

Effective population size, reproductive success and sperm precedence in the butterfly, *Bicyclus anynana*, in captivity

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Abstract

A pedigree approach is used to estimate the effective population size in two population cages of the butterfly, *Bicyclus anynana*. Each cage was founded with 54 individually marked adults of each sex. Matings were recorded over a 3-day period. Eggs were then collected from each female over a similar period before the numbers of hatching larvae were counted to assess progeny number. The males showed a higher variance in reproductive success than the females. Since about one-quarter of all females mated more than once, we also examined the pattern of sperm precedence using molecular markers or, in separate crossing experiments, wing pattern mutants. Both instances of complete first and last male sperm precedence, as well as of sperm mixing, were found. In some crosses a 'leakiness' was found in which some of the early eggs laid by a female were fertilized by a male partner which was subsequently completely unsuccessful. However, the estimates of effective population size were largely unaffected by the pattern of sperm precedence. Estimates for $N_e : N$ in each cage were close to 0.60. The possibility of obtaining comparable estimates in selected natural populations of butterflies is discussed.

Introduction

Captive populations provide a primary resource in many reintroduction programmes, including those involving a variety of butterfly species which have recently become extinct in particular regions or countries (e.g. Herms *et al.*, 1996; Witkowski *et al.*, 1997; Nicholls & Pullin, 2000). Information about effective population sizes is important to such programmes so that, if necessary, measures can be taken to minimize the rate of loss of genetic variation due to genetic drift. It may, for example, be possible to manage the populations to increase the proportion of breeding individuals and thus to reduce the rate of loss of genetic variation whilst in captivity.

The rate of loss of genetic variation from a closed population due to random genetic drift is determined by its effective size N_e . This is the number of individuals in an ideal population that would have the same genetic properties (in terms of random genetic drift) as an actual population with its own complicated pattern of variance in family size, sex ratio, etc. (Lande & Barrowclough, 1987). We have used laboratory stocks of the tropical butterfly *Bicyclus anynana* to make extensive genetical studies using artificial selection (see Brakefield, 1998; Brakefield & French, 1999). The extent to which genetic drift will contribute to divergence of selected lines will depend on N_e in these populations.

The present study uses a pedigree approach adapted from the experiments of Van Oosterhout *et al.* (2000) to provide some direct estimates of N_e for captive populations of this butterfly of known census size. We also discuss how the methodology we have used in the laboratory could be applied to certain natural populations of butterfly. There are few direct estimates of N_e for natural populations making generalizations about N_e difficult to establish for any species or group of organisms (e.g. Nunney & Elam, 1994; Frankham, 1995). Indirect

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estimates from analyses of fixation indices and variances in allele frequencies are always open to several sources of potential error. They are based on assumptions or interpretations about population, fitness and migration parameters, all of which may, furthermore, change over time. It is generally considered that N_e can be much smaller than the census size of a population, N . The issue of how N_e relates to census size is particularly relevant in conservation genetics and the management of both natural and captive populations but it remains controversial (see Mace & Lande, 1991; Nunney & Campbell, 1993; Frankham, 1995, 1996). If N_e is generally substantially lower than N in populations of concern to conservationists, extra attention from the genetic perspective will be necessary even when census numbers are hundreds or even thousands of individuals, especially if the time horizon is long (see Lande, 1995).

One important component of variation in reproductive success, and hence in N_e , is male mating success. We have observed that females will remate in our laboratory populations of *B. anynana* (e.g. Saccheri & Bruford, 1993). Multiple spermatophores also occurred in about one-third of females in a sample collected in Malawi (Brakefield & Reitsma, 1991). Although in many polyandrous species of lepidoptera the last male partner tends to fertilize the subsequent eggs laid by a female (see Walker, 1980; Watt *et al.*, 1985), in other species more complex patterns of paternity are found (e.g. Wedell & Cook, 1998). Since in the presence of multiple mating by females, the variance in male reproductive success will depend on the pattern of sperm competition we have also used crossing experiments in combination with molecular markers or visible genetic polymorphisms to examine patterns of paternity in *B. anynana*.

Materials and methods

The butterflies

Our outcrossed stock of *B. anynana* was founded from over 80 gravid females collected at a single locality in Malawi in 1988. It is maintained at a census size of several hundred adults in each generation. Adults feed on mashed banana and larvae on maize plants.

