The effects of parasitism and inbreeding on the competitive ability in *Daphnia magna*: evidence for synergistic epistasis

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Abstract

Synergistic epistasis for fitness is often assumed in models of how selection acts on the frequency and distribution of deleterious mutations. Evidence for synergistic epistasis would exist if the logarithm of fitness declines more quickly with number of deleterious mutations, than predicted by a linear decline. This can be studied indirectly by quantifying the effect of different levels of inbreeding on fitness. Here, six sets (different genetic backgrounds) of three increasingly inbred *Daphnia magna* clones were used to assess their relative fitness according to changes in frequency in a competition experiment against a tester clone. A novelty of the mating procedure was that the inbreeding coefficients (F) of the three clones belonging to each set increased in steps of 0.25 independent of the (unknown) inbreeding coefficient of the common ancestor. The equal increase of the inbreeding coefficients is important, because deviations influence the quantification of inbreeding depression, its variance and the detection of epistasis. In a simple mathematical model we show that when working with a partially inbred population inbreeding depression is underestimated, the variance of fitness is increased, and the detection of epistasis more difficult. Further, to examine whether an interaction between inbreeding and parasitism exists, each inbred clone was tested with and without a microsporidium infection (*Octosporea bayeri)*. We found a nonlinear decrease of the logarithm of fitness across the three levels of inbreeding, indicating synergistic epistasis. The interaction term between parasitism and inbreeding was not significant. Our results suggest that deleterious mutations may be purged effectively once the level of inbreeding is high, but that parasitism seems not to influence this effect.

Introduction

Inbreeding, the mating between relatives, leads to increased homozygosity of alleles that are identical by descent. Deleterious consequences of inbreeding on fitness, known as inbreeding depression, have been studied extensively and are found in almost all normally outbreeding organisms (for review see Charlesworth & Charlesworth, 1987). Typically, it has been found that inbreeding depression increases with an increase in the inbreeding coefficient F (Willis, 1993; Dudash *et al.*, 1997; Koelewijn, 1998; Ouborg *et al.*, 2000), which is a measure of the degree of inbreeding of an individual and gives the probability of identity by descent of two alleles at a locus (Wright, 1922). Apart from changes in mean genotypic values, inbreeding may also increase genetic variance for fitness (Willis & Orr, 1993; Deng & Lynch, 1997) and thus influence natural selection. However, inbreeding depression and its variance may not only be influenced by the degree of homozygosity but may also be influenced by the interactions among loci and/or by interactions with the environment.

Deleterious mutations act independently when the effect of a mutation does not depend on the existence of other mutations in the genome. If their effects are not independent, they may interact with each other. This might be either through antagonistic epistasis, where

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each additional mutation has a smaller effect than previous mutations, or through synergistic epistasis, where each deleterious mutation added to the genome has a greater effect than the preceding mutations (Peters, 1999). Synergistic epistasis leads to a decrease of the logarithm of fitness, with the slope declining more steeply as the number of mutations increases.

The presence of synergistic epistasis has important consequences for selection against deleterious mutations. As selection acts against individuals with low fitness, deleterious mutations are purged more efficiently with synergistic epistasis than without. The strongest effect is reached in truncation selection, where selection acts against individuals with more than a threshold number of deleterious mutations. The maintenance of sexual reproduction, despite its two-fold cost compared with asexual reproduction, may be explained if mutations interact synergistically (deterministic mutation hypothesis; Kondrashov, 1988).

Although it is difficult to study epistasis directly, the relationship between the number of mutations and their effect on fitness can be examined indirectly by monitoring the effect of different levels of inbreeding on fitness or fitness components. If it holds that inbreeding depression is mainly due to recessive deleterious mutations, a nonlinear relationship between the logarithm of fitness and the inbreeding coefficient indicates epistasis of harmful mutations (Crow & Kimura, 1970).

Recently it has been suggested that synergistic epistasis could even be stronger when individuals with large numbers of deleterious mutations suffer disproportionately more from parasitic diseases (Howard & Lively, 1994, 1998; Lively & Howard, 1994; West *et al.*, 1999). Again, this is particularly relevant for the maintenance of sex. So far the maintenance of sexual reproduction has been explained by either environmental or mutation-based models. Environmental models suggest that sex accelerates adaptations to a changing environment (for instance coevolutionary interactions with parasites) by creating new gene combinations (Red Queen hypothesis, Hamilton, 1980; Bell, 1982). Mutation-based models argue that sexual reproduction is advantageous because it allows populations to purge deleterious mutations more efficiently (deterministic mutation hypothesis, Kondrashov, 1988). The assumptions of these models may not always hold (e.g. highly virulent parasites, synergistic epistasis and high mutations rates). However, if an interaction between parasitic diseases and mutations exists, which would strengthen synergistic epistasis, it could render sex evolutionarily stable against repeated invasion by clones with strongly relaxed assumptions (Howard & Lively, 1994).

