

Inbreeding uncovers fundamental differences in the genetic load affecting male and female fertility in a butterfly

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Inbreeding depression is most pronounced for traits closely associated with fitness. The traditional explanation is that natural selection eliminates deleterious mutations with additive or dominant effects more effectively than recessive mutations, leading to directional dominance for traits subject to strong directional selection. Here we report the unexpected finding that, in the butterfly *Bicyclus anynana*, male sterility contributes disproportionately to inbreeding depression for fitness (complete sterility in about half the sons from brother–sister matings), while female fertility is insensitive to inbreeding. The contrast between the sexes for functionally equivalent traits is inconsistent with standard selection arguments, and suggests that trait-specific developmental properties and cryptic selection play crucial roles in shaping genetic architecture. There is evidence that spermatogenesis is less developmentally stable than oogenesis, though the unusually high male fertility load in *B. anynana* additionally suggests the operation of complex selection maintaining male sterility recessives. Analysis of the precise causes of inbreeding depression will be needed to generate a model that reliably explains variation in directional dominance and reconciles the gap between observed and expected genetic loads carried by populations. This challenging evolutionary puzzle should stimulate work on the occurrence and causes of sex differences in fertility load.

Keywords: inbreeding depression; genetic load; fertility; egg hatching; Lepidoptera

1. INTRODUCTION

The genetic load of deleterious alleles segregating in a population is a major determinant of fitness variation (Crow 1993), having consequences for genome variation (Charlesworth 1994), population persistence (Saccheri *et al.* 1998), the evolution of inbreeding avoidance mechanisms (Pusey & Wolf 1996; Tregenza & Wedell 2002), and heterosis-assisted gene flow (Ingvarsson & Whitlock 2000; Saccheri & Brakefield 2002). Mutation–selection balance theory provides the framework for understanding the evolutionary dynamics of genetic load (Crow 1993), but there remains considerable uncertainty as to the distribution of mutational rates and effects (Keightley & Lynch 2003). Even in the intensively studied *Drosophila melanogaster* there is a discrepancy between the observed genetic load and the prediction of a purely mutational model, suggesting that the recessivity and antagonistic pleiotropy of detrimental mutations has been underestimated (Charlesworth & Charlesworth 1999).

Inbreeding experiments are the most common device for quantifying the genetic load affecting components of fitness in a population. Variation in inbreeding depression among traits provides an opportunity for examining the factors maintaining genetic load and testing the assumptions of the mutation–selection model. The general pattern is for inbreeding depression (or directional dominance) to be most pronounced for traits closely associated with fitness, typically those involved in reproductive physiology

and early development, as opposed to traits whose contribution to fitness is conditional on environmental circumstances, often morphological characters (Crnokrak & Roff 1995). The traditional explanation for this pattern of inbreeding depression among traits is that strong directional selection on major components of fitness efficiently consumes additive genetic variation but allows deleterious recessives to persist: resulting in a relatively large component of directional dominance. No such directional dominance arises for traits under weak or balancing selection (Lynch & Walsh 1998). The available data fit this model quite well but, as we will show, our results caution against overly simplistic categorization of traits based on their presumed selection regime.

The butterfly *Bicyclus anynana* (Satyridae) carries one of the largest genetic loads reported in any animal (Saccheri *et al.* 1996; Oosterhout *et al.* 2000; Joron & Brakefield 2003). The strongest effects of inbreeding in *B. anynana* are seen for egg hatching rate (the proportion of eggs that hatch into larvae), a trait that accounts for 98% of the variation in egg-to-adult survival in captivity (Brakefield *et al.* 2001). In a previous study (Saccheri *et al.* 1996) we found that the offspring from a single generation of brother–sister mating (coefficient of inbreeding $F = 0.25$) produced eggs with a 30–70% reduction in average hatching rate. The equivalent reduction in *Drosophila* is 5–10% (see Saccheri *et al.* 1996). This level of genetic load is hard to reconcile with conventional mutation–selection balance theory (Crow 1993) and the population ecology of *B. anynana* (Brakefield & Reitsma 1991), which would require both implausibly high