Butterflies for the following experiments were reared in climate rooms at 27 °C and high humidity. We used only virgin, young butterflies (females 2–4 days old; males 2–7 days – males can live and reproduce for over 100 days but freshly eclosed butterflies do not readily mate). Two pure-breeding mutant lines were also used in experiments on sperm competition. One line is homozygous for the recessive *yellow* mutant (pupal colour) and the dominant *Spotty* mutant specifying two additional forewing eyespots (Brakefield & French, 1993). The other line is fixed for the recessive *melanine* allele yielding dark, ebony-coloured adults (previously unpublished).

Effective population size

Two replicate mating cages (I and II) were used for the N_e experiment. Each cage was a hanging cylinder of black cotton netting (30 cm diameter). Butterflies of each sex from the outcrossed stock were separated at eclosion and marked with individual numbers on their ventral wings. These numbers could be read on butterflies *in copula* without disturbing them. When sufficient butterflies were available, 54 males and 54 females were released together in each mating cage at lights on day 0. This adult density is typical for our experiments, especially those involving artificial selection. Adult food, but no egg-laying plant, was freely available in each cage. During the periods of light (each 9 h long) in the following 3 days the cages were inspected for new matings at least every 15 min. Under similar conditions, the time spent *in copula* averaged 31.3 min with a minimum of 15 min ($N = 111$; SD = 10.0). Courtship tended to peak towards the beginning and end of each day. Many matings occurred in the first hour of the experiment during which each cage was continuously monitored. Thereafter, notes were kept on those pairings of comparatively long duration.

Males were removed from the two cages at the end of day 3. After one further day, all females were placed individually in egg-laying pots containing adult food and a grass cutting for oviposition (males were stored at –80 °C). Eggs laid over the next 3 days were allowed to hatch after removal and storage of the female parent. Larvae were then counted on day 12 to yield estimates of progeny number. Any infertile or unhatched eggs were thus excluded from estimates of N_e .

In an additional experiment, the number of eggs an individual female lays during her first 3 days of oviposition was found to be well correlated to her lifetime egg production (Fig. 1a; the comparable correlation coefficient based on numbers of hatching larvae for the same set of 19 females was 0.80). On average, a female lays 29% of her total eggs over the first 3 days of oviposition. Furthermore, observations from another experiment in which families were raised in separate cages (excluding very high larval densities), indicate that numbers of hatching larvae are highly correlated with those of eclosing adults (Fig. 1b). These additional data sets demonstrate that our experimental data for early, partial fecundity of individuals in the parental generation provide good predictions of their lifetime reproductive success under our laboratory conditions.

Patterns of paternity and sperm precedence

Initial observations on sperm precedence were made by rearing offspring from females in the N_e experiment which had mated more than once. The adult offspring together with female parent and her mating partners were analysed by cellulose–acetate gel electrophoresis to