The present study consists of a theoretical and an experimental part. In the theoretical part we use a mathematical model to examine how inbreeding depression, epistasis and variance of fitness are influenced, when experiments are conducted with individuals from a population, which vary in their degree of inbreeding. Studies that deal with inbreeding and its consequences sometimes work with base populations that are assumed to be outbred or at least have low variance in the inbreeding coefficient across individuals (e.g. Willis, 1993; Dudash *et al.*, 1997; Koelewijn, 1998; Ouborg *et al.*, 2000). This assumption is often fulfilled, but we show here that if within-population variation in the inbreeding coefficient is high, it may lead to substantial deviations from expectations. Therefore, in the present experiment great care was taken to avoid this problem.

In the experimental part, we assess and compare the fitness of six sets of increasingly inbred *Daphnia magna* clones in a competition experiment vs. a tester clone. With three different levels of inbreeding, epistasis can be inferred from the shape of the curve. The experiment is designed so that the inbreeding coefficients of the three inbred clones differ from the lower to the next higher inbred clone exactly by 0.25, independent of the inbreeding coefficient of their ancestors. Thus, the results are not influenced by the unknown history of the genetic material used. To test for interactions between parasites and inbreeding, each line is tested with and without a microparasitic infection. Testing for epistasis across different levels of inbreeding only includes the natural variation in mutational load, rather than artificially elevated mutation levels as they occur when mutagens are used to create lines with different genetic loads.

Materials and methods

The model

We investigate the consequences of using individuals from a presumably outbred, but in reality partially inbred population, for the quantification of inbreeding depression, synergistic epistasis and the variance of fitness in relation to the inbreeding coefficient, F. For this we assume a base population in which a proportion (p) of individuals in each generation reproduces by selfing, whereas a proportion of $1-p$ individuals outcross. The individuals which self in a given generation are random, and for simplicity, no selection against inbred individuals is assumed. Such a situation may be found in self-compatible plants, hermaphroditic animals or many cyclic parthenogenes, such as *Daphnia,* in which one clone may produce males and females.

For each individual in the population fitness (W) was calculated as:

$$\ln W = B * F + C * F^2, \qquad (1)$$

where F is Wright's inbreeding coefficient relative to the base population, B measures the net decline in fitness (Charlesworth & Charlesworth, 1987); and C, when 0, accounts for no epistasis, when negative for synergistic epistasis.

After many generations with a constant p, the mean natural logarithm of fitness ($\overline{\ln W}$) of the entire base population is then:

$$\overline{\ln W}_{\text{base Pop.}} = \sum_{x=1}^{\infty} \left\{ \left[B*(1-(0.5)^x) + C*(1-(0.5)^x)^2 \right](1-p)p^x \right\}. \quad (2)$$

Additionally, in an experiment one might artificially self-fertilize members of this base population to quantify their fitness relative to the fitness of the base population. The mean natural logarithm of fitness of the selfed offspring of the base population is given by:

$$\overline{\ln W}_{\text{selfed Offspring}} = \sum_{x=1}^{\infty} \left\{ \left[B*(1-(0.5)^x) + C*(1-(0.5)^x)^2 \right](1-p)p^{x-1} \right\}. \quad (3)$$

The difference between eqns 2 and 3 is the exponent of the term that accounts for the increasingly inbred individuals [$(1-p)p^x$ in eqn 2, $(1-p)p^{x-1}$ in eqn 3, respectively]. Without epistasis ($C = 0$), the entire term containing C does not affect the fitness of the populations.

Comparing the fitness of the selfed offspring with the fitness of the base population, one can calculate inbreeding depression by using Lande & Schemske's (1985) equation:

$$\delta = 1 - \frac{\overline{\ln W}_{\text{selfed Offspring}}}{\overline{\ln W}_{\text{base Pop.}}}. \quad (4)$$

The variance of fitness of the base populations can be calculated as:

$$\text{var}_{\text{base Pop.}} = \sum_{x=1}^{\infty} \left\{ \left[B*(1-(0.5)^x) + C*(1-(0.5)^x)^2 - \overline{\ln W} \right]^2 (1-p)p^x \right\} + (1-p)\overline{\ln W}^2. \quad (5)$$

The variance of fitness of the selfed offspring is:

$$\text{var}_{\text{selfed Offspring}} = \sum_{x=1}^{\infty} \left\{ \left[B*(1-(0.5)^x) + C*(1-(0.5)^x)^2 - \overline{\ln W} \right]^2 (1-p)p^{x-1} \right\}. \quad (6)$$

This equation is equivalent to eqn 5. The term for the initially outbred individuals is now integrated into the equation due to the same reasons applicable to the difference between eqns 2 and 3.

As will be seen in the Results section, the model suggests, that if $p > 0$, the magnitude of inbreeding depression and the variance of fitness will be influenced. Further, due to the larger variances, the likelihood of detecting significant effects for inbreeding and epistasis is reduced. Thus, when quantifying these traits experimentally one should be aware of the variance in the level of inbreeding in the base population. Alternatively, one uses an experimental design in which the effect of variation in the inbreeding coefficient can be cancelled out. This leads us to the design of the following experiment, which, in addition to cancelling out ancestral variation in the inbreeding coefficient, also allows to combine sets of individuals from different populations. This makes the conclusions from the experiment more powerful, as the results are representative of more than one population and allows to use this method in cases where populations are difficult to define, which is often the case in subdivided populations.

