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Captivity masks inbreeding effects on male mating success in butterflies

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Small isolated populations are frequently genetically less diverse than core populations, resulting in higher homozygosity that can hamper their long-term survival[1–4]. The decrease in fitness of organisms owing to matings between relatives is well known from captive and laboratory animals. Such inbreeding can have strongly deleterious effects on life-history traits and survival[5–11], and can be critical to the success of population conservation[2,4,12]. Because pedigrees are hard to follow in the wild, most field studies have used marker loci to establish that fitness declines with increasing homozygosity[1,13,14]. Very few have experimentally explored the effects of inbreeding in the wild[15], or compared observations in the laboratory with field conditions[8,9]. Here, using a technique involving the transfer of marker dusts during copulation, we show that a small decrease in mating success of captive inbred male butterflies in cages is greatly accentuated in conditions with unconstrained flight. Our results have important implications for conservation and for studies of sexual selection because they show that the behaviours underlying patterns of mating can be profoundly influenced by a history of inbreeding or by any restraining experimental conditions.

Inbreeding depression in insects has been studied mainly in early life stages, especially with regard to egg-hatching and larval survival[7,8], and the issue of how behavioural characters are affected by inbreeding is largely unknown[16,17]. Yet mating behaviour and courtship, with choosy females selecting for honest signalling by males, together with intense male–male competition, are likely to have central roles in variation in lifetime reproductive success[9,14,18], and could therefore represent an important part of the genetic load of populations in the wild.

Populations of the small African butterfly *Bicyclus anynana* have a high genetic load, especially for egg-hatching rates[6,10]. The expression of inbreeding depression is important in determining the patterns of diversity in laboratory metapopulations[10]. Here we explore how the dynamics of inbreeding can directly influence pairings in a local population by performing mate-competition experiments, both as mating trials based on the choice of single females in the laboratory and at the population level in less constrained flight conditions in a large tropical greenhouse.

From the same outcrossed population, we first derived replicate groups of males of synchronized adult emergence with one of three inbreeding coefficients, $F = 0$ (F_0), $F = 0.25$ ($F_{0.25}$) and $F = 0.375$ ($F_{0.375}$). We then performed replicated mating trials in small cages in the laboratory, each involving competition between three males for an unmated, outbred female. Outbred (F_0) males showed a weak but repeatable advantage in mating success over either class of inbred male. This was significant when the inbred males were pooled ($G = 4.83$, d.f. $= 1$, $P = 0.027$; Fig. 1). Individual mating success in these constrained, artificial conditions was therefore dependent on the inbreeding history of the males. However, although we observed rejection behaviour by females, it is unclear whether they are able to assess the male's inbreeding coefficient as such. No difference in mating success was observed between the two levels of inbred males (Fig. 1).

Such mating trials enable the study of individual mating success, but the small volume of the cages is unlikely to allow the full expression of courtship characters. The courtship of *B. anynana*

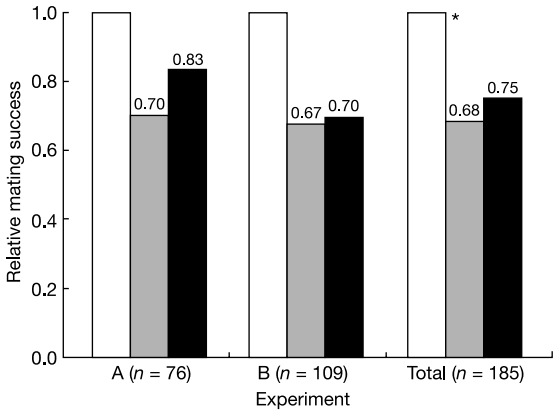

Figure 1 Mating success of inbred males relative to that of outbred males in laboratory trials. Two rounds of experiments, A and B, were performed with butterflies drawn at random from different sets of independent lines: seven F_0 lines (white bars), seven $F_{0.25}$ lines (grey bars) and six $F_{0.375}$ lines (black bars) were used in experiment A, and nine F_0, six $F_{0.25}$ and six $F_{0.375}$ lines in experiment B. There was no detected line effect on mating success (Supplementary Information). F_0 male mating success is set at 1. The total number of matings (n) is given for each experiment. G-test significance: asterisk, $P < 0.05$.

