Conservation Genetics

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Guidelines in conservation genetics and the use of population cage experiments with butterflies to investigate the effects of genetic drift and inbreeding

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Summary. Bottleneck experiments were performed on the tropical butterfly *Bicyclus anynana*. The reasons for choosing a species of butterfly are described together with the methodology used in the population cage experiments. Replicated lines founded by single pairs and then measured in generation 2 or 3 showed strong phenotypic differentiation, declines in heritabilities of morphological traits, dramatic reductions in egg fertility (due to inbreeding depression) and an increased sensitivity to insecticide applications (probably due to a loss of alleles conferring tolerance) relative to unbottlenecked control lines. Egg fertility showed some recovery in further generations. An experiment involving repeat bottlenecks provided support for the hypothesis that a purging of deleterious alleles contributed to this "fitness rebound". The preliminary results are examined in the context of developing guidelines in conservation genetics.

Introduction

The central purpose of conservation biology theory is to understand what determines the survival of natural populations or of communities of such populations. It is well established that catastrophes (natural or man-induced), environmental stochasticity, demographic stochasticity, and genetic stochasticity are the major causes of extinction. However, a quantitative description of the importance of each of these processes and how they interact in different species and environments is only at an early stage of development (Nunney and Campbell, 1993), such that the management of rare species is based on largely untested theoretical rules of thumb with little knowledge of the long-term consequences. In particular, the importance of genetic variation remains essentially unquantified for almost all organisms.

The dual environmental pressures of habitat fragmentation and contraction are tending to produce a greater isolation and smaller size of populations. Small population size can lead to genetic change and deterioration either through (1) introducing the stochastic sampling effects of genetic drift to produce a loss of heterozygosity, rare alleles

and additive genetic variance; or (2) increasing the incidence of matings between individuals related by descent and thus of inbreeding depression due to higher levels of homozygosity of rare recessive deleterious alleles (Lande and Barrowclough, 1987; Falconer, 1989). Any intermittent reduction in numbers in an already small population will result in a "bottleneck" event and a greatly increased chance of genetic drift and inbreeding. Heterozygosity at polymorphic loci and additive genetic variance for quantitative characters are expected to decline because of drift by one over twice the effective population size *per* generation of a bottleneck. Isolation restricts the possibility of migration and gene flow introducing novel genetic diversity; mutation will only provide slow or very slow recoveries in small populations (Lande and Barrowclough, 1987). Isolation will also lower the chance of recolonization following any local extinctions. Genetic diversity is likely to be directly related to the variability in individual fitness within a population and the ability of a population to produce an adaptive response to an environmental perturbation or change (Brakefield, 1991). Genetic diversity must also play a role in discussions of conservation priorities where choices need to be made in applying limited resources to the preservation and management of scattered populations of a rare species.

Local populations of many organisms are probably commonly established from small numbers of founder individuals (for examples involving butterflies see Oates and Warren, 1990; Pavlicek-van Beek et al., 1992). In these sorts of situations one can ask, and attempt to quantify, how the probability of establishment and persistence is related to the genetic diversity within the founders. Furthermore, an active conservation policy which is becoming increasingly important for butterflies in European countries such as The Netherlands (J. v. d. Made, personal communication) and Britain (Oates and Warren, 1990) is the reintroduction of a species into habitats where it has become extinct; such a policy also involves choices with respect to the founders. More specific questions need to be answered to produce adequate and rigorous guidelines to maximize the chance of success of such introductions; they include: how many, and which individuals (including which source when local adaptation and differentiation have evolved). Another policy which is being pursued particularly actively in The Netherlands is the establishment of landscape corridors and networks. In this case one question is: how much movement should be encouraged?; too much gene flow may swamp any processes of local adaptation. In rare species conservation where only small numbers of fully isolated local populations are extant, individuals could be moved around artificially; in which case, how many and how often? These types of questions all have a genetic component. The theory of population genetics can provide partial theoretical answers or guidelines (Lande and Barrowclough, 1987). Furthermore, computer simulations can examine the conse-