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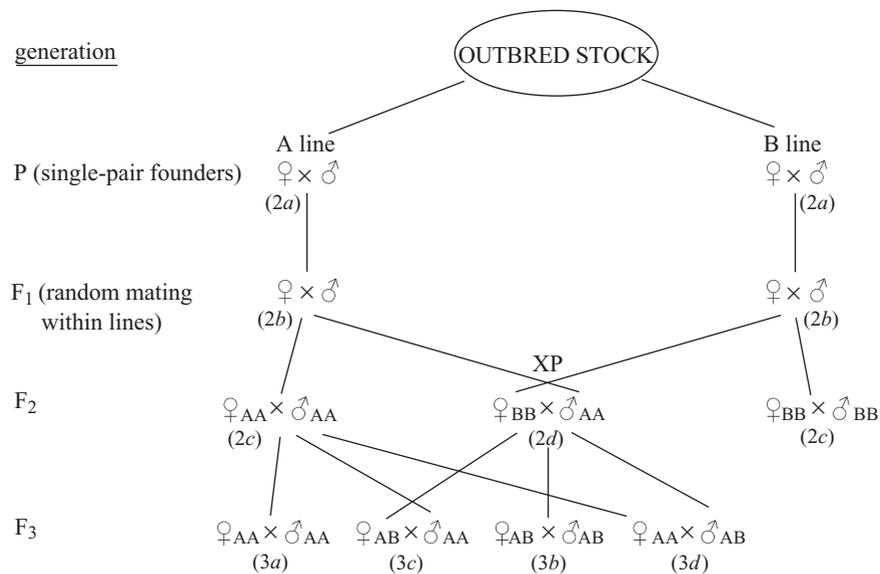


Figure 1. Mating scheme for one pair (A/B) of inbred lines used to produce different combinations of inbred and hybrid crosses (not shown: C/D and E/F). Generation refers to parents rather than offspring. For each pair of inbred lines the parental cross (XP) was performed in one direction only. Letter and number codes in parentheses denote crosses used, together with equivalent crosses from the other pairs of lines, for figures 2 and 3.

rates of mutation to alleles with large deleterious effects and implausibly large effective population sizes.

An interesting feature of the distribution of egg hatching rate among mothers within partly inbred lines of *B. anynana* is their bimodality: a large class of completely inviable or sterile egg batches, and a second, more dispersed, class with intermediate to high hatching (Saccheri *et al.* 1996). Superficially at least, this distribution qualitatively resembles the classic viability distribution of *D. melanogaster* made homozygous for chromosome II (Greenberg & Crow 1960), reflecting lethal and sub-lethal (detrimental) effects. However, the comparison with viability is compromised by the fact that egg hatching rate represents a composite trait determined by the fertility of both parents and the viability of embryos. In common with our own early inbreeding experiments in *B. anynana*, very few inbreeding studies have been designed to separate maternal, paternal and offspring effects. As we shall see, failure to do so can mask asymmetries in the components of genetic load that may signal complexities in the maintenance of genetic load, and lead to an examination of the universality of the directional selection–directional dominance prediction. To begin unravelling the cause of the high genetic load for egg hatching rate in *B. anynana*, we performed a series of breeding experiments to quantify the genetic loads associated with each of its components.

2. MATERIAL AND METHODS

(a) *Base population and experimental design*

The Leiden stock population of *B. anynana* was established in 1988 from 80 wild gravid females collected at a single locality (Nkhata Bay) in Malawi. Since that time, over 100 generations have elapsed during which the stock has been maintained at 500–1000 adults with some overlap of generations. High levels of molecular (Saccheri *et al.* 1999) and quantitative genetic variation (Saccheri *et al.* 2001) in the Leiden stock suggest that the standard husbandry regime has retained a large part of the initial genetic

variation. All butterflies in this study were reared and mated at 27 °C, 70% relative humidity, and a 12 L : 12 D cycle. Larvae were reared on potted maize plants of uniform quality and adults were fed on wet mashed banana.

For the main experiment, six inbred lines (A to F) were established with single pairs of mating butterflies taken from the Leiden stock population. In addition to these six inbred lines, maintained at an effective adult population of 30 mating pairs with non-overlapping generations, a random sample of 30 F₂ individuals of one sex from one inbred line was crossed to an equivalent opposite sex sample from another inbred line, resulting in three paired sets of inbred line crosses (A × B, C × D and E × F), referred to as the parental cross generation or XP (figure 1). In the next generation (F₃) we obtained samples of four types of crosses for each pair of lines: (i) inbred females × inbred males; (ii) inbred females × crossbred males; (iii) crossbred females × inbred males; and (iv) crossbred females × crossbred males. A second experiment, in which 12 inbred lines were established in the same way, provided additional data for the F₁ and F₂. The data from different lines within each type of cross and generation have been combined to focus on the main effects. Average inbreeding coefficients of mothers, fathers and offspring in each experimental cross are listed in table 1.