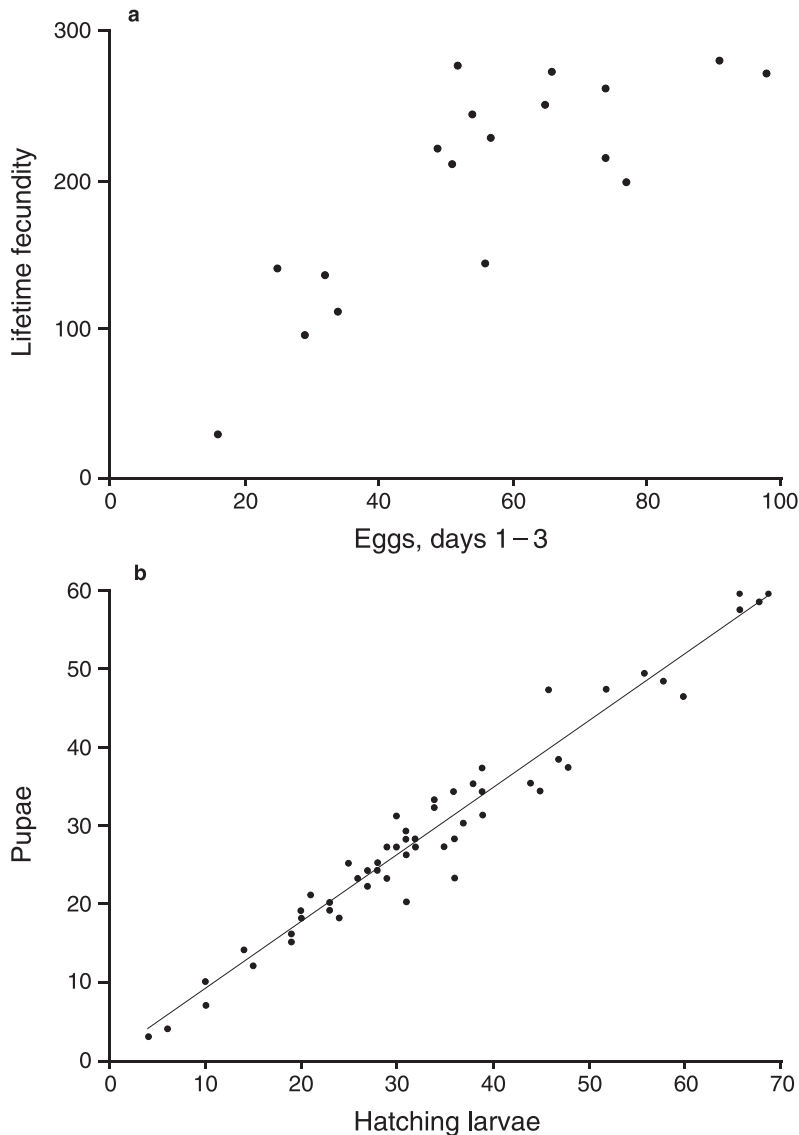


Fig. 1 (a) The relationship between the number of eggs laid by individual females of *Bicyclus anynana* over the first 3 days of oviposition and the total number of eggs laid during their lifetime. Mating and oviposition were carried out as in the main experiments. The correlation coefficient is 0.82. (b) The relationship between number of hatching larvae and individuals surviving to pupation for 56 families from the outcrossed stock. Eggs were laid over a single period of 12 h and larvae were reared in individual sleeves at 27 °C. The linear regression line equation is: $Y = 0.475 + 0.843X$ with a correlation coefficient of 0.98.

survey seven polymorphic enzyme markers (see Saccheri, 1995).

The application of the molecular markers demonstrated variation in the outcome of sperm competition. Therefore, two additional experiments (1 and 2) were performed using visible markers to examine whether the pattern of paternity was stable through the period of oviposition and to obtain more extensive data on the relative frequency of the different outcomes of sperm competition. Mating cages were initially set up as above and with comparable adult densities. Females from one of the mutant stocks were mated with two different males, one from the outcrossed, wild-type stock and the other from the same mutant stock. Both directions of the cross were made in approximately equal numbers to yield the reciprocal mating orders. An excess of males

was used in the cages for second matings to try to maximize their number. Experiments 1 and 2 used the *yellow-spotty* and *melanine* stocks, respectively. Neither of these stocks has been found to have an effect on pre-adult viability (although mean developmental time is longer for both *yellow* and *melanine* homozygotes, data from this study). Females were separated after the second mating and their offspring raised. In the crosses obtained from these experiments, only offspring sired by the mutant male would have the mutant pupal or adult phenotype.

Females in expt 1 mated with a virgin male on day 1 and then for a second time on day 3 or 4. A limited availability of virgin males led to the use in this experiment of second male partners which had already mated once (but 2 or 3 days earlier). A low proportion of

double-mated females was obtained probably because females were isolated from males from the end of mating on day 1 until day 3. This may then have resulted in females being subsequently less willing to mate, or more effective in rejecting courting males. Because of the comparatively small number of successful double matings, we reared more offspring from each female to examine whether sperm precedence remained stable through time after mating. We collected from each female four groups of eggs laid in consecutive periods. The first three periods were 3–5 days in length whilst the fourth extended until female death or 21 days by which time oviposition had usually ceased.

Experiment 2 involved only virgin males. In addition, second matings were allowed to occur immediately after the first copulation. Eggs from the first 2–3 days of oviposition were not reared because the results of expt 1 showed that paternity could change after the early eggs had been laid. Those from the next 5–7 days were reared. A selection of the second male partners in this experiment were tested for fertility by making a repeat mating to a virgin wild-type female and checking for egg hatching.