The competition experiment

Study system

The cladoceran *Daphnia magna* Straus is a planktonic crustacean common to freshwater lakes and ponds. It reproduces by cyclical parthenogenesis. Under favourable conditions females reproduce asexually through diploid parthenogenetic eggs whereas in the presence of environmental cues related to stressful conditions, they produce males and haploid eggs, which are then fertilized (Ferrari & Hebert, 1982). These fertilized eggs (ephippia) are able to survive harsh conditions and hatch when conditions return to normal. Males are produced parthenogenetically and are genetically identical to their mothers (Hebert & Ward, 1972). Therefore, sexual reproduction within a *Daphnia* clone leads to the genetically equivalent of selfing. Negative consequences of inbreeding are well known for *Daphnia,* and are usually quite strong (Innes, 1989; De Meester, 1993; Deng & Lynch, 1997; Haag *et al.*, 2002).

The *D. magna* clones used in this study originate from a metapopulation system in southern Finland (Pajunen, 1986). Several sets of three increasingly inbred clones were created from two half-sib clones (Fig. 1, $G1$-generation). One clone of the half-sibs is the outbred offspring ($G1$x, $F = 0$) of a cross between clones P_x and P_s. P_x and P_s were collected from different, but close by, sub-populations from the metapopulation system, because sub-populations are genetically poor and highly inbred (Ebert *et al.*, 2002). The other half-sib ($G1$s) is a selfed offspring of the P_s clone. As the history of P_s is unknown (it might have inbred itself), its selfed offspring has an inbreeding coefficient of $F = 0.5$ or higher. Likewise, the inbreeding coefficients of all offspring in subsequent generations will be affected by how inbred P_s was. Furthermore, the inbreeding coefficient of P_s of the different sets used here may differ.

The parasite. Octosporea bayeri is a microsporidium that parasitizes *D. magna* naturally (Ebert *et al.*, 2001). It is transmitted vertically from mothers to offspring with 100% fidelity, and horizontally via spores released from dead infected hosts. *Octosporea bayeri* decreases the fitness

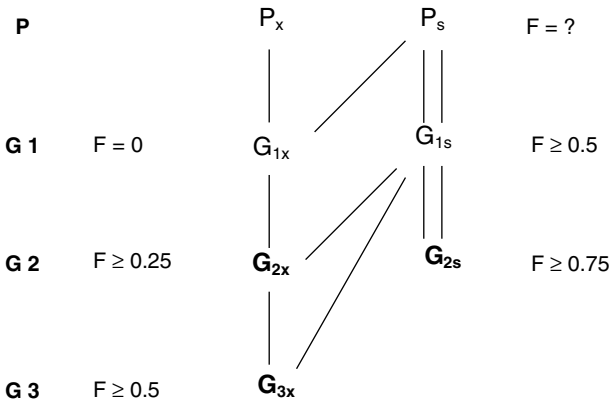

Fig. 1 The mating procedure to produce the three increasingly inbred clones (*G*2x: $F \geq 0.25$; *G*3x: $F \geq 0.5$; *G*2s: $F \geq 0.75$). The capital letters (*P* and *G*) refer to parental (*P*) and the following generations (*G*), the small letters to selfed (s) or crossed (x) matings. The common ancestor is the P_s clone and most inbreeding coefficients will be affected by the inbreeding coefficient of P_s.

of its host by increasing mortality and by reducing lifetime reproduction (D. Ebert, unpublished data). The parasite strain used in this study was isolated from a *D. magna* clone from the same metapopulation but a different sub-population than all the *P* clones.

Experimental design

The procedure to produce clones with three increasingly inbred genotypes is summarized in Fig. 1. As mentioned above, several sets of an outbred (*G*1x, $F = 0$) and a selfed (*G*1s, $F \geq 0.5$) clone, sharing one parent (P_s), were available at the beginning of the study. Further clones were produced by sexual reproduction: The one time selfed clone (*G*1s) was selfed a second time (*G*2s, $F \geq 0.75$). Clones *G*1x and *G*1s were crossed, leading to the offspring of a half-sib mating (*G*2x, $F \geq 0.25$). Subsequently, this offspring (*G*2x) was backcrossed to the one time selfed clone (*G*1s), giving the *G*3x generation (*G*3x, $F \geq 0.5$). Their coefficients of inbreeding were calculated with the following equations (Falconer & Mackay, 1996; Hartl & Clark, 1997):

$$F_{G1s} = 0.5 + 0.5F_{P_s},$$

$$F_{G2s} = 0.75 + 0.25F_{P_s},$$

$$F_{G2x} = 0.25 + 0.25F_{P_s},$$

$$F_{G3x} = 0.5 + 0.25F_{P_s},$$

where F_{P_s} is the unknown inbreeding coefficient of the P_s clone.

