treated lines were classed as 'M2' lines, whereas the remaining 24 single-treated lines constituted the 'M1' group. A further 24 untreated lines were maintained as unmutagenized controls. Importantly, this methodology means that the additional mutations were added separately in each line, making them real replicates (West *et al.*, 1998).

The experiments were carried out approximately 11 (experiment 1) and 14 (experiment 2) *Daphnia* generations after the mutagenesis treatments. Other experimental work conducted in the intervening period demonstrated that the effects of the mutagenesis were heritable and had been retained during this time (S.C. Killick, unpublished data). However, the number of mutant lines was significantly reduced during the first few post-treatment generations, largely because of high levels of sterility and juvenile mortality. As a result, only fourteen M1 lines and eight M2 lines were available for further study. In order to keep the number of lines used for each mutation level fairly even, 12 of the untreated control lines were randomly selected for use in the experiments.

Experimental procedure

For the pre-experimental maternal generation, the required number of jars was set up for each of the 34 lines, each containing six 2nd brood females. These were maintained at 20 °C, in a 12 L: 12D cycle, fed 5×10^6 algal cells Daphnia^{-1} day^{-2} and the medium was changed every other day.

Experimental replicates were established using 2nd, 3rd and 4th brood offspring, depending on the number of neonates (<24 h old) produced per brood. As such, the different parasite spore treatments were always applied between offspring from the same maternal line but a split-brood design was not possible because of insufficient clutch sizes.

For each replicate, the required number of neonates were initially placed into a 60 ml jar containing a small quantity of sand, 50 ml of *Daphnia* medium and 1 ml of the appropriate spore solution (1 ml of *Daphnia* medium was added to the controls). This constituted the infection period. Each jar was randomly allocated a position within a tray and placed into an incubator. Jars were rotated within trays and trays were rotated among shelves daily to minimize positional effects.

Following the infection period, the animals were moved to larger jars containing 200 ml of *Daphnia* medium. This constituted the post-infection period. Again, to remove positional effects, jars were randomized within trays and both jars and trays were rotated daily. Medium was changed every other day, and the number of offspring present and number of adult females remaining in each jar was recorded.

Experimental design

Experiment 1 – mutation, parasitism and food level

For each of the 34 lines, two maternal lines were established and assigned to either the 'Low' food or 'High' food group. Offspring from these lines were then randomly assigned to one of four different parasite spore treatments: control (no spores), Sp1, Sp7 or Sp13. Each replicate was started with six neonates and 1 ml of the appropriate spore solution (2.5×10^4 spores ml^{-1}) was added to each jar. The animals in both treatments were fed 5×10^5 algal cells *Daphnia*$^{-1}$ day^{-2} during the infection period, which lasted for 7 days. During the post-infection period, replicates in the 'High' and 'Low' food groups were fed 3×10^6 algal cells *Daphnia*$^{-1}$ day^{-2} and 1×10^6 algal cells *Daphnia*$^{-1}$ day^{-2} respectively. The animals were maintained at 20 °C in a 12 L: 12D cycle throughout, and each replicate was run for a total of 28 days.

Experiment 2 – mutation, parasitism and temperature

For each of the 34 lines, three maternal lines were established and each was assigned to one of three temperature treatments (15, 20 and 25 °C). Offspring

from these lines were then randomly assigned to one of four different parasite spore treatments: control (no spores), Sp1, Sp7, Sp13. Each replicate was started with eight neonates and 1 ml of the appropriate spore solution (5×10^4 spores ml^{-1}) was added to each jar. Following Mitchell *et al.* (2005), the length of the infection period and the amount of food provided was adjusted to compensate for temperature-associated differences in metabolism and feeding rate. As such, the infection period lasted 9 days at 15 °C, 7 days at 20 °C and 5 days at 25 °C. During this time, the animals were fed 5×10^5 algal cells *Daphnia*$^{-1}$ day^{-2} at 15 and 20 °C, and 7.5×10^5 algal cells *Daphnia*$^{-1}$ day^{-2} at 25 °C. For the post-infection period, food levels were also adjusted to accommodate temperature differences: 3×10^6 algal cells *Daphnia*$^{-1}$ day^{-2} at 15 °C, 3.5×10^6 cells *Daphnia*$^{-1}$ day^{-2} at 20 °C and 4.5×10^6 cells *Daphnia*$^{-1}$ day^{-2} at 25 °C. The length of the experiment was calculated using the 'degree-day' method described by Mitchell *et al.* (2005). Each replicate was run for a total of 500 degree-days: 33 days at 15 °C, 25 days at 20 °C and 20 days at 25 °C.