quences of this theory in terms of heterozygosity indices (see de Jong et al., this volume). However, more empirical data are necessary to be able to apply the theory and simulations with any real confidence to populations in the wild. Moreover, the relevance of heterozygosity and molecular-based indices of genetic diversity to variation in ecologically-important quantitative traits and to processes of adaptation in natural populations are essentially unexplored in a conservation context. A reliance solely on estimates of heterozygosity from molecular surveys could lead to incorrect decisions when developing conservation priorities or management policies.

Our laboratory experiments using butterflies in population cages are designed to describe some of the potential processes involved in genetic deterioration and to investigate how they influence variation in fitness within populations. Some of the preliminary results will be outlined here together with the rationale of performing these experiments. The first series of experiments involves examination of the effects of single-generation bottlenecks of differing size on genetic variances and fitness parameters in independent lines. A second series will investigate and describe empirically the effect of gene flow in groups of interacting small subpopulations ("metapopulations"); these will not be discussed further (C. van Oosterhout et al., unpublished).

Aspects of the initial experiments to be covered in this preliminary report are:

- (1) why butterflies?;
- (2) the methods of working with butterfly populations in cages;
- (3) the experimental design and objectives;
- (4) a summary of some of the results from bottleneck experiments (Saccheri, unpublished data).

Materials and Methods

Why butterflies?

Butterflies attract a high level of public awareness and conservation activity. The ecology and food plant specialization of many butterflies makes them especially sensitive to habitat disturbance or change. Many species in temperate communities have shown severe declines (see, e.g., reports in Pavlicek-van Beek et al., 1992). In Britain, this decline is considered to have been more extreme than for any other well known group of plants or animals; nearly half of the 59 resident species have experienced major range contractions, and four have recently become extinct (Thomas, 1991; Warren, 1992). The record is even worse in The Netherlands, where 15 of the 75 species have become extinct this century and a further 26 species have an endangered or vulnerable status (Tax, 1989).

The reasons for these types of changes are diverse, but both the contraction and fragmentation of habitat are undoubtedly major factors (Thomas, 1991; Warren, 1992). Many of the remaining populations of endangered species are both of small size and isolated from any other local populations. Butterfly populations are also frequently susceptible to population crashes which may be climatically-induced (Thomas, 1983). Bearing in mind that the effective population size is likely to be substantially smaller than the census number (Brakefield, 1991), genetic processes associated with small numbers are likely to be a major factor influencing genetic diversity in populations of butterflies of concern to conservationists.

Thus, many species of butterfly are likely to be particularly prone to genetic stochasticity and deterioration which is likely to be exacerbated by demographic and environmental stochasticity. Laboratory population cage experiments to investigate empirically the effects of population demography and bottlenecks on genetic diversity and fitness have, to date, mostly involved species of insects, especially *Drosophila* and *Musca*. These species have a more open population structure than most butterflies and other organisms of major concern to conservationists which are characterized by low dispersal and discontinuous distributions. A few similar experiments have been attempted with other animal species including mice (Brewer et al., 1990). Some of the experimental work with diptera has suggested that even severe repeated bottlenecking of populations may not necessarily lead to (substantial) lowering of evolutionary potential (López-Fanjul and Villaverde, 1989; Bryant and Meffert, 1993); theory indicates that a transformation of non-additive components of genetic variance into additive components may contribute to such results (Goodnight, 1987, 1988). These considerations led us to develop an experimental system based on a non-migratory species of butterfly, *Bicyclus anynana*, with a short generation time.