(b) *Egg hatching distributions*

In each generation, from the parental base population (P) to F₃, the distribution of egg hatching rate was estimated for each line from samples of singly mated male–female pairs. Age at mating for both sexes ranged from 1 to 8 days (mode, 3 to 4 days), and was equivalent between lines and generations. Singly mated male–female pairs were obtained by mixing equal numbers of virgin females and males in large netting cages, and isolating pairs *in copula*. After separation, females were placed into communal population cages (one cage per line) with a potted maize plant to induce egg laying. This initial egg-laying period of 24 h served to eliminate any eggs that had been held in the ovarioles, and which might have biased egg hatching estimates. Each female was then transferred to a net-covered pot containing a watered cutting of

Table 1. Average inbreeding coefficients of mothers, fathers and offspring in each experimental cross. Cross type refers to codes in figure 1.

cross type	mothers	fathers	offspring
P (2a)	0	0	0
F ₁ (2b)	0	0	0.25
F ₂ (2c)	0.25	0.25	0.25
F ₂ (2d)	0.25	0.25	0
F ₃ (3a)	0.25	0.25	0.25
F ₃ (3b)	0	0	0.125
F ₃ (3c)	0	0.25	0.125
F ₃ (3d)	0.25	0	0.125

the preferred host plant for oviposition, *Oplismenus compositus*. One sample of eggs was collected from each female, for a period of 24 h in P and F₃, for 48 h in F₁ and 72 h in F₂. This difference in egg collection period does not affect intergeneration comparisons as hatching rates of eggs laid during the first 6 days are highly correlated ($r^2 = 0.98$, $n = 30$). The modal number of eggs laid by individual females over 24 h was 35. Females that laid less than five eggs were excluded from the analysis. Eggs were incubated a further 4 days before counting unhatched eggs and early instar larvae to estimate egg hatching rates. Total numbers of matings varied between generation and type of cross but, with the exception of F₁ and F₂ where data from two separate experiments were combined (see table 2), the contribution of each line to the total sample within any particular cross type was the same. Total numbers of matings used to estimate each egg hatching distribution are given in figure legends.

(c) Genetic load estimation

The genetic load, in lethal or sterile equivalents per zygote, was estimated as twice the slope (B) of the regression of the ln-transformed trait values on the coefficient of inbreeding F (see Morton *et al.* 1956; Saccheri *et al.* 1996; Keller & Waller 2002). The base population was used as the $F = 0$ reference. Depending on whether the inbred sample refers to the zygote offspring, the parents, or both, B represents, respectively, the zygote viability load (V), the fertility load (S) or the sum of both (T). The slopes are based on only two levels of parental or offspring inbreeding ($F = 0$ and $F = 0.25$), but with egg hatching estimates for several lines per regression. We have previously shown that the relationship between egg hatching rate and F is linear, at least within the range 0–0.3 (Saccheri *et al.* 1996).

(d) Causes of male sterility

Lepidoptera produce two distinct types of sperm which differ in morphology and behaviour: nucleate eupyrene sperm, capable of fertilization, and apyrene sperm lacking functional nuclear material (Meves 1902; Silberglied *et al.* 1984). A sample of singly mated females from the inbred lines were dissected to determine whether the cause of egg hatching failure was associated with lack of spermatophore transfer by males or differences in the numbers of eupyrene and apyrene sperm.

3. RESULTS AND DISCUSSION

(a) Separating viability from fertility effects

We first present the changes in the distribution of egg hatching rate from P to F₁, F₁ to F₂, and F₂ to XP (figure 2). Eighty-five per cent of matings in the outbred stock produced egg batches with hatch rates higher than

Table 2. Variation in the proportion of sterile matings among partly inbred lines.

(Each line was established from a different single female–male pair taken from the outbred base population. Effective population sizes in F₁ were at least 40, and so will have had negligible effect on the inbreeding coefficient in F₂, estimated to be 0.25. The proportion of sterile matings was estimated in F₂.)

inbred line	n^a	proportion of completely sterile matings
A	119	0.36
B	26	0.19
C	110	0.47
D	37	0.24
E	128	0.41
F	40	0.40
1	18	0.61
2	22	0.77
3	23	0.22
4	19	0.53
5	24	0.00
6	22	0.55
7	19	0.58
8	21	0.43
9	17	0.71
10	15	0.27
11	9	0.67
12	23	0.26

^a Total number of matings per line used to estimate the proportion of sterile matings (i.e. no eggs hatched).

80% (overall mean hatch rate = 85%), with only 3% of egg batches being totally sterile or inviable. In the F₁ generation, in which parents have an F of 0 and offspring an F of 0.25, there was a general shifting down of the distribution, with a marked decline in the 100% class and a small increase in the zero hatching class (overall mean = 70%). The zero hatching class increases sharply in the F₂, where parents as well as offspring have an F of 0.25, which together with a further decline in hatch rates at the upper end of the distribution, leads to a large decline in average hatch rate (37%). In the XP, the inbreeding status of parents is unchanged, but the offspring have an F of zero. Here, the size of the zero hatching class is essentially identical to that in F₂ but the second mode shifts to 100% hatching, resulting in a slightly improved average (44%). This indicates that the large fraction of egg batches in F₂ and XP from which no larvae hatch is related to the inbreeding status of parents and not offspring: fertility load is substantially larger than viability load. Moreover, complete sterility (zero hatching class) accounts for two-thirds of the 48% reduction in egg hatch rate because of infertility, the remainder being a result of partial sterility.