Data analysis

At the end of each experiment, the mean total number of offspring produced per female (i.e. the total number of offspring produced over the study period divided by the number of females in the jar) was calculated for each replicate. Data from the two experiments was analysed separately because of differences in experimental treatments. Our focal response variable was offspring production because this is the main fitness consequence of *P. ramosa* infection (Ebert *et al.*, 1996). All data was ln-transformed for analysis, as this is needed to test for epistatic interactions between mutations (Charlesworth, 1990; West *et al.*, 1998). First, we looked at the factor effects and their interactions by running generalized linear mixed models (GLMMs) fitted using the expected mean squares (EMS) method. For experiment 1, mean total offspring production was analysed using food level, mutation level and parasite treatment as fixed effects, with mutant line entered as a random effect and nested within mutagen level. For experiment 2, mean total offspring production and mortality per replicate were both analysed using parasite treatment and mutagen level as fixed effects, and temperature as a continuous variable. Line was again entered as a random effect and nested within mutagen level. Parasite treatment was entered as a fixed effect as the differences in infectivity and virulence caused by these parasite isolates (which represent different *P. ramosa* genotypes) is well established in our laboratory (Carius *et al.*, 2001; Little, 2006), and one of our main aims was examine potential parasite genotype-by-environment interactions, and how these in turn may affect the outcome of parasitism on mutation load. For both experiments we also analysed the effect of parasite treatment with the control (parasite-free) lines removed, in order to see if the effects of the different strains varied.

If deleterious mutations are acting independently, fitness is expected to decline in a linear fashion when plotted on a log scale. However, under synergistic epistasis, in which each additional mutation leads to a greater reduction in fitness than the last, the fitness function is expected to be nonlinear, with the slope declining more steeply as the number of mutations increases (Charlesworth, 1990). To test for nonlinearity, we compared the least square means for each mutation level using the contrast methods described in Underwood (1997). If fitness was declining in a linear fashion, then the difference between the LS means of the M1 and M2 lines should not be significantly different from the difference between the control and M1 lines, i.e. (C − M1) = (M1 − M2). However, if mutations were interacting synergistically the M1 − M2 difference would be expected to be significantly higher than the Control - M1 difference, i.e. (C − M1) < (M1 − M2).

If we found evidence for epistasis, we analysed the data further using standard regression analysis in order to verify nonlinearity and examine the effects of the individual parasite strains. The linear and quadratic terms were fitted sequentially to the data. If the quadratic term ($\ln Y = c+\alpha m+\beta m^2$; where Y is fitness and m is the number of mutation episodes) was significant (when the linear term was also included in the model), the fitness function was deemed to be nonlinear and the interaction between mutation levels was considered to be epistatic (Elena & Lenski, 1997; West *et al.*, 1998; Peters & Keightley, 2000; Rivero *et al.*, 2003; Fry, 2004). Under these circumstances mutagen level had to be entered as a continuous variable, meaning the random effects of line could not be nested within mutagen level. However, we found that our results were still in agreement with the contrast analysis.