Estimates of effective population size

Lande & Barrowclough (1987) provide expressions to estimate N_e for a discrete generation of a single panmictic population given data on numbers of males and females and the variance in progeny numbers. Separate estimates for N_e for animals of each sex in a parental population of size N are obtained from

$$(N \cdot \bar{k} - 1) / [\bar{k} + (V_k / \bar{k}) - 1]$$

where \bar{k} is average progeny per male or female (in our experiment, the number of hatching larvae), and V_k the variance in the progeny number among males or females. These estimates are then combined according to

$$N_e = 4[(1/N_{e,\text{males}}) + (1/N_{e,\text{females}})]^{-1}.$$

Results

Effective population size

Mating history in females

Over one-quarter of females were observed to mate more than once (Table 1). Ten females laid no fertile eggs (including two with no eggs). Five of the six females not observed to mate nevertheless produced fertile eggs (these females are excluded from analysis of male reproductive success unless stated otherwise). Some copulations may have been shorter than 15 min or, more likely, matings also occurred in darkness. Observations of inactivity in cages at 'night' suggested that this must be rare. However, pairing can occur at night in the laboratory since a low proportion of fertile eggs was

Table 1 Variation in the observed number of mating partners obtained by the males and females in the two captive populations of *B. anynana*.

Cage	No. of mating partners				
	0	1	2	3	4/5
(A) Females:					
I	4	36	10	3	1*
II	2	37	10	5	0
(B) Males:					
I	11	27	10	6	0
II	7	28	13	6	0

*Single female mated five times but died in the mating cage before laying any eggs; these matings are excluded from the data given here for males in cage I and from all other analyses of mating success.

obtained after a large group of highly receptive virgin females had been mixed with unmated males only over a single period of darkness.

Of the total of 137 matings observed in both cages, 94 were on day 1 (57 in the first hour), with 21 and 22 on days 2 and 3, respectively. Eighty-nine of the 102 mated females were observed to pair on day 1, with only two first matings on day 3. Five males mated for the first time on day 3.

Mating success of males

Table 1 shows the variation in the observed number of female partners obtained by males. The data are similar for each cage. Only 18 of the 108 males were not observed to mate. In contrast, about one-third of males obtained two or more mates. Males show a higher variance in observed numbers of copulations than females (Levene's Test Statistic = 5.49; $P = 0.02$; using data pooled across cages after testing for homogeneity).

Males could be highly successful in matings made on consecutive days (paternity demonstrated by electrophoresis or assumed because no other potential male parents were observed *in copula*). There were also 17 males which obtained repeat matings on day 1. Both pairings were successful for at least six of these males (one male also mated successfully for a third time on day 2). All such second matings involved very long copulations (even extending overnight in one case). This suggests that the males needed time before they could form and transfer a second spermatophore. The extended mating time may thus reflect a period of mate guarding before sperm is transferred.

Estimates of effective population size

Figure 2 shows the variation in progeny number at the early larval stage for the 54 females and males in each experimental mating cage (assuming complete last male sperm precedence). Variance in reproductive success is