These results are consistent with our previous interpretation of the pattern of heterosis in this species (Saccheri *et al.* 1996), but also allow the separate quantification of zygote viability load and fertility load. The zygote and embryo viability load (V) estimated from P and F₁ egg hatching distributions was 1.75 ± 1.29 (95% confidence interval) lethal equivalents per zygote, which is about twice the viability load in *Drosophila* (when calculated in an equivalent way and assuming that two-thirds of the load affecting egg to adult survival in *Drosophila* affects viability

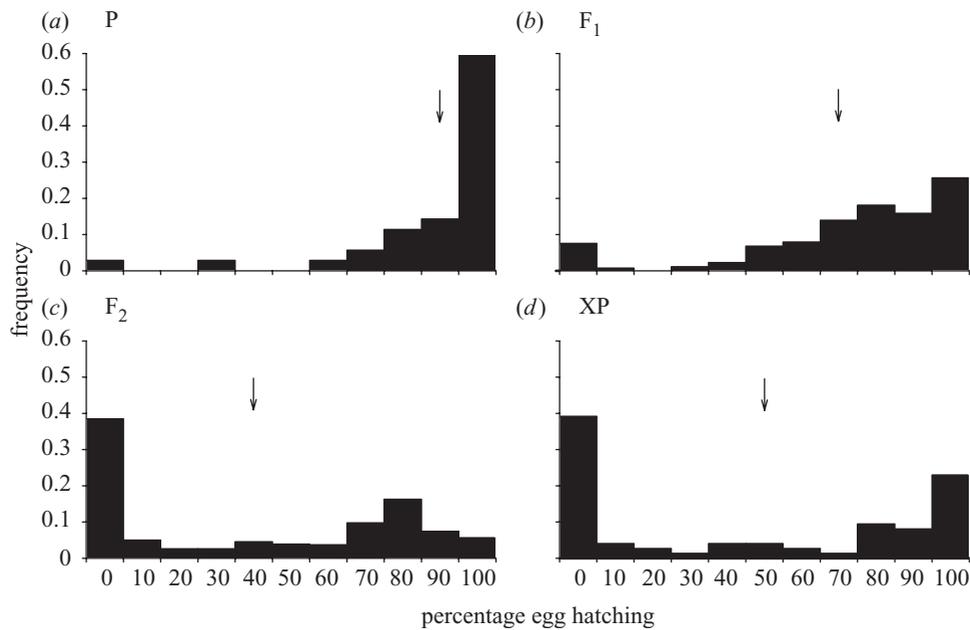


Figure 2. Frequency distributions of the proportion of eggs hatching per mated female in the outbred base population and three classes of daughter lines (see figure 1): (a) outbred base population or P ($n = 35$); (b) F₁ ($n = 265$); (c) F₂ ($n = 692$); and (d) F₂ cross or XP ($n = 74$). Arrows indicate the mean hatch rate.

rather than fertility (see Pavan *et al.* 1951; Temin 1966; Saccheri *et al.* 1996)). The fertility load (S) estimated from P and XP egg hatching distributions, where in XP parents are inbred but their offspring are outcrossed, was 5.47 ± 0.24 . Rather than summing S and V , a more accurate estimate of the total zygote viability and sterility load (T) was obtained from P and F₂ data (8.74 ± 2.76), which is closely similar to the estimate from a subset of the same lines in F₃ (8.80 ± 0.78). By comparison, the range of T in *Drosophila* is 1–2 (Dobzhansky *et al.* 1963; García *et al.* 1994). $T - V$ gives an estimate of S ($8.74 - 1.75 = 6.99$) that should be more representative of the base population, by virtue of the larger sample of lines for F₁ (18 lines) and F₂ (18 lines). Under the assumption that males and females contribute equally and additively to the sterility of non-hatching eggs, S should be interpreted as approximately twice the fertility load for either sex. Assuming that both parents are equally sensitive to inbreeding depression for fertility, this equates to 3.5 sterile equivalents per zygote, irrespective of sex. However, we will show that this assumption is incorrect, and that one should derive sex-specific estimates.

(b) Sex differences in fertility load

Frequency distributions of egg hatching rates for each class of cross in F₃ are shown in figure 3. The striking and quite unexpected result is that the inbred female \times crossbred (i.e. hybrid) male distribution (figure 3d) is statistically indistinguishable from that of the double hybrid cross (figure 3b). These distributions (figure 3b,d) are very similar to the F₁ distribution (figure 2b), and all have average hatching rates of *ca.* 70%. In terms of inbreeding, the only difference between the double hybrid cross (figure 3b) and F₁ cross (figure 2b) is in the inbreeding coefficients of the offspring ($F = 0.125$ and 0.25 respectively). By contrast, the hybrid female \times inbred male distribution (figure 3c) is the same as the double inbred

cross (figure 3a), and both have hatching rates of less than 40%. Thus, while female inbreeding clearly has a minimal effect on egg hatching, *ca.* 70% of the inbreeding depression in *B. anynana* is due to male fertility load.