Methodology of butterfly population cage experiments

B. anynana is a small satyrine butterfly which occurs in seasonal environments in sub-Saharan Africa. Brakefield and Reitsma (1991) studied the population biology of several species of *Bicyclus*, including *B. anynana*, over a transition from wet to dry seasons at a forest-edge site in Malawi. The adults are rather sedentary, long-lived, feed only on fallen fruit, and lay eggs on grasses including species of *Oplismenus*. The population structure consists of an unstable mosaic of rather small concentrations of butterflies. Movements of adult butterflies between local habitat patches were detected. Rates of movement may vary with seasonal changes in biotope.

In the laboratory, net population cages ($34 \times 64 \times 47$ cm) comfortably house over 300 adults or 500 larvae. Current experiments suggest that, in these conditions, the effective population size is of the order of one-third to one-half of the absolute number of adults (van Oosterhout et al., unpublished). Adults are fed on mashed banana. Most females mate only once. Mating is allowed to occur for about 1 week following peak adult eclosion before eggs are collected on grass plants or cuttings rooted in water introduced into the cage. Alternatively, butterflies *in copula* or individual gravid females can be removed from mating cages and eggs obtained by holding the females in net-covered small plastic pots containing a grass cutting. Females lay 10–20 eggs *per* day up to a total of over 300 eggs. Families of larvae can be reared on young maize plants to pupation in small net sleeves for offspring-parent analyses of heritability. The generation time at 26°C in the experimental climate room with 12L:12D and ca. 70% RH is about 6–7 weeks. A captive stock established in 1988 from approximately 80 gravid females collected at a single site (Nkhata Bay, Malawi) has been maintained at about 500 adults with some overlap between generations. High levels of heterozygosity remain in the stock (Saccheri and Bruford, 1993).

We were concerned to be able to analyze different modes of genetic variation and types of traits in our bottleneck experiments:

Molecular polymorphism. Saccheri and Bruford (1993) have developed fingerprinting techniques for the detection of hypervariable minisatellite DNA markers. Allozyme variation at six polymorphic loci is also being surveyed using a cellulose acetate system. These data will be discussed elsewhere (Saccheri, unpublished data).

Visible polymorphism. The stock is polymorphic for a gene determining pupal color; a recessive allele at a frequency of about 5% changes the normal green color to yellow.

Morphological variation. Heritabilities and additive genetic variances have been obtained for quantitative variation in wing size and a number of wing pattern elements including the size of a particular eyespot and of a pale band (Holloway et al., 1992; Windig, 1993). Measurements are made quickly and accurately on wings removed from frozen bodies (retained for molecular analysis) using a computerized image analysis system. The full multivariate data matrix for all traits can be simplified by applying principal component analysis to produce linear and orthogonal combinations of the individual traits. The different components can be readily interpreted in terms of the traits and can themselves be treated as separate genetic characters.

Variation in insecticide tolerance. We were also concerned with measuring a trait of potential involvement in the ability of a population to withstand a novel environmental stress or challenge. Individual final instar larvae vary in their susceptibility to topical applications of the synthetic pyrethroid insecticide, deltamethrin. A dose-response curve

was determined for controls to select an LD_{50} application rate to survey the experimental lines. The proportion of samples of larvae which survive to produce healthy adults is scored.

Variation in egg hatching. Eggs laid on grass cuttings either by populations or individual females can be easily counted. Viable eggs hatch after 4 days when the number of first instar larvae is counted.

Design of the bottleneck experiments

Bottleneck lines were established from single, three, and 10 pairs of fertile founders. Four or six (for the single-pair bottleneck treatment) replicates were used. Each replicate was established using the progeny from a random selection of the requisite number of fertile pairings taken from mating cages of stock butterflies. Four control lines of 300 butterflies (two established from about 75 fertile pairings) were also reared. All treatment lines were allowed to freely increase in size up to a carrying capacity of 300 adults (for single-pair lines this took two to three generations). The five types of traits described above were measured in generation 2 or 3 (some also at additional times). Heritabilities were estimated from about 12 families reared *per* replicate line with five offspring of each sex *per* family.