Important for our design is that the F_{G2s}, F_{G2x} and F_{G3x} values increase equally with a slope of 0.25 with increasing F_{P_s}. Thus, the absolute difference in the inbreeding coefficients of these three clones is the same throughout the entire range of possible inbreeding coefficients of their common ancestor P_s. This is important for our study because it is unknown whether the different P_s clones are already inbred and whether they differ in this respect. It is known that clones collected from the metapopulation system where the *P* clones were taken from are often strongly inbred (Ebert *et al.*, 2002). By choosing the *G*2s, *G*2x and *G*3x clones we can ignore the unknown history of the P_s clone, which also allows us to use sets of clones with different genetic backgrounds.

Clonal fitness was assessed during a competition experiment vs. a tester clone starting from equal representation of the tester and the tested clones. The change in frequency was used to calculate the relative fitness (*W*) of the inbred clones (relative to the tester clone) according to the following equation (modified after Hartl & Clark, 1997, for the case that $q_o = p_o = 0.5$):

$$\ln W = \ln \frac{q(t)}{(1 - (t))}, \qquad (7)$$

where *q(t)* is the frequency of the inbred clone (either infected or uninfected) at time *t*.

Capaul & Ebert (2003) have shown that competitive ability is a good measure of fitness in *D. magna* and that it is largely insensitive to the tester clone. The advantage of assessing fitness in a competition experiment is that overall fitness is assessed rather than fitness components.

General experimental conditions

Throughout the entire study the *D. magna* clones were kept in artificial culture medium (Klüttgen *et al.*, 1994), modified after Ebert *et al.* (1998), and were fed the unicellular algae *Scenedesmus gracilis* from chemostat cultures. The breeding was conducted under ambient room temperature and light conditions. The competition experiment was carried out in climate chambers with constant temperature (20 ± 1 °C) and a light : dark cycle of 16 : 8 h.

Breeding of experimental lines

The production of males was induced by keeping monoclonal cultures at high densities. Selfed ephippia (the sexually produced resting eggs of *Daphnia*) (*G*2s clones) were produced in monoclonal mass cultures. For the production of outcrossed offspring (*G*2x and *G*3x clones) males and females of the two clones to be crossed were exchanged and allowed to mate. At least every 3 days the newly produced offspring was removed to avoid selfing by male offspring. Mating was induced to produce several ephippia. However, for the competition experiment and for further matings, only one arbitrarily chosen hatchling was used to establish a clonal line.

The sexually produced ephippia were placed in artificial culture medium and kept in the dark at 5 °C. After

2 weeks the culture medium was removed and the ephippia were allowed to dry on a sunny window sill (temperature: 20–30 °C) with additional artificial light from above (light : dark cycle of 16 : 8). This resembles the natural conditions in the metapopulation, where drying of the small rockpools is very frequent. After 1 week culture medium was added. Ephippia hatched within 1–3 weeks. After hatching, clones were kept in replicated monoclonal mass cultures with exclusive asexual propagation.

The *G*2s clones hatched *c.* 7 months, the *G*2x clones 5 months and the *G*3x clones 3 months prior to the start of the competition experiment.

Of the 14 half-sib pairs (*G*1s and *G*1x) available for this study (in the following each pair is referred to as 'origin'), in only six cases was it possible to obtain the three increasingly inbred clones. In the other cases the hatching success of the resting eggs was too low.

From each clone, several individuals were exposed to spores of one strain of *O. bayeri*. The spore solution of *O. bayeri* was prepared by homogenizing infected individuals. Two *Daphnia,* 3–5 days of age, were placed in 3-mL medium and were exposed to 0.1 mL of a 800 000 spores mL^{-1} spore solution for 6 days. As the parasite is transmitted vertically to all offspring with 100% fidelity, a line will not loose the parasite once it is infected.

The tester clone, used in the following experiment, originated from a small pond near Leuven in Belgium and was grown in large numbers in 12-L tanks. To reduce tank effects, individuals from different tanks were mixed before being used in the competition experiment. The tester clone was known to differ from all other clones at the malate dehydrogenase enzyme (Mdh) locus (EC 1.1.1.37).

The competition experiment

All clones (uninfected and infected, as well as the tester clone) had been kept under the same environmental conditions 4 weeks prior to the start of the competition experiment. Each replicate of the competition experiment consisted of a 1.5-L jar with initially 30 inbred and 30 tester individuals (a total of 60 individuals). The number of replicates per origin ($n = 6$ origins), inbreeding coefficient ($F = 0.25$, $= 0.5$ and $= 0.75$) and infection state (uninfected and infected) varied between three and six; a total of 186 replicates were analysed. The competition experiment lasted 28 days, which is approximately two to three *D. magna* generations. A pilot study showed that 28 days was an adequate time for the frequencies of the inbred and tester clone to change. Sixty-six individuals were randomly taken from each jar and the clone frequencies were determined by allozyme electrophoresis at the Mdh locus (Hebert & Beaton, 1993).

To be able to compare the relative fitness between the uninfected and infected inbred clones, the tester clone had to remain uninfected during the competition experiment. This was possible because horizontal transmission of *O. bayeri* occurs only through spores released from decaying hosts. To avoid this, dead individuals were removed daily from the competition jars (before they released infective spores).