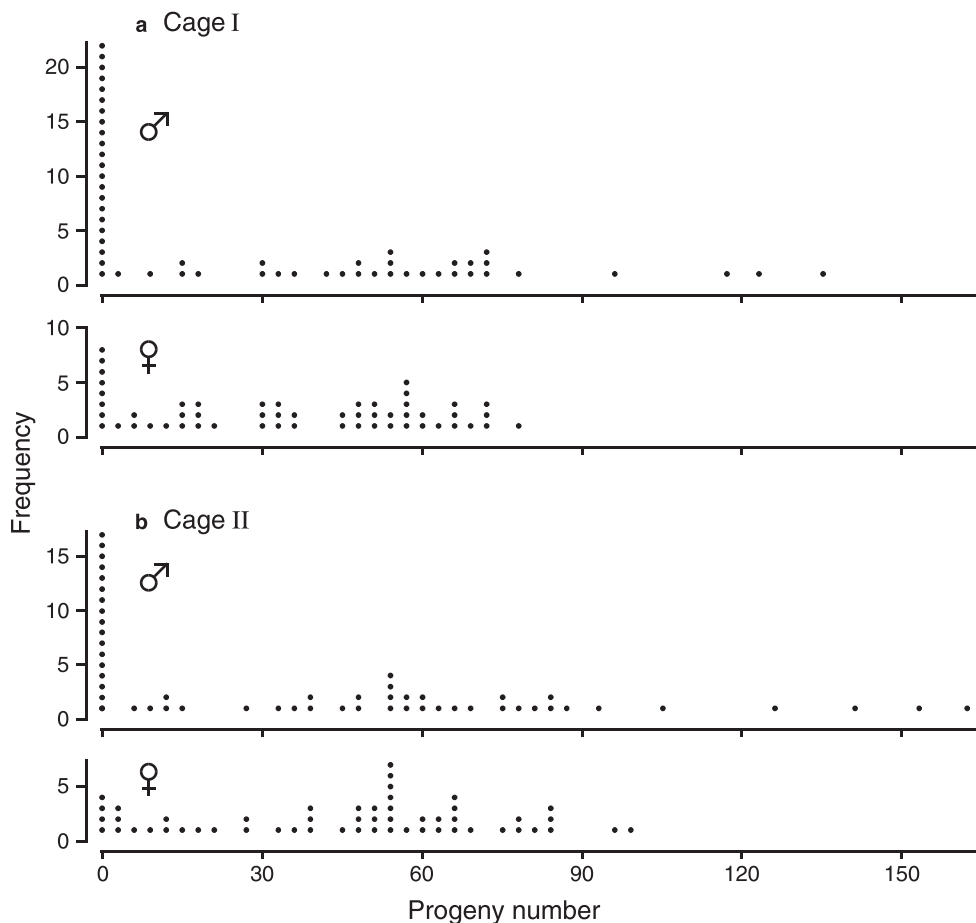


Fig. 2 Variance in reproductive success for butterflies of each sex in the two captive populations of *B. anynana* measured as progeny number at the hatching larval stage and assuming last male sperm precedence.

higher in males with highly significant differences in each cage (I: Levene's Test Statistic = 7.59, $P < 0.001$; II: LTS = 10.25, $P = 0.002$; variances for each sex are homogeneous across cages). About one-third of males have no reproductive success under these conditions of equal sex ratio and high density. Substantially fewer females had no reproductive success. In contrast, some males mate with more than one female, which tends to yield a comparatively high reproductive success. Thus the five (in cage I) or seven (II) individual males with the highest progeny numbers as shown in Fig. 2 were all the final mating partners of either two or three different females.

The two replicate cages yielded closely similar estimates for effective population size (Table 2). The estimates were largely unaffected by the model of sperm precedence (Table 2). Allocating five unmated males to the five fertile females with no recorded partners yields only slightly higher estimates. As expected from the difference across the sexes in the variance of reproductive success, the estimates for the effective numbers of

Table 2 Estimates of effective population size for the two captive populations of *B. anynana* under assumptions of three models of sperm precedence: first = first male sperm precedence; last = last male sperm precedence; mix = equal sperm mixing from all male partners. Additional estimates given in parentheses assume that the five fecund females not observed to mate had male partners from within the pool of unsuccessful males. The effective population size relative to census size ($N = 108$) is also given.

Cage	Model	$N_{e, \text{males}}$	$N_{e, \text{females}}$	N_e	N_e/N
I	first	27.74 (30.34)	37.24	63.59 (66.88)	0.59 (0.62)
	last	24.67 (27.17)	37.24	59.36 (62.84)	0.55 (0.58)
	mix	29.31 (31.94)	37.24	65.61 (68.77)	0.61 (0.64)
II	first	27.87 (29.57)	40.12	65.78 (68.09)	0.61 (0.63)
	last	27.64 (29.34)	40.12	65.46 (67.79)	0.61 (0.63)
	mix	24.81 (26.52)	40.12	61.32 (63.86)	0.57 (0.59)

females are consistently higher than those for males. In the mating and rearing conditions of our experiments, N_e was about 60% that of the census number (Table 2).

Sperm precedence

Observations from application of enzyme markers

We were able to screen the parents and offspring of 19 families from our cage experiments in which the female parent had mated two or three times. Data for five families were uninformative. Of the remaining 14 families, the data were either conclusive or highly supportive of a particular outcome of sperm competition. Only two families had fewer than 10 offspring (mean $N = 20$; max. = 30).