Most lines were discontinued at generation 4. The single-pair lines were reared on to generation 8, when they were rebottlenecked with several replicates *per* original line. Two of these replicates were allowed to flush up to 300 adults again before repeat measurements were made of each trait (including heritabilities; not discussed below). A similar procedure was also performed on replicated lines established by single pair crosses between three of the original bottleneck lines, and on new single-pair bottleneck lines and a control line all established at the same time as the rebottlenecking.

Results

Polymorphism

Four of the six single-pair lines became fixed for the green pupal color allele. The other two lines had homozygote frequencies indicating that one of the original four alleles at this locus was yellow. The two replicates of one of these latter lines each lost this allele at the second bottleneck. These results and those of the molecular markers (Saccheri, unpublished data) are broadly consistent with the expected effects of genetic drift. It will be interesting to examine how the changes in heterozygosity within and among treatments are correlated with observed changes in fitness-related parameters.

Morphological variation and heritabilities

Fig. 1 illustrates how the single-pair lines produced substantial morphometric differentiation among replicates, while there was little differentiation for the controls. The pattern among 10-pair lines was indistinguishable from the controls, while the three-pair lines yielded an intermediate amount of differentiation. There is also evidence from an analysis of variance in principal component values that the single-pair (but not three-pair) lines tend to show a reduced phenotypic variation within lines.

Fig. 2 shows that the single-pair lines after their flush period were associated with reduced heritabilities for the principal components describing most of the morphological variation relative to the control and 10-pair lines. Again, the three-pair lines appear to be rather intermediate. In general, the additive genetic variances follow a similar pattern (not shown).

Fig. 1. Phenotypic differentiation among replicated single-pair bottleneck lines (left side) and unbottlenecked controls (right side) of the butterfly *Bicyclus anynana*. Means with standard error bars are shown for each of three pattern characters on the ventral hindwing of males: an index for the area of a pale band across the wings; a measure of the average color in gray values of a standard area of the wing; the area in square mm of the largest eyespot.

Fig. 2. Mean heritabilities with standard errors attached for each treatment in the bottleneck experiments. Values are for each of the first three principal components: PC1 (explaining 31% of total variance), PC2 (18%) and PC3 (13%) which are primarily measures of eyespot size, color and band with wing size, respectively (see also Fig. 1).

Insecticide tolerance and genetic drift

A summary of the data for mortality of larvae after standard application of insecticide is given in Tab. 1. Two or three of the six original single-pair lines show evidence of reduced tolerance after the first bottleneck-flush cycle. All except one of these lines were highly susceptible (in each of the two replicates tested) to the insecticide after a second

Table 1. Percentage survival in pooled samples from the single-pair bottleneck lines after standard application of the insecticide, deltamethrin: lines A to J were bottlenecked at generations 0 and 8, and lines 4 to 9 were bottlenecked once when established at the time of generation 8. Approximate sample size *per line* is indicated for each group (n.d. = no data)

Generation	F3	F7	F10
A	3% (N = 60)	23% (N = 30)	9% (N = 60-80)
C	n.d.	73%	56%
F	17%	53%	3%
G	0%	30%	0%
H	n.d.	57%	3%
J	40%	47%	7%
4	—	—	43% (N = 40)
7	—	—	42%
8	—	—	3%
9	—	—	45%

severe bottleneck and flush. One of the four new single-pair bottlenecks was also susceptible. These results can be explained by the existence of genetic variation for tolerance in combination with loss of tolerance alleles in many bottleneck lines due to genetic drift. However, we have no independent evidence for such genetic variation.

Egg hatching, inbreeding depression and the purging hypothesis

A dramatic decline in the proportion of eggs which hatch occurs following single-pair bottlenecks. Fig. 3 shows data from population

Fig. 3. Population fertility of the single-pair bottleneck lines (symbols connected by lines) and the unbottlenecked controls (x). Changes through time are shown for the bottlenecked lines; each symbol represents an individual replicate.