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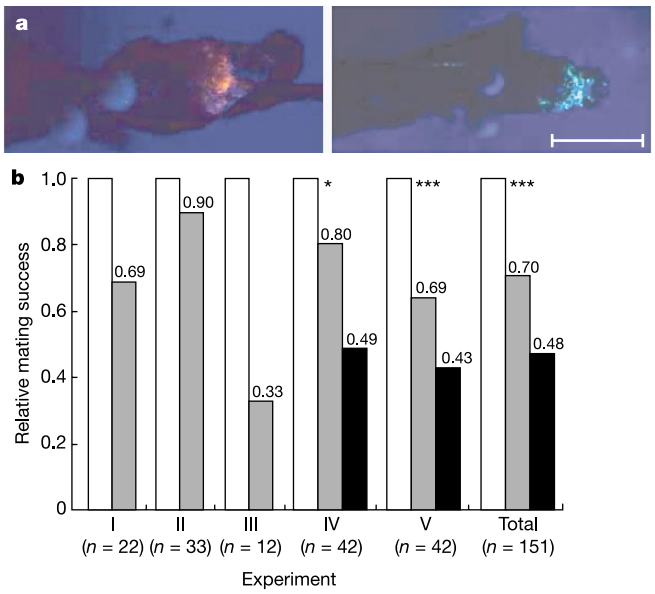

Figure 2 Mating success of inbred and outbred males in free-flight conditions. **a**, Ventral abdominal tips of mated females showing transferred orange (left) and green (right) fluorescence under ultraviolet illumination. Scale bar, ~3 mm. **b**, Mating success of inbred males relative to that of outbred males (F_0, set at 1), standardized by the relative disappearance of males. Five replicate experiments, I to V, were performed using butterflies from 15 F_0 and 14 $F_{0.25}$ independent lines in experiments I–III, and from 15 F_0, 12 $F_{0.25}$ and 6 $F_{0.375}$ lines in experiments IV and V. No $F_{0.375}$ males were released in experiments I–III. *G*-test significance: asterisk, $P < 0.05$; three asterisks, $P < 10^{-4}$. White bars, F_0 lines; grey bars, $F_{0.25}$ lines; black bars, $F_{0.375}$ lines.

involves a perch-and-chase strategy[19,20]. Males take off from chosen perches to investigate any flying object they detect. Females are then pursued, such chases being interrupted by repeated alightings. Close-range courtship signals then come into play, involving flickering of the male's wings and the release of pheromones from exposed androconia, followed by attempts of the male to latch onto the female's abdomen[19]. In small cages, the opportunity for 'normal' mate detection and chase are effectively absent; courtship can often be initiated by chance encounters, and females might also be unable to reject copulation attempts.

We therefore performed large-scale experiments in semi-natural conditions, allowing free flight and a fuller expression of mate location and courtship behaviour, by releasing temporary populations of butterflies in a spacious tropical greenhouse. The butterflies behaved naturally in a vegetation structure and at an adult density comparable to many local field populations[20]; perching behaviour and male–male interactions seemed to us closely comparable to those in the field. Equal numbers of males of differing inbreeding coefficients were used in each experiment. Males had their genitalia dusted with fluorescent dusts, a different colour being used for each inbreeding level, before being released and allowed to establish themselves in the vegetation of the greenhouse. They then competed over a period of 36–48 h for about half as many virgin females. Dust was transferred to the female's abdomen during mating; after recapture this enabled the identification of the class of male with which each female had mated (Fig. 2a, Table 1) (occasional double matings were detected). Again, outbred males showed a higher rate of mating than either class of inbred male (F_0:$F_{0.25}$ comparison, $G = 8.91$, d.f. $= 1$, $P < 2 \times 10^{-3}$; F_0:$F_{0.375}$ comparison, $G = 18.6$, d.f. $= 1$, $P < 2 \times 10^{-5}$). Highly inbred males achieved only 14% of all matings, representing a mating success about one-half that of outbred males. Males of the intermediate level of inbreeding had an intermediate success equivalent to 70% of outbred males (Fig. 2b; overall F_0:$F_{0.25}$:$F_{0.375}$ comparison, $G = 21.36$, d.f. $= 2$, $P < 2 \times 10^{-5}$). More interestingly, the deleterious effect of inbreeding on mating success was, overall, significantly stronger in free-flight conditions than in the laboratory ($\chi^2 = 9.36$, d.f. $= 2$, $P < 0.01$), strongly suggesting that the cages allowed only a partial estimation of inbreeding depression.