The results showed three examples of complete first male sperm precedence, seven of complete last male sperm precedence, one where the second of three male partners fathered all offspring, and three of sperm mixing. There was at least one conclusive example of each outcome. Furthermore, for first male sperm precedence the second male involved was in two cases successful in another pairing and therefore not sterile. In one family with sperm mixing the numbers of offspring fathered by the three consecutive male partners was 0, 6 and 20. For the other two examples of sperm mixing in which the paternity of some offspring was uncertain, application of a maximum likelihood method (Dickinson, 1986) estimated the proportion of offspring fertilized by the second male partner (P_2) as 0.14 and 0.21 (with a third male being unsuccessful in the latter), respectively. There was no example of a triple-mated female in which all three males were shown to have fathered offspring ($N = 5$).

Experiments 1 and 2 with colour markers

The main results of expt 1 are shown in Fig. 3. A higher proportion of crosses were obtained in which the first male partner was homozygous for *yellow* and *Spotty* (A), than the reverse (B). Again the results from the 14 families show examples of both first and second male sperm precedence. Overall the latter outcome is most frequent. Only family A3 shows substantial sperm mixing throughout most of the period of oviposition. However, there are several families in which either some of the earlier offspring are sired by the first male (e.g. A1 and A8), or while the majority of offspring in each period are sired by one male, a few eggs have been fertilized by the alternative male (e.g. A4 and B4). These results indicate that the system is 'leaky' in the sense that it tends to yield first or last male sperm precedence but that occasionally sperm from the other male partner are successful, especially shortly after the final mating. A further family (B12) shows some indication for a switch-over about halfway through oviposition from sperm mixing to exclusive use of sperm from the first mating.

The families from expt 2 excluded the eggs laid shortly after the second of the two matings. Each set of crosses include examples of both first and second male sperm precedence, and several clear examples of sperm mixing

(Table 3). In some of the former cases a 'leakiness' was again observed. Such examples also demonstrate that both male partners were fertile. The results of both expt 1 and 2 suggest that once copulation has begun the homozygous mutant males or their sperm tend to be less effective in competition than wild-type males (see, e.g. total offspring numbers given in Table 3).

Discussion

Over one-quarter of the females in our mating cages mated more than once over 3 days. Our data on patterns of paternity for multiply mated females of *B. anynana* demonstrate a wide variation in the outcome of sperm competition. Either male partner of double-mated females may be completely successful, or some form of sperm mixing can sometimes occur. There may also be a 'leakiness' in the system such that while the last male fertilizes the great majority of the eggs, some of those oviposited may be fertilized by an earlier male partner. Instances when this effect is restricted to eggs laid soon after the final mating may indicate that at least some of the mechanisms actively involved in sperm precedence occur after separation of the second pairing.

The overall results suggest that last male sperm precedence is the most common outcome of sperm competition in *B. anynana* although all possible outcomes occur. Last male sperm precedence appears to dominate in the lepidoptera when polyandry occurs (Walker, 1980; Watt *et al.*, 1985). The outcome is more variable in some species including *Pieris rapae* (Wedell & Cook, 1998). However, the extreme variation found in the present study with rather frequent sperm mixing has rarely been reported (although see for the moth *Spodoptera frugiperda*, Martin *et al.*, 1989; for the ladybird beetle *Adalia bipunctata*, De Jong *et al.*, 1993, 1998).

In *B. anynana* under the conditions of a high density of receptive butterflies (with no protandry) in our mating cages and with about one-quarter of females mating more than once, the impact of sperm mixing on effective population size is small. This holds even when sperm mixing is assumed to be maximized with all male partners of a female fertilizing an equal proportion of her eggs (Table 2). The data shown in Fig. 1(b) suggest pre-adult development after egg hatching would also make little contribution to variance in reproductive success in our laboratory populations. The most important contribution to reducing N_e relative to N in our two population cages was variance among individuals in progeny production at the stage of egg hatching. Males show a substantially higher variance in reproductive success than females, resulting in lower values for N_e in that sex (Table 2). This difference between the sexes while already apparent in terms of numbers of mating partners (Table 1) is much more substantial at the stage of hatching larvae (Fig. 2).