Results

Experiment 1 – mutation, parasitism and food availability

There were highly significant effects of mutation load ($F_{2,39} = 54.74$, $P < 0.0001$, $n = 188$), parasite treatment ($F_{3,39} = 129.67$, $P < 0.0001$) and food availability ($F_{1,47.27} = 244.83$, $P < 0.0001$) on offspring production (Table 1; Fig. 2). With the control (parasite-free) lines removed from the analysis the effect of parasite treatment remained significant ($F_{2,22} = 58.43$, $P < 0.0001$), confirming that levels of virulence differed between strains. However, parasite treatment showed no significant interaction with either food treatment ($F_{3,39} = 0.95$, $P = 0.427$) or mutation load ($F_{6,74.14} = 1.37$, $P = 0.250$). A similar result was obtained with the control lines removed (parasite × food: $F_{2,22} = 0.03$, $P = 0.975$;

Table 1 Generalized linear mixed model examining the effects of mutation load, parasite infection and food treatment. Mutagen level, parasite treatment and food level are fixed effects, with line as a random effect nested within mutagen.

Source	Df_{Num}	Df_{Den}	SS	MS	*F*-value	*P*-value
Mutagen	2	39	14.11	7.06	54.74	0.0002
Parasite	3	39	50.15	16.72	129.67	<0.0001
Food	1	47.27	31.56	31.56	244.83	<0.0001
Mutagen × parasite	6	74.14	1.06	0.18	1.37	0.250
Mutagen × food	2	43.88	3.37	1.69	13.08	<0.0001
Parasite × food	3	39	0.37	0.12	0.95	0.427
Mutgen × parasite × food	6	39	0.81	0.14	1.05	0.408
Line (mutagen)	26	28.56	17.49	0.67	5.22	<0.0001
Line (mutagen) × parasite	73	39	17.87	0.24	1.9	0.015
Line(mutagen) × food	26	39	3.22	0.12	0.96	0.533

Table 2 Generalized linear mixed model examining the effects of mutation load, parasite infection and temperature. Mutagen level and parasite treatment are fixed effects, with line as a random effect. nested within mutagen, and temperature as a continuous effect.

Source	Df_{Num}	Df_{Den}	SS	MS	*F*-value	*P*-value
Mutagen	2	86.35	46.53	23.27	54.78	<0.0001
Parasite	3	227.77	69.28	23.09	54.38	<0.0001
Temperature	1	192	5.26	5.26	12.39	0.0005
Mutagen × parasite	6	171.15	8.06	1.34	3.16	0.006
Mutagen × temperature	2	47.26	15.43	7.72	18.17	<0.0001
Parasite × temperature	3	192	12.29	4.10	9.65	<0.0001
Mutagen × parasite × temperature	6	192	7.58	1.26	2.97	0.008
Line (mutagen)	30	107.62	59.39	1.98	4.66	<0.0001
Line (mutagen) × parasite	89	192	41.38	0.46	1.09	0.301
Line (mutagen) × temperature	30	192	18.98	0.63	1.49	0.058

parasite × mutation: $F_{4,40.77} = 1.22$ $P = 0.331$). Food treatment showed a significant interaction with mutagen level ($F_{2,43.88} = 13.08$, $P < 0.0001$). This appears to be due to mutational load having less of an impact under low food conditions; as can be seen in Fig. 2, the decline in fitness with increasing mutation load is much more pronounced when food is plentiful [linear regression (all parasite treatments pooled) – high food: $P < 0.0001$, slope = −0.56; low food: $P = 0.019$, slope = −0.19].

The contrast analysis found no significant difference between the LS mean differences (C – M1 = 0.4048 ± 0.102, M1 – M2 = 0.3322 ± 0.122; $F = 0.737$, NS), suggesting that mutations were not acting epistatically and fitness declined in a linear fashion.