Statistical analysis

The frequencies of the tested clones at day 28 were used to calculate the fitness for each clone in each replicate according to eqn 7. The data were analysed with JMP-IN (JMP-IN version 3.2.6, SAS Institute Inc. 1996). A full-factorial analysis of variance (ANOVA) was conducted using origin, infection and inbreeding coefficient as factors and the natural logarithm of fitness as the dependent variable; origin and all its interactions were treated as random effects (Snedecor & Cocerham, 1967). The assumptions of ANOVA were met. The differences between the natural logarithm of the fitness of the three levels of inbreeding were tested with the Tukey–Kramer procedure (Dunnett, 1980).

Results

The model

The mean natural logarithm of fitness of the base population and the selfed offspring decreases with increasing selfing rate of the base population due to the increased proportion of inbred individuals (eqns 2 and 3) (Fig. 2a). The difference between the fitness values of the base and the offspring population also decreases with increasing selfing rate of the base population. Thus, using material from a base population that is partially inbred will lead to an underestimation of inbreeding depression in comparison with entirely outbred populations (Fig. 2b). This effect is found regardless of the presence or absence of epistasis.

The variance of fitness of base and the selfed offspring population increases with increasing selfing rate of the base population up to a maximum at around $F = 0.7$ and then decreases again (eqns 5 and 6). Clearly, entirely outbred or inbred populations have a variance of fitness of 0. With epistasis the variance reaches much higher values than without epistatic gene interactions.

For the model with epistasis, when plotting the natural logarithm of fitness vs. different inbreeding coefficients, curves become flatter, making it more difficult to detect deviations from linearity (Fig. 3). This flattening of curves together with the increase in the variance of fitness reduces the power to detect epistasis when the base population is partly inbred.

The competition experiment

The natural logarithm of fitness decreased significantly across the three levels of inbreeding (Table 1; Fig. 4). The

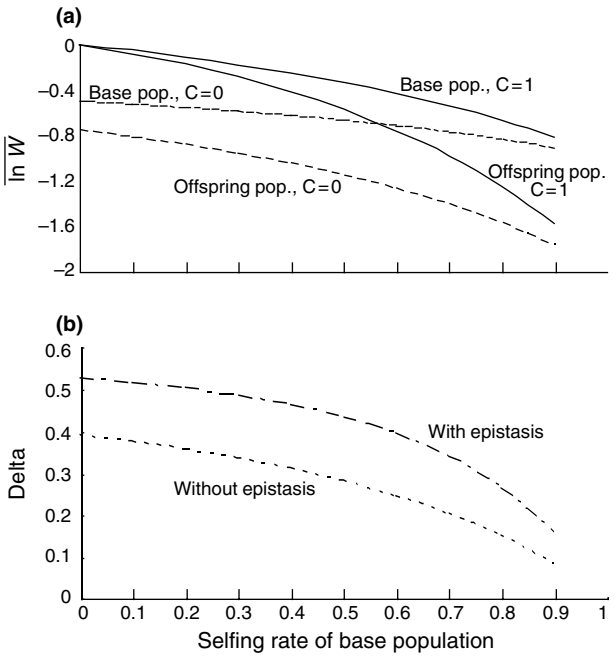

Fig. 2 (a) Mean fitness ($\overline{\ln W}$) of the base and the selfed offspring population with increasing selfing rate of the base population [without epistasis ($B = -1, C = 0$) and with epistasis ($B = -1$, $C = -1$)]. (b) Decrease in inbreeding depression (δ) with increasing selfing rate of the base population according to the equation by Lande & Schemske (1985) $\delta = 1-(\overline{\ln W}_{\text{add. selfed Pop.}}/\overline{\ln W}_{\text{base Pop.}})$. With epistasis inbreeding depression gets larger, but decreases almost equally compared with the calculations for the populations without epistasis.

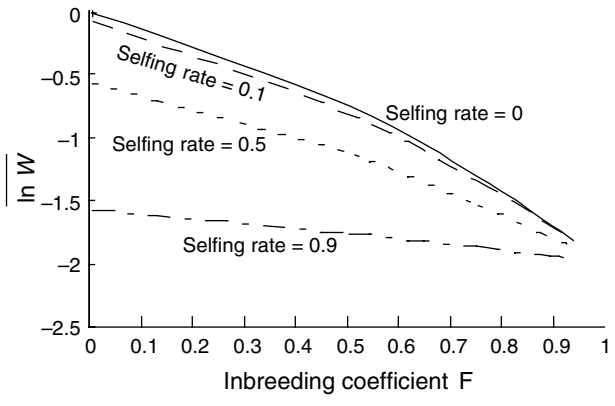

Fig. 3 Decline of fitness in relation to the inbreeding coefficient *F*, when epistasis is included. Fitness decline is shown for four different levels of selfing in the base population. Working with inbred populations makes it more difficult to detect epistasis, because when plotting the natural logarithm of fitness vs. different *F* values, curves become flatter and therefore reduce the likelihood of detecting deviations from linearity.