A direct estimate of the male fertility load was obtained from the difference in slopes (B) between inbred male \times hybrid female cross and inbred female \times hybrid male cross regressions. Given that the female fertility load is negligible and that the average inbreeding coefficient of offspring from both crosses is the same (table 1), this procedure corrects for viability load effects in the inbred male \times hybrid female cross. The estimate is 8.55 ± 1.78 male sterility equivalents per zygote, which is somewhat larger than the estimates obtained from F₁ and F₂ data (which included a larger sample of lines), but with overlapping 95% confidence intervals. We have yet to determine the proximate cause of inbred male sterility in *B. anynana*, but have established that it is not a result of a lack of sperm transfer, at least as far as the spermatophore in the bursa copulatrix (H. Lloyd, unpublished data).

The qualitative difference observed between male and female fertility loads indicates that this species carries recessive alleles which, when homozygous, cause male sterility, but no equivalent recessives affecting female fertility. That male sterility recessives do not cause sterility in females is not surprising considering the differences in egg and sperm development, matching the sex-specificity of sterility (but not inviability) factors in *Drosophila* (Chung 1962; Temin 1966; Lindsley & Tokuyasu 1980, p. 273). The real puzzle of these results is twofold: why is the magnitude of the male fertility load so great, and why, by contrast, is the female fertility load negligible? Under the reasonable assumptions that natural selection acts to maximize the fertility of eggs and sperm, and that mutations affecting these traits arise at approximately the same rate (though mutagenesis experiments in *Drosophila* suggest 50% greater potential for mutation to male than female

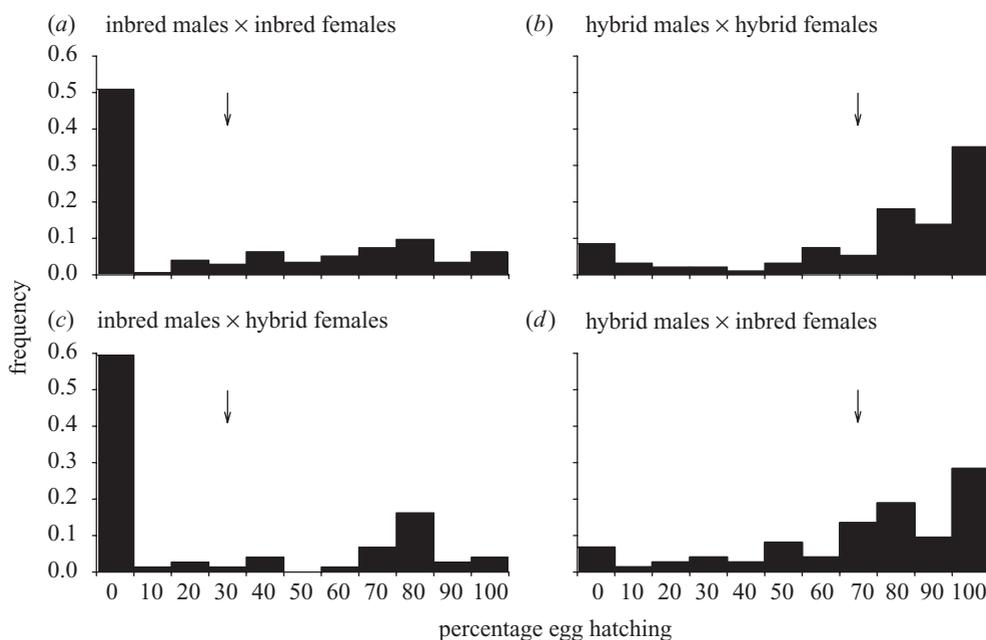


Figure 3. Frequency distributions of the proportion of eggs hatching per mated female in different classes of cross at the F_3 level (see figure 1): (a) inbred males \times inbred females ($n = 175$); (b) hybrid males \times hybrid females ($n = 94$); (c) inbred males \times hybrid females ($n = 74$); (d) hybrid males \times inbred females ($n = 74$). Arrows indicate the mean hatch rate.

sterility (see Lindsley & Tokuyasu 1980, p. 273)), classical evolutionary genetics predicts that male and female fertility loads should be approximately equal (Lynch & Walsh 1998). The much higher number of gametes produced by males than by females is expected to lead to weaker fertility selection per sperm than per egg, as partial infertility will have less impact on male reproductive success. But deleterious mutations inducing sterility of all sperm, such as those detected in *B. anynana*, should be subject to intense selection. Clearly, the standard model used to account for patterns of directional dominance among traits purely in terms of their contribution to fitness, which gives a measure of the influence of directional selection (Crnokrak & Roff 1995), is inadequate here.