Fig. 4. The relationship between the fertility of females taken from generation 8 of the single-pair bottleneck lines and fertility in full-sib crosses of their offspring (means for 1–5 females). Each symbol indicates one of the six replicates (symbols as in Fig. 3). Heritability ($\pm$ standard error) estimated from the pooled data is 0.69 ± 0.23 ($P < 0.01$).

counts for the six original single-pair lines. There is a 50% or greater reduction relative to controls. Once again, the three-pair lines were intermediate while the 10-pair lines are indistinguishable from the controls. These results are consistent with an extreme inbreeding depression and the existence of many genes with deleterious recessive alleles in our stock population.

This interpretation is supported by the observed “fitness rebound” in some single-pair lines in later generations (Fig. 3). Three of the six replicates recovered much of their egg viability by the seventh generation. These results parallel similar observations in Bryant et al.’s (1990) experiments with houseflies, and are consistent with selection favoring genotypes less affected by inbreeding depression and thus with a purging of some deleterious alleles.

Females *in copula* were taken from the eighth generation of the original single-pair lines to describe individual variation in egg hatching and attempt to demonstrate heritability by rearing and scoring their F1 offspring (after mating of the full sibs). The data for the parent butterflies in Fig. 4 show that substantial phenotypic variation (still) occurred within each line. Furthermore, the pooled offspring-parent regression provides some evidence of a genetic component and thus of the potential for “fitness rebound” (Fig. 4).

Many individual females taken from the eighth generation of each of the single-pair lines were sterile, failing to produce any viable eggs (pooled data: 82 of 129 females). The overall proportion was higher

than for females collected at the same time from the stock (1 of 9). A similar difference (but where all scored females were collected *in copula* and each laid eggs) was found two generations after the second bottleneck of each original line (pooled data: 77 sterile of 179 females) relative to the unbottlenecked controls (4 of 32). Thus, any fitness rebound had possibly not led to a full recovery in fertility of matings, at least in part, because some deleterious alleles had become fixed.

The repeat bottleneck experiment examined the purging hypothesis by comparing the loss of fertility in the different treatments (Fig. 5). On the basis of initial analysis of the untransformed data for all fertile F2 females, the second-bottleneck of the pure lines produces a smaller loss of egg hatching (mean with SE: $21.9 \pm 3.2\%$, $n = 137$) than in either the new single-generation bottlenecks ($43.6 \pm 2.8\%$, $n = 68$) or in the single-pair crosses of original bottleneck lines ($38.1 \pm 4.3\%$, $n = 59$; $F = 10.96$, $df = 2 \text{ \& } 261$, $P < 0.001$). The lower average drop in fertility after the repeat bottlenecks of the pure lines is consistent with some previous purging of deleterious alleles. The behavior of the crosses suggests that there were differences among the original bottleneck lines with respect to the identity of the deleterious alleles.

The high inbreeding depression suggests a large genetic load and that the effective population size of the field population in Malawi is very large, with a history lacking founder or bottleneck effects.

Discussion

These experiments show how laboratory bottlenecked-populations of a species of butterfly are susceptible to effects of genetic drift and inbreeding. Inbreeding depression effects on fertility are especially strong. However, these may not be (fully) representative of those expected in wild populations of species with naturally low effective population sizes and low rates of gene flow, or in species with a prolonged recent history of small fragmented populations. Such conditions are likely to be associated with the type of purging of deleterious alleles phenomenon for which we have some evidence in our bottlenecked populations. The effects of genetic drift leading to loss of genetic diversity have been detected in the bottleneck lines for each of the surveyed modes of genetic variation. The demonstration of an increased susceptibility to insecticide is of particular interest in illustrating the potential of such effects to lower the ability of populations to adapt to environmental change or to tolerate any perturbation or stress (see also Krebs and Loeschcke, this volume).