Outbred males were recaptured in higher numbers than inbred males at the end of the experiments (Table 1). Although the difference is not significant, the trend could contribute to the decreased net mating success of inbred males. Nevertheless, inbred butterflies still mated significantly less often when the relative loss of males was controlled for (pooled data: $G = 8.52$, d.f. $= 2$, $P = 0.014$). Thus, the slightly faster disappearance of inbred males during the experiments (due to effects on longevity[9,10,15,16], escape or lethargy[16,18]) cannot fully explain the decreased mating success in free-flying *B. anynana*; at least 60% of the estimated genetic load can be accounted for by differences in mating ability, excluding survivorship (Table 2). Inbreeding must therefore affect other traits that are expressed, or detected, only in free-flying conditions. The genetic load estimated for male mating success in the greenhouse (Table 2) is comparable to that for egg hatching (2.70 lethal equivalents per gamete in ref. 10).

Table 1 **Female mating and male recapture in greenhouse experiments**

	Experiment					
Parameter	I	II	III	IV	V	Total
Test	F_0:$F_{0.25}$	F_0:$F_{0.25}$	F_0:$F_{0.25}$	F_0:$F_{0.25}$:$F_{0.375}$	F_0:$F_{0.25}$:$F_{0.375}$	F_0:$F_{0.25}$:$F_{0.375}$
Dust colour	O:G	O:G	O:G	G:O:Y	G:O:Y	
Females (F_0)						
Released	50	72	50	90	90	352
Recaptured (%)	44.0	48.6	24.0	56.7	56.7	48.6
Mated with						
F_0	13	17	9	20	25	84
$F_{0.25}$	6	14	3	14	12	49
$F_{0.375}$	–	–	–	7	5	12
$F_0 + F_{0.25}$	3	2	0	1	0	6
Unmated	0	2	0	9	9	20
Males						
Released	47:47	59:59	48:48	60:60:60	60:60:60	274:274:120
Recaptured (%)	44.7	44.1	29.2	47.2	34.4	40.3
F_0	24	27	14	33	28	126
$F_{0.25}$	18	25	14	29	21	107
$F_{0.375}$	–	–	–	23	13	36

For males, the figures are absolute numbers after recapture for each inbreeding coefficient. For females (all $F = 0$), the figures are the numbers of recaptured females that mated with each *F*-class of male. Dust colours were orange (O), green (G) and yellow (Y) (see Fig. 2a). Overall recapture rates (%) are given for each experiment.

Table 2 **Genetic load for mating success in caged and free-flying male butterflies**

Experiment	Comparison	Genetic load	s.e.	*P*
Trials in captivity	F_0:$F_{0.25}$	1.50 (1.17,1.83)	0.07	0.002
	F_0:$F_{0.25}$:$F_{0.375}$	0.83 (−0.03,1.70)	0.31	0.055
Free-flight experiments	F_0:$F_{0.25}$ (I–III)	2.58 (0.41,4.76)	0.78	0.029
		2.10 (−0.71,4.91)	1.01	0.107
	F_0:$F_{0.25}$:$F_{0.375}$ (IV, V)	3.37 (1.53,5.21)	0.66	0.006
		1.96 (0.57,3.36)	0.50	0.017
	Total (I–V)	2.75 (0.92, 4.59)	0.82	0.007
		1.73 (0.49,2.98)	0.55	0.010

Lethal equivalents per gamete are estimated from the slope of the regression of the natural-log-transformed mating success on the inbreeding coefficient, for the given classes of inbreeding and experiments[10,30]. The mating success in cage experiments is calculated on the basis of the proportion of matings in each replicate experiment. The mating success in greenhouse (free-flight) experiments is calculated as the number of matings divided (in the upper line of each pair) by the number of males at release or (in the lower line) after recapture. Lower and upper 95% confidence interval boundaries are given in parentheses. Note the wider confidence intervals for greenhouse estimates; these are likely to result from additional sources of variation, such as the amount of sunshine, inherent in more natural experimental conditions.

The marked decrease in mating success in male *B. anynana* due to inbreeding could have different causes. Disruption of close-range courtship signals (such as pheromone profiles) could act both in captivity and in the semi-natural conditions. However, other components of male mating success are likely to be expressed only or mainly in free-flying populations. Locomotor or athletic abilities strongly affect male mating success in some inbred *Drosophila* lines[16,18], and these effects could also be important in our greenhouse experiments. They could, for example, affect the alertness and responsiveness of perching males, their persistence in chasing females once courtship is initiated, and the outcome of male–male interactions, which are all expected to lead to an overall lower mating of inbred males. The apparent genotype–environment interaction results mainly from the poor performance of the most inbred males in the greenhouse (Figs 1 and 2). Although the use of more classes of inbreeding would be needed to confirm this interaction, it could result from different behavioural characters showing inbreeding depression at different levels of inbreeding in combination with conditions under which characters are expressed differentially[3,7].