There are apparently no other reports of such marked differences in fertility load between the sexes, which in part reflects a lack of appropriate experiments to partition out sex effects. The historical focus of *Drosophila* (Simmons & Crow 1977) and mammalian (Ralls *et al.* 1988) studies on quantifying viability loads has led to effects on fertility being sidelined. Two notable exceptions are Chung (1962) and Temin (1966), showing that, in *D. melanogaster*, chromosome II homozygotes causing complete sterility were two to three times as common for males than females. Also, the ratio of partial to complete male sterility loads in *Drosophila* (0.5) is similar to that in *B. anynana* (0.45, obtained by excluding the zero class). In contrast to our results, however, in *Drosophila* the female fertility load was not negligible and the total fertility load was about half the size of the viability load. Large genetic loads on egg hatching rate are known in several other species of Lepidoptera (Higashiura *et al.* 1999), suggesting that the phenomenon of a large male-biased fertility load may be quite general, though the magnitude of the effect detected in *B. anynana* may represent an extreme case. Indeed, it seems unlikely that it would be exclusive to *B. anynana*, which is by no means

exceptional from a phylogenetic perspective, occupying a central clade within the genus (Monteiro & Pierce 2000).

The peculiarly lepidopteran characteristic of producing anucleated (apyrene) as well as nucleated (eupyrene) sperm (Drummond 1984) is not obviously involved in the high male fertility load. Eupyrene and apyrene sperm counts (Gage & Cook 1994) from spermatophores recovered from eight sterile and four fertile matings revealed no significant differences among sterile and fertile matings in the number of eupyrene or apyrene sperm, or in the eupyrene:apyrene ratio. However, the fact that in Lepidoptera males are the homogametic (ZZ) and females the heterogametic (ZW) sex does suggest a simple though implausible explanation for the lack of an inbreeding effect on female fertility. If all or most of the genes controlling egg fertility were located exclusively on the Z chromosome, mutants would always be expressed and therefore efficiently selected against, and crucially inbreeding would have no effect on the frequency with which they were expressed in the population. Z-linkage of sperm fertility, however, would lead to relatively higher equilibrium frequencies of recessive male sterility mutations and inbreeding depression (see mutation–selection balance predictions in Appendix B). But it seems highly unlikely that in an organism with 28 autosomes (A. van't Hof, unpublished data) all the critical stages of oogenesis should be Z-linked. The alternative is that such genes also occur on autosomes but that normal egg development is somehow sheltered from the effects of mutation of the underlying genes.

The other side of the puzzle is the magnitude and distribution of the male sterility effect. Comparison of the egg hatching distribution for XP (figure 2d), in which parents but not offspring are inbred, with the outbred base population (figure 2a) indicates that most recessives affecting male sterility act in a threshold manner: males are either completely sterile or fully fertile. This distribution of effects and the high variance in the size of the zero hatching class

among inbred lines (table 2) suggests that inbred male sterility is caused by recessives of large effect at relatively few loci. Our observations also indicate a low frequency of each male sterility recessive in the stock population, since male sterility is very low in the outbred base population (figure 2a); however, male sterility recessives must be very common given that out of several tens of independent inbred lines only one has failed to show inbreeding depression for egg hatching. Assuming that homozygosity for a male sterility recessive at one or more loci results in complete sterility, a genetic model with 4–10 loci, each carrying tens of different rare male sterility recessives, is more consistent with our data than a system in which the recessives are distributed over fewer or many more loci, or are individually more common but less diverse (see Appendix A).

A detailed analysis of the underlying genetics is needed to assess whether mutation pressure alone is maintaining these sterility alleles, or whether it is necessary to invoke antagonistic pleiotropy (Charlesworth & Charlesworth 1999). Selection in only one sex retards the elimination of deleterious alleles, leading to higher equilibrium allele frequencies compared with an equivalent bi-sexually selected trait. A simple mutation–selection balance model (see Appendix B) suggests that, while sex-limited selection may well favour higher fertility loads in general, it is insufficient, on its own, to account for the magnitude of the male fertility load in *B. anynana*, or a fertility : viability load ratio of 4 (6.99/1.75)—as opposed to an equivalent ratio of 0.5 for *D. melanogaster* chromosome II (Temin 1966). Certainly in the case of *Bicyclus* this ratio reflects more than differences in the number of mutable genes affecting fertility versus viability, which *Drosophila* data suggest is at least three times greater for viability (Lindsley & Lifschytz 1972). A viability load comparable to that in *Drosophila* further indicates that a higher genomic mutation rate in *B. anynana* is unlikely to be an important factor shaping the male sterility load (as well as the apparent absence of female fertility load).