The declines in heritabilities of morphometric traits suggest that the *B. anynana* genome responds to a single bottleneck event in a more classical manner (Falconer, 1989) than as described in some experi-

Percent of eggs hatching per new line or cross

Fig. 5. Variation in fertility pooled across replicates for each of the three treatments in the rebottlenecking phase of the experiment: (a) "singles" = four new single-pair bottleneck lines; (b) "doubles" = single-pair rebottlenecks of the six original single-pair bottleneck lines (giving a total of two bottlenecks); (c) "crosses" = lines founded by a single pair with the parents taken from different original single-pair lines. Each P value shows the fertility of the original female parent; each F1 value is the mean egg hatch for several F1 full-sib pairings and each F2 value is the mean for many individual females from the second generation after the bottlenecks (no sterile females are included; only about one-half of F1's were continued on to F2's). Arrows show overall mean values in each generation of each treatment.

ments with *Musca* or *Drosophila* which reported increases in additive genetic variances following single or repeated bottlenecks (López-Fanjul and Villaverde, 1989; Bryant and Meffert, 1993; although additive genetic variance for fertility may have increased in our lines). Recent theoretical treatments have also shown how bottlenecks may lead to a transformation of non-additive into additive components of genetic variance (Goodnight, 1987, 1988), an affect which could partially "compensate" for the loss of heterozygosity and allelic variation due to drift. However, there is no evidence for this phenomenon in our experiments; indeed, the declines we have measured in additive genetic variances are greater than one would expect given our estimates of effective population size associated with the bottleneck episode (Saccheri, unpublished data).

Given that genetic diversity should be maximized, some generally accepted management policies for populations can be listed (Brakefield, 1991):

- (1) maintain population size minimizing the incidence and duration of any bottlenecks;
- (2) maximize the proportion of breeding adults;
- (3) preserve relevant habitat and environmental heterogeneity;
- (4) minimize the rate and extent of any (man-induced) environmental change;
- (5) retain natural patterns of gene flow.

The experiments reported here are concerned with the development of more specific and, in practice, probably more useful guidelines, and illustrate how quantitative experiments can be designed to investigate questions of genetic management. There is an urgent need for well designed experiments in a wide range of taxa. Our preliminary results support one specific recommendation, namely, that reintroductions of butterfly species, or of similar organisms, which use 10 or more gravid females and which are followed by a rapid flush are likely to be more persistent than those based on fewer founders. Although rare alleles will be lost with a founder size of 10 pairs and this may be of practical importance for the persistence of natural populations, we were unable to detect any detrimental effect resulting from genetic impoverishment due to such a founder/bottleneck event: there was no measurable loss of morphological or physiological variation and no inbreeding depression (in a constant environment with a limited array of stresses).

Some suggestions can be made with respect to the types of experiments which will be useful in focussing attention on the unanswered questions such as those outlined in earlier sections. Experiments could include ones designed to investigate:

- (1) the effect of bottleneck or founder events on realized heritabilities as measured by applying artificial selection;

- (2) the interactions between adaptive potential and both genetic diversity and inbreeding history by employing arrays of realistic environmental challenges;
- (3) the relationships between the (genetic) identity of the founders and the rates of inbreeding depression and recovery ("fitness rebound");
- (4) the effects of periods of population "flush" following bottlenecks on genetic variances and the frequencies of deleterious recessives;

Numerous man-made introductions of butterflies have been documented in European countries (Oates and Warren, 1990; Warren, 1992; various papers in Pavlicek-van Beek et al., 1992). Some of these, including examples which involved very few founders, have become established in the short term, but it is not known what overall proportion was unsuccessful in establishment, or whether those established in the short term will persist in the longer term. Our laboratory results suggest that introductions involving at least 10 gravid females from an outcrossed captive stock or a large natural population will be more likely to be successful than when very few founders are used, but will be as likely to persist as those involving many more founders. However, although survival or fitness differences may not occur or be measurable in laboratory environments, the results may be very different in nature. Thus, field-conducted replicate experiments involving different numbers and sources of founders are needed to properly substantiate such laboratory-based guidelines.

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