parasite reduced the fitness significantly. However, the interaction between infection state and inbreeding coefficient was far from being significant, indicating that the shape of the curve of uninfected clones is not significantly different from the shape of the curve of infected clones. Therefore, a synergism between parasites and inbreeding is not supported by this study. Synergism between the inbreeding coefficient and parasitism would be indicated when the mean natural logarithm of fitness of parasitized *Daphnia* with $F = 0.75$ would be lower than that found in the present study. To obtain an indication of how much lower this fitness value would have to be to reveal a significant effect, we reduced its value stepwise by −0.05 until the interaction term of the ANOVA was significant. Significance was reached for $\ln W = -2.46$. Thus, the fitness difference between parasitized and nonparasitized *Daphnia* would have to be twice as high at the highest inbreeding coefficient as to the lower inbreeding coefficients to reach significance. Thus, given the variation in the data, small synergistic effects between inbreeding coefficient and parasitism would have been difficult to detect, suggesting a low statistical power for the interaction term. Low statistical power for interaction terms is a well-known problem in experimental biology.

The fitness between the *G*2x ($F = 0.25$) and the *G*3x ($F = 0.5$) clones did not differ significantly, whereas the fitness of the *G*2s clone ($F = 0.75$) was significantly lower than the fitness of both, the *G*2x and *G*3x clones (Tukey–Kramer procedure, Table 2; Fig. 4). Thus, there was a nonlinear decrease in the natural logarithm of fitness across the three coefficients of inbreeding, strongly indicating the existence of epistasis. This pattern is the same for uninfected and infected clones.

Discussion

The model

Working with populations that are assumed to be homogeneously outbred, but in fact contain a mixture of inbreed and outbreed individuals, has effects on results concerning the degree of inbreeding depression, variance of fitness and on the detection and quantification of epistasis. Inbreeding depression is smaller in partially inbred populations compared with outbred populations. Thus, by wrongly assuming a population to be homogeneously outbred, the magnitude of inbreeding depression can be underestimated. The model shows, that assuming no selection, fitness will decrease with increasing selfing rate of the base population; the higher the proportion of inbred individuals within a population, the lower its mean fitness. The underestimation of inbreeding depression is due to a decreased difference in the mean fitness of the base and the selfed offspring populations. The difference decreases because the mean fitness of highly inbred base populations decreases

Table 1 Three-way ANOVA for fitness ($\ln W$) with origin, infection and inbreeding coefficient as dependent variables. Origin and all its interactions are treated as random effects.

	Denominator					
Source	d.f.	Type III MS	d.f.	MS Error	*F*	*P*
Origin (O)	5	6.8416	2.83	1.1671†	5.8619	0.0963
Infection (I)	1	30.8859	5.02	1.4401‡	21.4478	0.0056
Inbreed. Coeff. (IC)	2	10.9671	10.21	0.4079§	26.8838	0.0001
O*I	5	1.4469	10.06	0.6875¶	2.1045	0.1478
O*IC	10	0.4076	10.00	0.6887††	0.5919	0.7894
I*IC	2	0.1003	10.12	0.6863‡‡	0.1461	0.8659
O*I*IC	10	0.6887	150.00	0.4399§§	1.5657	0.1220
Error	150	0.4399				

†MS(O*I) + 0.9953*MS(O*IC) −0.9953*MS(O*I*IC).
‡0.9932*MS(O*I) +0.0068*MS(Error).
§0.9905*MS(O*IC) +0.0095*MS(Error).
¶0.9953*MS(O*I*IC) +0.0047*MS(Error).
††MS(O*I*IC).
‡‡0.9905*MS(O*I*IC) +0.0095*MS(Error).
§§MS(Error).

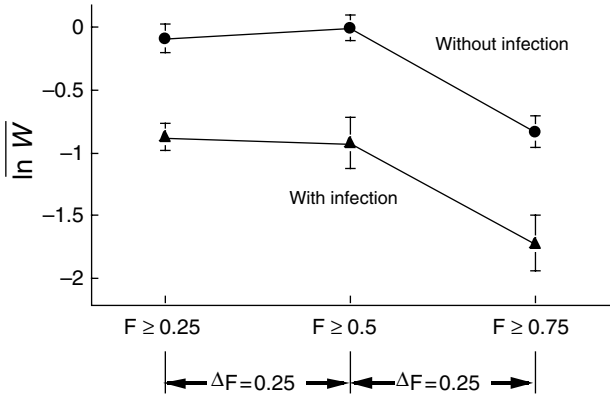

Fig. 4. Mean fitness (natural logarithm of fitness, $\overline{\ln W}$) of the three increasingly inbred uninfected and infected clones of the six origins plotted against their inbreeding coefficient ($F = 0.25$; $= 0.5$; $= 0.75$). Inbreeding coefficients used on the *x*-axis assume that $F_{P_s}=0$. Real inbreeding coefficients are more likely to be higher, but do not differ within origin.

Table 2 Results of the experiment's Tukey–Kramer procedure. Positive values between the absolute difference and the least square deviation (LSD) show pairs of mean values that are significantly different ($P < 0.05$).