Experiment 2 – mutation, parasitism and temperature

In this experiment there were highly significant effects of mutation load ($F_{2,86.35} = 54.78$, $P < 0.0001$, $n = 365$), parasite treatment ($F_{3,227.77} = 54.38$, $P < 0.0001$) and temperature ($F_{1,192} = 12.39$, $P = 0.0005$) (Table 2; Fig. 3). Again, with the control (parasite-free) lines removed the effect of parasite treatment remained significant ($F_{2,193.63} = 44.43$, $P < 0.0001$), indicating that virulence varied between strains. Parasite treatment showed a significant interaction with both temperature ($F_{3,192} = 9.65$, $P < 0.0001$), and mutation level ($F_{6,171.15} = 3.16$, $P = 0.006$). With the control lines removed the parasite treatment-by-temperature interaction remained significant ($F_{2,135} = 11.73$, $P < 0.0001$) but the interaction between parasite treatment and mutation did not ($F_{4,159.47} = 1.1$, $P = 0.360$). The significance of the interaction between parasite treatment and temperature in this case indicates that the effects of the individual strains varied with temperature. Mutation load was also found to significantly interact with temperature ($F_{2,47.26} = 18.17$, $P < 0.0001$); the mutation load-associated decline in fitness observed in experiment 1 was also apparent here, although its severity varied depending on the temperature at which the lines were maintained.

We investigated the interactions (including the significant three-way interaction between mutation, parasitism and temperature) further using two-way ANOVAs for each of the three temperature treatments, with mutation load and parasite treatment as main effects. Mutation load was found to impair offspring production at 15 °C ($F_{2,107} = 26.32$, $P < 0.0001$) and 20 °C ($F_{2,118} = 12.93$, $P < 0.0001$) but not at 25 °C ($F_{2,104} = 2.80$, $P = 0.066$). By contrast, parasite treatment impaired offspring production at 25 °C ($F_{3,104} = 56.84$, $P < 0.0001$) and 20 °C

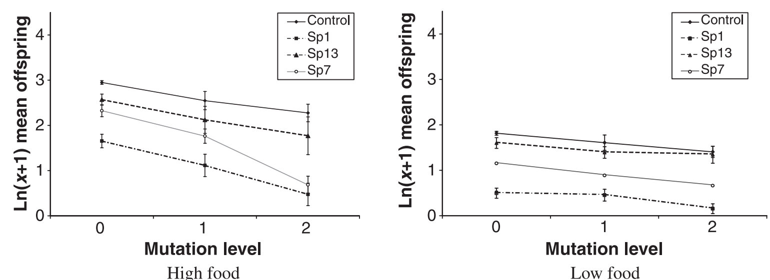

Fig. 2 Ln(x + 1)-transformed mean number of offspring produced by females from each of the three mutation treatments under different feeding regimes. Bars represent the standard errors.

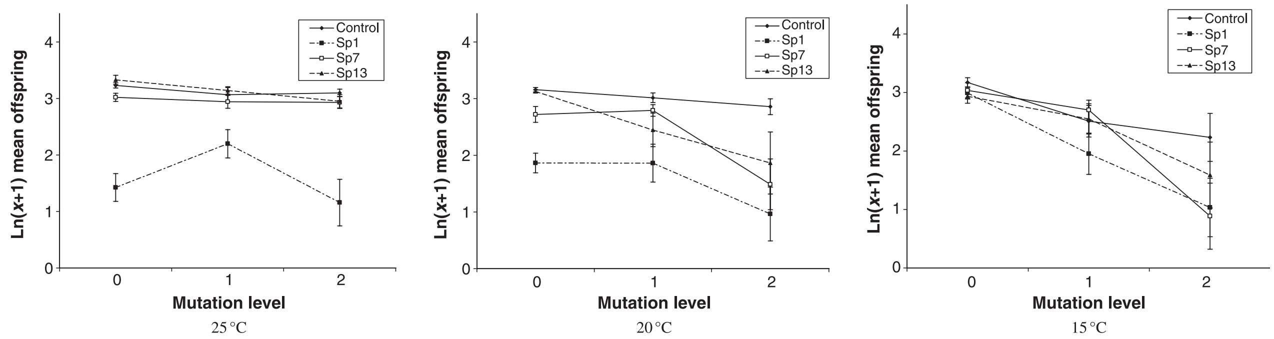

Fig. 3 Ln(x + 1)-transformed mean number of offspring produced by females from each of the three mutation treatments maintained at different temperatures. Bars represent the standard errors.