Inbreeding can influence the survival of populations[2,3], for example through a direct effect on individual survival[4]. The poor mating vigour of inbred males in *B. anynana* indicates that the fragmentation of populations with a high genetic load[6] might lead to a general decrease in mating activity. Outbred immigrants into small, isolated populations would then enjoy an immediate mating advantage, thereby enhancing the heterosis-assisted[21,22] restoration of mean population fitness unless offset by ecological effects or outbreeding depression[23]. If, as is likely, long-distance migrants are more outbred, this process could be important in the frequency-dependent maintenance of genetic diversity at a regional scale in fragmented landscapes[24].

Our results highlight the necessity for a field validation of behavioural data from captive animals, especially those involving interactions and signalling, as in mate choice experiments (such as ref. 19). Captivity constrains behavioural responses and may truncate the expression of key courtship characters. Similarly, estimates of inbreeding depression in invertebrates are taken almost exclusively from laboratory experiments[1,3,6,16]. However, these are likely to be severe underestimates, particularly when stressful field conditions[15,25] or relaxed constraints on the expression of behavioural traits[9], as in our study, magnify inbreeding depression (but see ref. 8 for a case in which it is not magnified). A low mating success of inbred males, as occurred in our greenhouse experiments, could directly affect the success of reintroduction programmes from captive stock[26]. Conserving genetic diversity through such programmes might therefore be a risky enterprise unless backed up by observations from the wild[12].

The technique we have developed here for comparing the mating success of different cohorts of animals is widely applicable to many issues in sexual selection and mate choice[19,27], and could be used in natural environments. It could also be combined with additional studies for key individuals, for example to examine variation in mating success within cohorts[28], or to determine the outcome of sperm competition for double-mated females[27]. Such approaches would reveal more about the proximal reasons for differences in mating patterns and reproductive success, as well as the consequences of these differences. □

Methods

Inbred and outbred lines

An outbred stock of *B. anynana* from Malawi has been maintained with high genetic diversity in the laboratory for more than 100 generations; the adult population size is several hundred individuals with an effective population size ~60% of the total population[28]. Mating pairs were drawn from this stock and their progeny were reared at 27 °C, giving independent lines with an inbreeding coefficient $F \approx 0$. From these F_0 lines, brother–sister mating pairs were drawn, giving lines with $F = 0.25$. Again, from these $F_{0.25}$ lines, brother–sister mating pairs were drawn, giving lines with $F = 0.375$. Independent F_0, $F_{0.25}$ and $F_{0.375}$ lines were available simultaneously after three generations; all replicate experiments involved, for each class of males, individuals taken from 6–15 synchronous independent lines (see Figs 1 and 2; Supplementary Information). Larvae were reared with an excess of food in sleeves containing about 30 larvae each; inbred and outbred lines were interspersed in the rearing chamber. Rearing details are described elsewhere[28].

Cage trials

Each trial involved competition between three males, one of each inbreeding class, for a single unrelated, outbred female, in a 12 × 20 × 28 cm^3 mesh cage, at 27 °C. Virgin males 3–5 days old were introduced into each cage in the afternoon and left to settle overnight; about 60 min after the lights had been turned on the next morning, a single 2-day-old virgin outbred female ($F = 0$) was introduced, followed by monitoring of matings every 5–10 min. Mating in *B. anynana* lasts on average ~30 min (Supplementary Information). Butterflies from cages in which mating had occurred were not used again. If no mating had occurred after 120 min, the unmated female was discarded, whereas males were fed and tested on the following day with a new virgin female (but there was then no further re-use of males). Two replicate experiments were conducted with different sets of lines: 76 and 109 matings were recorded in 100 and 120 trials, respectively (Fig. 1).

Free-flight experiments

We treated the genitalia of 3–7-day-old virgin males with coloured 'rodent-tracking' fluorescent dust, using a different colour for each inbreeding class. Males were dusted on the same morning and released at midday in a greenhouse that provided a 15 × 15-m^2 flight area with a temperature of 24–32 °C, high humidity and tropical vegetation. A high and more open space covered a central, circular pond around which rotten fruit was placed as a food source to give an environment comparable to local habitat patches characterized by a high adult density in the wild[20]. Males were left to adapt and interact with one another. On the following morning, 2–5-day-old outbred ($F = 0$) virgin females were released, in numbers corresponding to a ~3:1 male:female ratio at release (Table 1; using $F = 0.25$ females gave similar results; see Supplementary Information). A second group of females, about half the size of the first group, was released 1 day later. All butterflies were netted 1.5 days after the last group of females was released, until no more butterflies were found. This procedure resulted in a high mating rate of recaptured females (88%) while ensuring a relatively high competition for pairings between males. Note that virgin females are very rare in the field[20], and the ratio of males to receptive females used here is probably substantially lower than occurs in nature. Dust is consistently transferred to the female's genitalia and abdominal folds during mating, and pilot experiments showed no effect of dust-colour on mating success (Supplementary Information). As an internal control for any possible effect, we switched colours used for F_0 and $F_{0.25}$ males between experiments (Table 1). Recaptured females were inspected for fluorescence with a binocular microscope under ultraviolet illumination to record the class of male(s) they had mated with. Colours were chosen so that double fluorescence could be detected and scored unequivocally. Recaptured males were also scored to monitor survivorship. Five experiments were performed sequentially (Table 1).