The *Drosophila* data and our own point to a greater sensitivity of spermatogenesis to mutational disruption than oogenesis, but it is difficult to avoid the conclusion that, additionally, direct or indirect fertility selection must be operating differentially among the sexes to produce such divergent sterility loads. Anecdotally, the magnitude of the genetic load on egg hatching rate in the stock population has declined by ca. 40% over 50 generations in captivity (Saccheri *et al.* 1996), no doubt because of selection against homozygotes in a relatively small laboratory population (ca. 600 adults per generation), but possibly also assisted by the relaxation of selection that somehow favours these alleles in nature.

Our results suggest that to understand the causality and evolutionary dynamics of higher-level genetic phenomena, such as dominance and inbreeding depression, the development and evolutionary history of individual traits in each sex needs to be modelled explicitly. The role of complex selection should become more tractable as large-scale studies of gene expression patterns help direct the analysis of pleiotropy and epistasis. The specific case of male-biased fertility load has several obvious evolutionary implications, including steep declines in the ratio of effective census population sizes with inbreeding (Saccheri *et al.* 1999), slowing the rate of purging (Hedrick 1994; Saccheri *et al.* 1996; Crnokrak & Barrett 2002), and shifting optimal mating strategies (Parker 1983). Its true significance,

however, will only be revealed through an understanding of the factors maintaining it.

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APPENDIX A: GENETIC MODEL FOR INBRED MALE STERILITY

What is the simplest genetic model consistent with the magnitude of the sterile class at different levels of inbreeding? From a series of experiments we have empirical estimates of the proportion of sterile matings at four levels of inbreeding (table 3).

Table 3. Proportion of sterile matings at four levels of inbreeding.

treatment	F^*	average proportion of sterile matings in F_2
outbred stock	0	0.02
three pairs	0.083	0.20
one pair	0.25	0.50
double one pair	0.38	0.75

* Inbreeding coefficient relative to outbred stock.

Under the assumption that homozygosity for a sterility recessive at one or more loci renders a male sterile, we have explored what combinations of allele frequency of sterility recessives (q), number of loci carrying sterility recessives (n), and number of different sterility recessives per locus (A) are most compatible with the data in table 3.

The probability of being homozygous for one or more alleles (Φ) is given by

$$\sum_{r=1}^n p_{\text{hom}}^r p_{\text{not hom}}^{n-r} \frac{n!}{r!(n-r)!}, \quad (\text{A } 1)$$

where p_{hom} , the probability of being homozygous for a sterility recessive, is given by $(q^2(1-F) + qF)A$; $p_{\text{not hom}}$, the probability of not being homozygous for a sterility recessive, is given by $1 - p_{\text{hom}}$; n is the total number of loci carrying sterility recessives; and r is the number of homozygous loci. The last term gives the number of possible combinations for any given n and r .

Table 4 shows series of Φ for five different combinations of q , n and A , which should be compared with the observed proportion of sterile matings in table 3. Values of q larger than 0.03 result in too high a fraction of steriles at zero inbreeding or, with small A , too low a fraction at higher F . This strongly suggests that male sterility recessives are individually rare but numerous. It is more difficult to estimate the number of loci segregating for male sterility recessives, as a large range of n can be made to approximately fit the data in table 3 by varying A . However, large variance in the size of the sterile class among equally inbred lines (see table 2) suggests fewer loci, perhaps less than 10. On developmental grounds it is also unlikely that such trait-specific all

Table 4. The expected probability of being homozygous for one or more male sterility recessives.

	<i>q</i>	0.01	0.04	0.04	0.01	0.01
	<i>n</i>	10	10	10	5	20
<i>F</i>	<i>A</i>	25	5	2	55	15
0.00		0.03	0.08	0.03	0.03	0.03
0.25		0.21	0.21	0.21	0.23	0.24
0.25		0.49	0.44	0.44	0.53	0.54
0.38		0.64	0.57	0.57	0.70	0.70

or nothing sterility could be achieved by mutations at large numbers of loci.

To infer the *minimum* number of loci carrying male sterility recessives, we have considered a population founded by a single unrelated female–male pair. The possible frequencies *q_i* of any allele in this sample of four founding haplotypes are 0.25, 0.5, 0.75 and 1 (assuming autosomal loci). The virtual absence of sterile males in F₁ matings indicates that the frequency of any male sterility recessive in the founding sample of genes must typically be 0.25. Under the extreme assumption that *all* alleles at ‘male sterility loci’ cause sterility when homozygous, how many such loci are required to produce the observed levels of sterility in F₂ of lines founded by a single pair?