($F_{3,118}$ = 16.55, P < 0.0001), but not at 15 °C ($F_{3,107}$ = 2.59, P = 0.057), although this was only barely nonsignificant. A significant interaction between mutation level and parasite treatment was found at 25 °C ($F_{6,104}$ = 2.86, P = 0.013).

In contrast to Experiment 1, the contrast analysis found a significant difference between the LS mean differences (C – M1 = 0.2768 ± 0.126, M1 – M2 = 0.7054 ± 0.141; F = 16.93, P < 0.001) indicating that mutations were interacting synergistically and fitness was declining in a nonlinear fashion. The results of the regression analyses are shown in Table 3. In agreement with the findings of the contrast analysis, the quadratic term was significant when applied to the whole data set, indicating a nonlinear fitness decline overall. Separating the data into 'parasitized' (all three strains pooled) and 'unparasitized' (control) classes found that negative epistasis was only observed under conditions of parasitism. This seems to be largely because of the influence of strain Sp7, which caused epistasis in both the 20 °C and 15 °C treatments and also had an epistatic effect overall.

Table 3 Probability and t-values obtained from fitting the linear and quadratic models to ln(x + 1)-transformed fitness measurements obtained from Experiment 2. Significance of the quadratic term would suggest epistatic interactions between mutations. Significant terms are highlighted in bold for clarity.

	Linear model		Quadratic model	
Treatment	t-value	P-value	t-value	P-value
All data	**−6.07**	**<0.0001**	**−2.16**	**0.031**
Parasite treatment				
Unparasitized (control)	**−3.20**	**0.002**	0.78	0.436
Parasitized (pooled)	**−5.64**	**<0.0001**	**−2.59**	**0.0102**
Parasite strain				
Sp1	**−2.85**	**0.006**	−1.89	0.063
Sp7	**−3.98**	**0.0001**	**−2.87**	**0.005**
Sp13	**−3.97**	**0.0001**	−0.36	0.722
Sp7 (25 °C)	−0.72	0.48	0.28	0.779
Sp7 (20 °C)	**−2.61**	**0.014**	**−2.57**	**0.015**
Sp7 (15 °C)	**−4.00**	**0.0005**	**−2.47**	**0.021**

Discussion

We found that the fitness consequences of deleterious mutations: (i) showed no consistent trend towards synergistic epistasis; (ii) depend upon environmental conditions (food availability and temperature) and parasite infection. In experiment 1 we found no evidence for synergistic epistasis. In Experiment 2 we found evidence for synergistic epistasis, and its extent was increased by parasite infection. The fitness consequences of parasite infection varied with genotype, and parasitism overall showed a significant interaction with mutation load. Considering environmental conditions, we found that the fitness consequences of deleterious mutations were greater when food was more plentiful (experiment 1) and at lower temperatures (experiment 2).

Although there was no consequence of food treatment on the effects of parasite infection, we did find a highly significant interaction between food treatment and mutation load. In both treatments fitness was found to decline in a linear manner, but with the effect of increasing mutation load more pronounced in the high food treatment. This stands in contrast to previous suggestions that the effects of mutation load should be exacerbated when individuals have to compete for resources (Kondrashov & Houle, 1994; Peck & Waxman, 2000; You & Yin, 2002; Rivero *et al.*, 2003). A possible explanation for this is that although food levels were classed as 'low', they may not have been low enough to lead to severe competition, although reducing food levels did clearly reduce fitness. Alternatively, it could be due to the way in which mutations were affecting the animals physiologically. If, for example, mutation load affected rates of nutrient uptake by reducing filter-feeding rates, then it is possible that the mutation-free lines would be less noticeably affected by changes in food concentration. Food uptake in *Daphnia* is known to follow a Type II

functional response (i.e. nutrient intake increases with increasing food concentration, but levels off because of processing constraints) (De Mott, 1982; LaMontagne & McCauley, 2001), and mutations may act to reduce the concentration at which ingestion rate asymptotes (i.e. limit food intake). Under such circumstances, the control (mutation-free) lines would be most likely to benefit from high food levels and the disparity between mutation levels would be more noticeable.