Statistical analyses

Significance was tested by using *G*-tests[29]. Laboratory mating distributions were compared with 1:1:1 or 1:(1 + 1) distributions. For free-flight experiments, replicates I–III were compared with a 1:1 distribution (two classes of males), and replicates IV and V with a 1:1:1 distribution (three classes). Detected double matings (that is, those involving males of differing inbreeding coefficients) were scored as 0.5 for each class of male. Although all replicates involved males in even numbers, the total is uneven because $F_{0.375}$ males were not always used. The overall distribution of matings was therefore compared with the actual distribution of released males: 274:274:120 (F_0:$F_{0.25}$:$F_{0.375}$). To correct for male disappearance, we compared the distribution of matings with the distribution of males after recapture, for example 126:107:36 (F_0:$F_{0.25}$:$F_{0.375}$) for the overall data set (Table 1).

We tested for a stronger effect of inbreeding in the greenhouse versus cages by using a 2 × 3 (treatments × inbreeding classes) heterogeneity test with a Williams' correction[29] (experiments IV and V only).

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Dosage sensitivity and the evolution of gene families in yeast

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According to what we term the balance hypothesis, an imbalance in the concentration of the subcomponents of a protein–protein complex can be deleterious[1]. If so, there are two consequences: first, both underexpression and overexpression of protein complex subunits should lower fitness, and second, the accuracy of transcriptional co-regulation of subunits should reflect the deleterious consequences of imbalance. Here we show that all these predictions are upheld in yeast (*Saccharomyces cerevisiae*). This supports the hypothesis[2,3] that dominance is a by-product of physiology and metabolism rather than the result of selection to mask the deleterious effects of mutations. Beyond this, single-gene duplication of protein subunits is expected to be harmful, as this, too, leads to imbalance. As then expected, we find that members of large gene families are rarely involved in complexes. The balance hypothesis therefore provides a single theoretical framework for understanding components both of dominance and of gene family size.

About 30% of yeast genes code for proteins that are involved in annotated (experimentally confirmed) protein complexes[4]. Consider a complex formed by the binding of proteins A and B. There are numerous reasons[1] why an excess of A, for example, might be deleterious: A could form homodimers with a function different from that of the AB heterodimer[5], it might be a regulatory subunit that competes with other regulatory subunits to bind the catalytic subunit B (ref. 6), it might be toxic by binding irreversibly to targets where AB should bind normally[7], or it could form toxic precipitates[8]. Additionally, subunits forming a bridge between parts of a complex can inhibit complex assembly if present in excess[1,9] (Supplementary Information).

If imbalance were deleterious[1] (the balance hypothesis) we would expect adaptations to minimize the degree of imbalance. Rapid degradation of unassembled ribosomal subunits[10] is likely to be one of these. The balance hypothesis also predicts that a greater decrease in fitness should be seen in cells that are heterozygotes for knockouts of single genes if the gene is involved in a complex than if it is not. A systematic mutagenesis experiment[11] allows comparison of the dosage sensitivity of many genes. For nearly all single-gene deletions in the yeast genome the growth rates of heterozygous and homozygous diploid strains are known[11]. To minimize any measurement biases we consider only essential genes (lethal homozygote deletion). The decrease in mean fitness of heterozygotes compared with the wild type is 5%, and only a few knockouts in essential genes have a large effect on fitness (for distribution see Supplementary Information). To test whether dosage-sensitive genes are more likely to be involved in protein complexes, we used an annotated list of known complexes in yeast[4]. Unfortunately, the list is not complete and might be biased, so an extended set of protein interactions was also used (Supplementary Information).

As predicted, genes with low heterozygote fitness tend to be in complexes: genes with less than 5% fitness deficiency constitute 52% of the 816 proteins investigated, but only 37% of them are involved in protein complexes, whereas of those with high fitness deficiency (more than 15%), more than 88% of them are known to interact with other proteins (Fig. 1). This implies that dosage-sensitive genes are at least twice as likely to be involved in protein