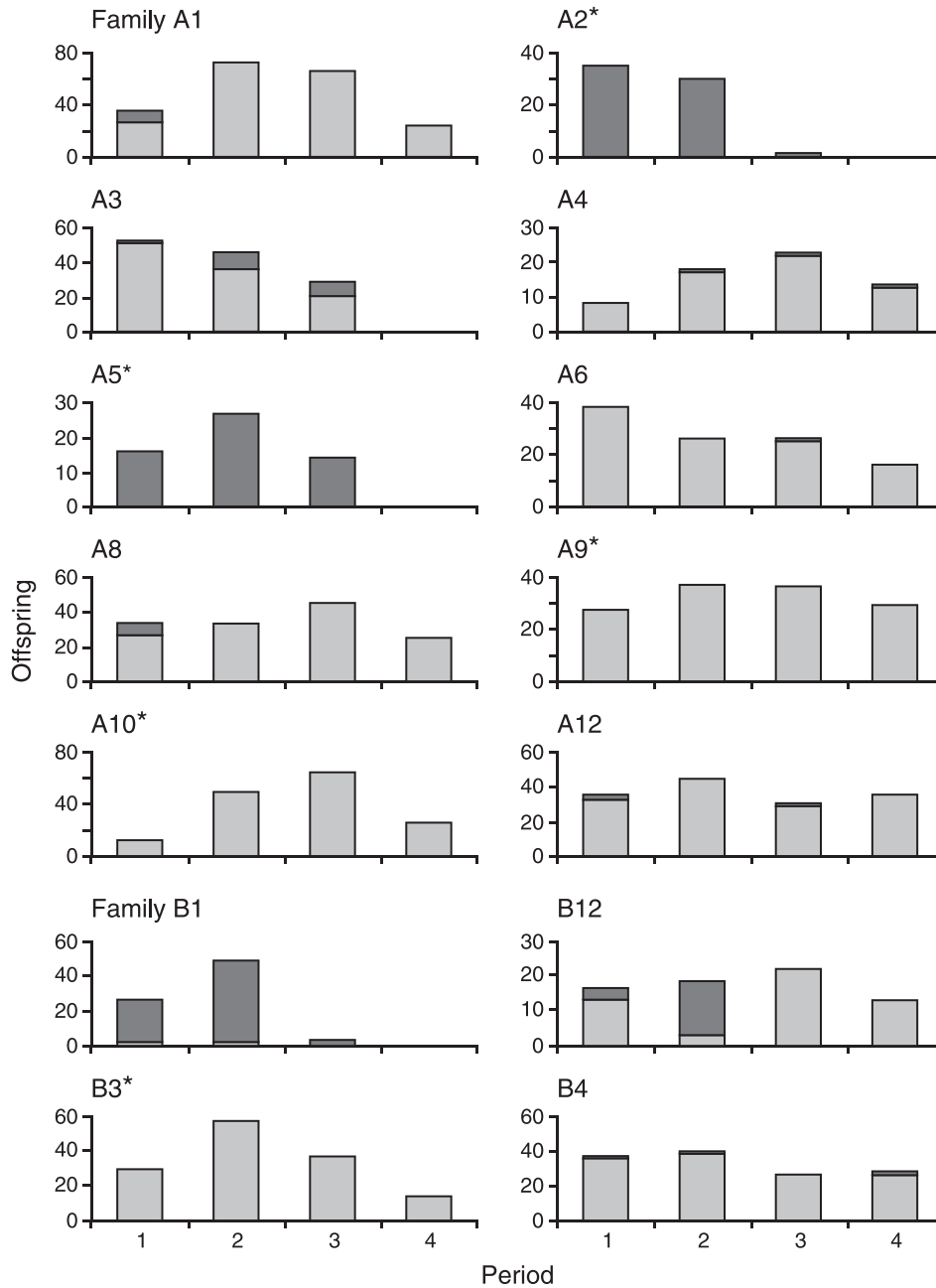


Fig. 3 Numbers of offspring of mutant (dark shaded) or wild type (pale shaded) phenotypes reared from double-mated females in Experiment 1 for the two directions of cross: A, first male, mutant; B, first male, wild type. The progeny are shown for each family for each of the four consecutive periods of oviposition. All offspring in those families with an asterisk attached were sired by the same male. Note that the scale of the y-axis is variable. For further details see text.