Mutation load also showed a significant interaction with temperature. Previous studies have shown that exposure to stressful temperatures can exacerbate the effects of deleterious mutations (Barnes *et al.*, 1989; Korona, 1999; Vassilieva *et al.*, 2000; Szafraniec *et al.*, 2001; but see Kishony & Leibler, 2003), although in this case none of the three temperature treatments can really be regarded as stressful; the animals are usually maintained at 20 °C and the remaining two temperatures are within the temperature range normally experienced by *Daphnia* under both natural and laboratory conditions (Mitchell & Lampert, 2000; Mitchell *et al.*, 2005). As such, we actually found that mutation load reduced fitness at 15 °C and 20 °C, but had no significant effect at 25 °C. Given that this is close to the optimal temperature for *D. magna* development (Mitchell & Lampert, 2000; Giebelhausen & Lampert, 2001), it is possible that the effects of mutation load were rendered benign under these optimal conditions, i.e. they displayed 'conditionally neutral' effects (Kondrashov & Houle, 1994; Elena & de Visser, 2003).

The effects of temperature on host-parasite interactions have been described in a range of other species [reviewed in Thomas & Blanford (2003)]. Of the most relevance is a recent study by Mitchell *et al.* (2005), which found strong evidence of temperature-related genotype-by-environment (G × E) interactions in the *D. magna* – *Pasteuria ramosa* system. Mitchell *et al.* (2005) exposed a range of *D. magna* clones to a 'mixed-bag' of *P. ramosa* spores (i.e. the spore solutions used were likely to contain a mixture of different parasite genotypes) under different temperature regimes, and found that not only did parasite virulence vary across the temperature range (highly virulent at high temperatures, less so at lower temperatures) but also that the susceptibility of the different clones changed between temperatures. In line with Mitchell *et al.* (2005), we found that, overall, parasitism had a high impact on host fitness at 25 and 20 °C, but less of an impact at 15 °C. In the earlier study, however, it was not possible to test for parasite genotype-by-environment interactions, thus the strong parasite G × E interactions found in the present study are of particular interest. For example, all parasite strains affected host reproduction at 20 °C, but at 25 °C both Sp7 and Sp13 had a limited effect while Sp1 had a large effect. This strain-specific effect at 25 °C indicates that temperature-dependent virulence is not likely to be due to an up-regulation of the host immune response under optimal conditions; rather, it would seem that these two isolates were simply not adapted to the higher temperature.

The main aim of this study, however, was to test the hypothesis that infection by parasites increases the extent of synergistic epistasis between mutations in their hosts. In this respect, we obtained a negative (experiment 1) and a positive (experiment 2) result. One of the parasite isolates (Sp7) was found to have negatively epistatic effects in experiment 2, although these effects were influenced by temperature; Sp7 had little effect at 25 °C but caused epistasis at 15 and 20 °C. This finding is of particular significance as it highlights the importance of examining pluralistic hypotheses under a diverse range of conditions. Previous empirical tests of the pluralist approach to sex have failed to find evidence that parasites are capable of causing or increasing the extent of synergistic epistasis between mutations in their hosts, but these studies have utilized a restricted range of environmental conditions and did not differentiate between parasite genotypes (Peters, 1999; Salathé & Ebert, 2003; Haag *et al.*, 2003; Cooper *et al.*, 2005).

To conclude, theoretical work is required to determine the implications of our results for the evolutionary maintenance of sex and recombination. We have shown that parasite infection can lead to an increased extent of synergistic epistasis between deleterious mutations, but only under some environmental conditions. The implications of this are not clear because such interactions have not been specifically modelled, and biological details can have strong effects on the form of selection for and against sex (Otto & Nuismer, 2004). In particular, while some studies have suggested that variation in epistasis reduces the advantage of sex (Otto & Feldman, 1997; Burt, 2000), others have suggested the reverse (Agrawal, 2001; Siller, 2001).

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