Uninfected clones [Abs (Diff)−LSD]		
Inbreeding coefficients	0.5	0.75
0.25	−0.29687	0.363079
0.5		0.442587
Infected clones [Abs (Diff)−LSD]		
Inbreeding coefficients	0.5	0.75
0.25	−0.55954	0.246918
0.5		0.181697

relatively less when selfed than when a completely outbred population is selfed.

In our simplified model, everything else being equal, the variance of fitness is 0 for completely outbred and completely inbred populations. However, for populations with some degree of selfing, the variance increases. The difference in the magnitude of variance in the model without and with epistasis is quite substantial, although expected. It is analogous to the effect of epistasis in Kondrashov's (1988) deterministic mutation hypothesis, which states that in the presence of synergistic epistasis, the variance of fitness of individuals in a sexual population is higher than without epistasis. The increased variance allows selection to act more efficiently against genotypes with many mutations.

The model further highlights a problem in assessing epistasis. It becomes more difficult to detect epistasis when populations are wrongly assumed to be homogeneously outbred. The fitness curve of increasingly inbred individuals flattens and the deviation from linearity is hard to discover. This decrease in statistical power is even amplified by the enlarged variances of fitness.

The model used selfing as a mechanism to create partially inbred base population. The described effects are also found with other breeding systems causing variation in the degree of inbreeding within populations, for example if populations are to some degree inbred, but receive occasional immigrants, which cause some individuals to outcross. The effects described here do not apply for study systems in which the entire base population is homogeneously inbred. It is the variation in the inbreeding coefficient in the base population, which causes the effects described here.

Although our model is very simple, it highlights the potential problems one might encounter during quantification of inbreeding depression and epistasis. To overcome the described problem, it is important to either start

with completely outbred populations, to know their exact inbreeding coefficient or to make sure that the absolute difference in their inbreeding coefficient stays the same in regard to their common ancestor. This latter approach was used here.

The competition experiment

The present study detected synergistic epistasis, as the natural logarithm of fitness decreased in a nonlinear way across six sets of three increasingly inbred *D. magna* clones (Fig. 4). The experimental detection of epistasis has been difficult, and only a few studies exist on the interaction of deleterious mutations. For example, Mukai (1969, *Drosophila melanogaster*) demonstrated the existence of synergistic interaction among newly arising mutant polygenes located in the same chromosome. Homozygous viability of these chromosomes was tested and the average viability decreased nonlinearly. Temin *et al.* (1969) studied viability effects of the second and third chromosomes of *D. melanogaster* at three inbreeding levels. Their data suggest slight (not significant) synergistic epistasis between chromosomes. Also working with *D. melanogaster,* Whitlock & Bourget (2000) showed that recessive mutations in a homozygous state are generally deleterious and that with respect to productivity (a combined measure of fecundity and egg-to-adult survivorship) mutations interact synergistically. Plants have also been examined for fitness components at different levels of inbreeding (Willis, 1993; Dudash *et al.*, 1997; De Visser & Hoekstra, 1998; Koelewijn, 1998; Ouborg *et al.*, 2000). Most of these studies only found limited evidence of epistasis in some fitness components; sometimes epistasis could only be found on the family level but not on the population level.

In most studies on inbreeding depression and epistasis, base populations are used that are assumed to be homogeneously outbred or at least have a low variance in the inbreeding coefficient across individuals. Individuals of these populations are then used to establish higher levels of inbreeding. Although this assumption may be appropriate for most populations, it is certainly not the case for the *Daphnia* metapopulation used in this study, for which frequent selfing seems rather common (Ebert *et al.*, 2002; Haag *et al.*, 2002). As shown in the mathematical model, working with partially inbred populations will underestimate inbreeding depression, increase the variance of fitness and make the detection of epistasis more difficult, unless one uses an appropriate experimental design.

In our experiment, the inbreeding coefficients of the three clones in each set depended on common ancestors with unknown history of inbreeding. Our breeding design allowed to keep the differences between the inbreeding coefficients consistent within each origin, as the difference in the inbreeding coefficients of the three inbred clones increases equally depending only on their common ancestor. Although this might not be a common approach for studies on inbreeding depression, our crossing scheme was not designed to show and quantify inbreeding depression itself, but to test for epistasis across three levels of inbreeding with and without a parasitic infection.

The inbreeding coefficient gives the probability of identity by descent of two alleles at a locus (Wright, 1922). The more inbred an individual is, the higher is the probability of having identical homozygous loci and thus, deleterious mutations become expressed and reduce the fitness of the carrier. The relationship between the inbreeding depression and the number of deleterious mutations depends on the underlying genetic mechanisms. For the simplest case of deleterious mutations being completely recessive and without epistasis, the relationship is linear. In other, more complex cases (e.g. partial dominance and overdominance), the relationship depends on the details of the genetic architecture. Nevertheless, the inbreeding coefficient is thought to be an approximation of the number of deleterious mutations in homozygous state in a genome and therefore studies on inbreeding depression can be used to study the relationship between the effect and the number of deleterious mutations. An advantage of studies testing for this relationship based on different levels of inbreeding is that they use the natural variation in genetic load and the natural degree of dominance. In contrast mutation accumulation experiments (e.g. Elena & Lenski, 1997; Peters, 1999) work sometimes with specific classes of mutations (depending on the mutagen), which may result in different effects.