The ratio of $N_e : N$ in our two captive populations can be conservatively estimated as about 0.60 (Table 2). The estimates are broadly consistent with studies of the loss of genetic variance in bottlenecked lines of *B. anynana* (Saccheri *et al.*, 1999). They indicate that effects of random genetic drift will be small in our artificial selection experiments on *B. anynana* when at least 30

mated females are used in each generation (see Brakefield, 1998). This conclusion has been supported by closely similar responses in replicated selected lines in more recent experiments in our laboratory (P. Beldade and P. M. Brakefield, in preparation). In some of these experiments we have also used a large excess of males in mating cages. More females will then have unique

Table 3 Numbers of offspring of wild-type (++) and *melanine* mutant (*mm*) phenotypes reared in expt 2 (minimum family size = 10) for the two directions of cross as indicated. An interpretation of the outcome of sperm competition is given together with specific comments regarding 'leakiness' and male fertility where tested in an additional pairing: first = first male sperm precedence; second = second male sperm precedence; mix = sperm mixing. Total offspring numbers are given for each direction of the cross including small families. For further details of the experiment see text.

Family	Offspring numbers		Outcome	Comments
	Wild-type	Mutant		
First male = <i>mm</i> ; second = ++				
C1	42	8	mix	
C2	12	1	second	'leaky'
C3	76	0	second	
C7	47	0	second	
C11	0	41	first	second male fertile
C13	14	0	second	
C14	30	0	second	
C15	36	0	second	
C16	38	1	second	'leaky'
C17	7	11	mix	
C18	35	0	second	
C19	1	62	first	'leaky'
C-all	338	150		
(B) First male = ++; second = <i>mm</i>				
D3	1	45	second	'leaky'
D5	50	0	first	second male infertile
D6	60	0	first	second male infertile
D7	59	18	mix	
D10	30	0	first	second male infertile
D11	30	9	mix	
D15	13	46	mix	
D16	57	1	first	'leaky'
D17	6	6	mix	
D18	73	2	first	'leaky'
D-all	333	131		

male partners so that N_e is expected to approach double that for females (from Table 2: $N_e : N$ will then approach 80 : 108). Management of other captive (or natural) populations to minimize the variance in male mating success will be expected to have similar consequences. It is important to note that our estimates are for a single generation. For short-term captive breeding programmes with other species of butterfly of broadly comparable biology and management, our results suggest that genetic drift will make only a small contribution to loss of genetic variation when N is of the order of 100 adults.

We believe that the approach we have used here to obtain estimates for N_e in two captive populations could be transferred to certain natural populations of butterfly. Indeed there are parallels with recent attempts to estimate heritabilities in natural populations by associating phenotypic comparisons with inferences of relatedness (Mousseau *et al.*, 1998; Ritland, 1996, 2000). Suitable candidates may include species of butterfly with a metapopulation structure of networks of small sub-populations characterized by quite low rates of interpopulation migration and nonoverlapping generations. *Melitaea cinxia*, as surveyed in Finland (Hanski *et al.*, 1994), may represent such a candidate. To obtain estimates of N_e for particular local populations would

then require the ability to apply mark–release–recapture methods to sample intensively two consecutive adult populations, combined with the noninvasive collection of material (e.g. punches of wing tissue) from all individuals in each sample. Analysis of the latter samples by molecular techniques to survey highly polymorphic markers, such as appropriate microsatellites, could trace the reproductive success for all individuals sampled from the first generation. This pedigree information in combination with analysis of the capture–recapture data could provide direct estimates for N_e as well as population size, N . The availability of sufficiently polymorphic molecular markers may at present constrain application of such an approach, especially bearing in mind the likely history of inbreeding and loss of genetic variance in such populations (e.g. Saccheri *et al.*, 1998). One way in which this problem might be minimized is to initially screen candidate local populations to preselect those which by chance have maintained polymorphism at the available molecular markers. Such estimates in the future will be invaluable not only in helping to resolve controversies about how much smaller effective population sizes tend to be than census number, but also in understanding the mechanism of maintenance of heterozygosity in structured populations (Hedrick & Gilpin, 1997).

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