The probability of being homozygous for one or more alleles (Φ^*) is given by

$$\sum_{r=1}^n p_{\text{hom}}^r p_{\text{het}}^{n-r} \frac{n!}{r!(n-r)!}, \tag{A 2}$$

where *p_{hom}* is the probability of being homozygous for *any* allele (and is equal to the inbreeding coefficient *F*); *p_{het}* is the probability of being heterozygous (1-*F*); *n* is the total number of loci carrying sterility recessives; and *r* is the number of homozygous loci.

Values of Φ^* for one to five loci (table 5) suggest that even under the extreme assumption of loci almost exclusively carrying large numbers of (rare) male sterility recessives, a minimum of three to four such loci are necessary to produce the levels of sterility observed in single-pair lines. Variance among lines would be a result of the presence of alleles that do not cause sterility in one or more of the founding haplotypes.

APPENDIX B: MUTATION–SELECTION BALANCE

At equilibrium the rates of gain and loss of deleterious mutations are equal. For an autosomal locus selected in both sexes, mutation–selection balance predicts

$$2Npu = 2Npqhs + 2Nq^2s \text{ or } pu = qs(ph + q), \tag{B 1}$$

where *N* is number of diploid individuals; *q* is the frequency of deleterious mutation *a*; *p* is the frequency of the allele that mutates to *a*; *u* is mutation rate to *a*; *h* is the coefficient of dominance; and *s* is selection coefficient against *aa* homozygotes.

The effect of sex-linkage for males and females

In the Lepidoptera, males are homogametic (ZZ) and females are heterogametic (ZW). Thus, we expect Z-linked female-specific mutations (irrespective of their degree of dominance) to be expressed in all females independently of

Table 5. The probability of being homozygous for one or more alleles with increasing numbers of loci in the F₂ of a single-pair line (i.e. *F* = 0.25).

<i>n</i>	Φ^*
1	0.25
2	0.44
3	0.58
4	0.68
5	0.76

the degree of inbreeding, but Z-linked male-specific recessives to be expressed only in homozygotes. Here we explore whether Z-linkage of sex-specific fertility loci could account for the qualitative difference among males and females in the effect of inbreeding on fertility.

For sterile recessives (i.e. *h* = 0 and *s* = 1), mutation–selection balance leads to, for males:

$$\frac{3}{2}Npu = Nq^2s \Rightarrow \hat{q}_{zz} = \sqrt{3u/2}, \tag{B 2}$$

and for females:

$$\frac{3}{2}Npu = \frac{1}{2}Nqs \Rightarrow \hat{q}_{zw} = 3u. \tag{B 3}$$

The equilibrium frequency is higher for Z-linked male specific sterility mutations, but in practice the difference between 3*u* and 3*u*/2 (*q*² for males) in the outbred population would be undetectable.

In order to compare the effects of inbreeding on female and male fertility, we require an estimate of the mutation rate *u* to Z-linked sex-specific sterility alleles that is compatible with the observed frequency of female sterility in the outbred population. Our data indicate that female sterility is rare (less than 1 in 100), and if we further assume that these sterility-inducing alleles can occur at 10 Z-linked loci, substitution into equation (B 3) gives *u* = 1/3000. For the same mutation rate to Z-linked male-sterility alleles at 10 loci, substitution into equation (B 2) gives $\hat{q}_{zz} = \sqrt{1/2000}$ and a frequency of 1 in 200 sterile males in the outbred stock, which is not detectably different from our data. To calculate the expected proportion of sterile males when inbreeding is imposed on this model we substitute into equation (A 1) for *q* = \hat{q}_{zz} , *n* = 10 and *A* = 1. The value obtained for *F* = 0.25 with these parameter estimates is 0.06, an order of magnitude below the observed value. Varying the mutation rate and numbers of loci within the constraints of rare female sterility does not materially alter this result. Thus, Z-linkage could in theory explain the lack of an inbreeding effect on female fertility, but cannot account for the magnitude of the male effect.

Can selection in only one sex account for the very high frequency of male sterility recessives in *Bicyclus anynana*?

For lethal or sterile recessives (i.e. *h* = 0 and *s* = 1):

$$pu = q^2, \text{ and if } p \approx 1 \text{ then } \hat{q} = \sqrt{u}.$$

If selection acts on one sex only (but mutations arise in both sexes):

$$2Npu = Nqs(ph + q) \text{ or } 2pu = qs(ph + q),$$

$$2u = q^2 \text{ and } \hat{q} = \sqrt{2u}.$$

The ratio of the equilibrium frequencies of lethal or sterile recessives in the one-sex and two-sex selection case is $\sqrt{2} = 1.41$. Thus, all other things being equal, we expect a higher mutation load for traits that are selected in only one sex, compared with those selected in both sexes. In reality, this simple prediction is complicated by substantial differences in n , s , h and u between fertility mutations, which are largely under sex-specific selection, and viability mutations, which are mostly selected in both sexes. In any case, male-only selection is not sufficient to account for the male fertility load in *Bicyclus anynana*.

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