Selection during breeding of experimental lines can distort the relationship between inbreeding depression and the inbreeding coefficient. For example it may be that only *D. magna* individuals with a reduced number of harmful mutations in the inbred lines did hatch, survive and reproduce. Of the three increasingly inbred clones, the $F = 0.25$ clones and $F = 0.75$ clones may have been most exposed to selection (See Fig. 1). The $F = 0.25$ clones, because they were required in the breeding of the $F = 0.5$ clones, and the $F = 0.75$ clones, because they hatched first out of the three inbred clones. The $F = 0.5$ clones hatched last and were not required in an additional breeding procedure. Therefore, if selection acted on the inbred clones, it concerned mainly the fitness of the $F = 0.25$ and $F = 0.75$ clones. In this case the fitness estimates would be too high. Synergistic epistasis could only falsely be detected if the fitness estimates were reduced in the $F = 0.25$ and $F = 0.75$ clones and elevated in the $F = 0.5$ clones, which is exactly opposite to the effect selection might have had. Thus, if selection acted as assumed above, it would have reduced the chances of finding synergistic epistasis. Likewise, if the effect of selection was simply proportional to the inbreeding coefficient (i.e. strongest on $F = 0.75$) it would reduce the likelihood to detect

epistasis, but not strengthen it. Therefore, the results of our experiment clearly indicate synergistic gene interactions.

Unlike most earlier studies, we assessed fitness in a competition experiment. In a competition experiment the effects of all fitness component contribute to the performance of a clone and the environmental conditions for the competitors within a replicate are equal. When single fitness components are examined, important factors could be neglected and the difference in these phenotypic traits between different levels of inbreeding might be small and difficult to detect. Further, competition might create some form of stress, which may strengthen the effect of inbreeding (Haag *et al.*, 2002, You & Yin, 2002). Koelewijn (1998) found inbreeding depression and some evidence for epistatic interactions under harsh field conditions, whereas in the greenhouse neither of the two was detected. Peters (1999) points out that some proportion of the mutations are deleterious only under harsh conditions.

Synergism between inbreeding and parasitism

There was no significant interaction, and thus, no evidence for synergism between the inbreeding coefficient and the consequences of parasitic infection found in this study. The parasite reduced the fitness of all inbred clones equally. Previously, Coltman *et al.* (1999) reported an interaction between inbreeding and parasitism. Inbred soay sheep showed a higher mortality when parasitized by gastrointestinal nematodes. They suggest that parasitism intensifies selection against inbreeding. Addressing similar questions, Stevens *et al.* (1997) examined the effect of a tapeworm parasite on inbred *Tribolium* lines. Although inbred beetles did not have significantly different infection intensities than noninbred individuals, inbreeding increased parasite prevalence significantly. In our study, the probability of becoming infected (prevalence) at different levels of inbreeding could not be studied, as within each replicate either all or none of the tested animals were parasitized. This is because we kept the conditions such that only vertical transmission of *O. bayeri* occurred.

With respect to parasitism, the result of the present study is consistent with the result of Peters (1999), who exposed *Arabidopsis thaliana* individuals to five levels of chemical (EMS) mutagenesis and three levels of *Pseudomonas syringae* infections and measured different fitness components. Although he found a negative effect of mutation and of the pathogen, there was neither a synergistic interaction of mutations, nor a synergistic effect of mutations and pathogens on the characters measured. Thus, as in the present study, the consequences of infection and of deleterious mutations seemed to be independent of each other. In contrast to Peters' (1999) study, the present study did show a nonlinear decrease in fitness with increasing numbers of mutations.

Consistent with our findings, another study on *D. magna* (Haag *et al.*, 2003), which was specifically designed to test whether parasitic infections increase selection against inbred genotypes, also found no evidence for a synergism between parasites and inbreeding. Haag *et al.* (2003) included 14 genetic backgrounds, but contrasted only controls with one level of inbreeding. Some of the clones they used were the *G*1x and *G*1s clones, used for breeding the experimental clones of the study presented here. In contrast to our study, Haag *et al.* (2003) had higher power to find a possible synergism between parasites and inbreeding, but their design did not allow to test for epistasis across different levels of inbreeding. Although their experiment clearly showed an absence of synergism between parasites and inbreeding, they found that the effect of parasites on inbreeding depression depended on the genetic background. In our experiment, this was tested by the three-way interaction, which was only marginally significant.

In conclusion, this study observed epistasis using three increasingly inbred clones of *D. magna* in a competition experiment. The clones with the highest inbreeding coefficient showed a significant decrease in their fitness compared with the other two, less inbred clones. However, no synergism between inbreeding and a parasite was found, the parasite reduced the fitness of all clones equally, independent of their inbreeding coefficient. Thus, this study suggests that mutations and one environmental factor (a parasite) may contribute to the maintenance of sex independently, rather than synergistically.

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