

Notes and Comments

Experimental Confirmation that Inbreeding Depression Increases Extinction Risk in Butterfly Populations

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Inbreeding depression—reduced fitness caused by mating among relatives—is well documented for a wide range of taxa (Charlesworth and Charlesworth 1987; Ralls et al. 1988; Thornhill 1993; Saccheri et al. 1996; Ballou 1997; Westemeier et al. 1998; Antolin 1999; Madsen et al. 1999; Morjan et al. 1999; Weeks et al. 1999; van Oosterhout et al. 2000). The most likely mechanism of inbreeding depression is the expression of deleterious recessive alleles in the offspring of close relatives (Charlesworth and Charlesworth 1987; Lande 1995; Ballou 1997; Bijlsma et al. 1997; Tanaka 1998). A much more controversial issue is the extent to which inbreeding depression influences the dynamics of natural populations, including the risk of population extinction. Following a period in the 1970s when much emphasis in conservation biology was placed on genetic causes of extinction (e.g., Frankel and Soulé 1981), a consensus emerged in the 1980s that emphasized the primary role of ecological factors in

reducing population size and causing the ultimate demise of small populations (e.g., Lande 1988). Nevertheless, integration of ecological, genetic and environmental factors remains critically important for a thorough understanding of the risk of population extinction (e.g., Dunham et al. 1999).

Though the primary role of ecological factors in population extinction is now widely accepted, some results, especially on plant populations, suggest that inbreeding and genetic drift in small populations may influence dynamics and ultimately increase the risk of population extinction (Charlesworth and Charlesworth 1987; Newman and Pilson 1997). Observations on reptiles and birds (Keller et al. 1994; Madsen et al. 1996, 1999; Westemeier et al. 1998) and results from small-scale field experiments on mammals and plants (Jiménez et al. 1994; Newman and Pilson 1997) also indicate that inbreeding may significantly increase extinction risk. Recent theoretical studies have suggested that slightly deleterious alleles may accumulate in small populations to the point that their impact on population dynamics cannot be ignored (Hedrick 1994; Frankham 1995; Lynch et al. 1995), though an experimental test using *Drosophila* failed to support this genetic “meltdown” model (Gilligan et al. 1997).

In a previous observational study on the Glanville fritillary butterfly (*Melitaea cinxia*), Saccheri et al. (1998) found that the extinction risk of small local populations increased with the degree of inbreeding, which they inferred from the average heterozygosity of the field populations. Laboratory results showed a reduction in several fitness components, such as larval survival, adult longevity, and egg-hatching rate, after just one generation of full-sib mating (Saccheri et al. 1998). Because the Glanville fritillary metapopulation is structured into many very small local populations, which often consist of just one family group of larvae (Hanski 1999), the observation that inbreeding increases extinction rate is not very surprising.

The purpose of this study was to experimentally test the hypothesis that inbreeding depression increases the risk of extinction of small populations. *Melitaea cinxia*

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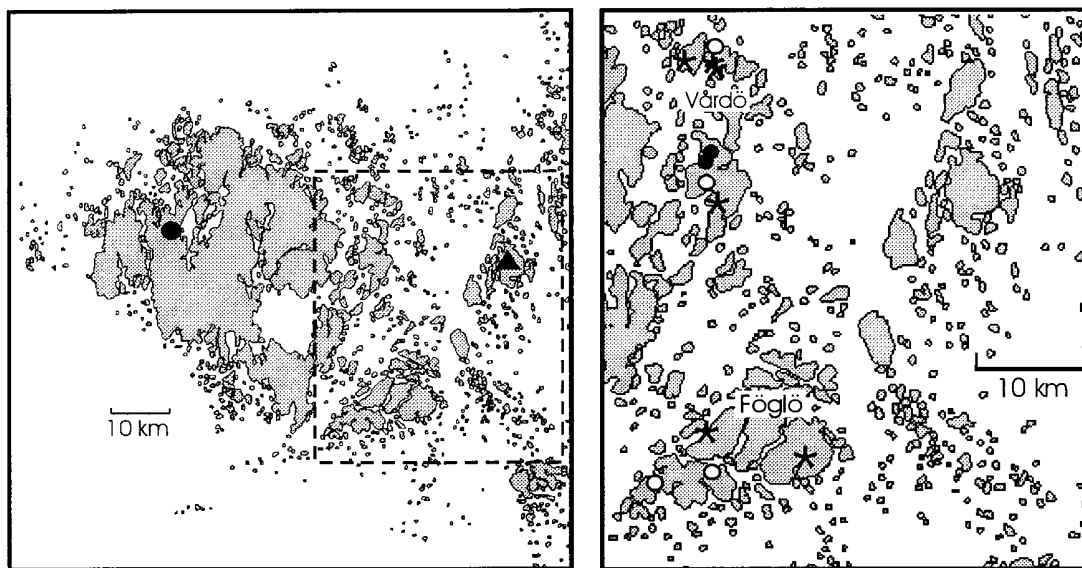


Figure 1: *Left*, Map of the Åland Islands in SW Finland. Symbols indicate the origins of the laboratory stock (*filled circle*, Hammarland, Sälis; *filled triangle*, Kumlinge). The dashed line shows the location of the map in the right panel. *Right*, Locations of the experimental populations: for crossbred experimental populations, *asterisks* = all produced offspring; for inbred experimental populations, *filled circles* = produced offspring, and *open circles* = no offspring).

is an appropriate species for such an experiment because of its metapopulation structure (Hanski 1999) and because of the previous observational findings (Saccheri et al. 1998). We established experimental populations in the field from laboratory-reared families of *M. cinxia*. Half of the populations were started with offspring of full-sib matings; the rest, with offspring of outbred matings. We recorded the subsequent performance of these experimental populations.

Material and Methods

Life Cycle of Melitaea cinxia

Larvae use two host plants in the Åland Islands in SW Finland, *Plantago lanceolata* and *Veronica spicata*. The habitat patches suitable for *Melitaea cinxia* (dry meadows) can be readily delimited based on the occurrence of the host plants (Kuussaari 1998). Eggs are laid in large batches, and the gregarious larvae spin a conspicuous web on the host plant. The suitable habitat patches are generally very small (0.14 ha on average; M. Nieminen, J. Pöyry, and I. Hanski, unpublished data), and hence, most local populations are also small, often consisting of just a single larval group (Hanski et al. 1995; Hanski 1999). The larvae diapause within densely spun webs called “winter nests,” which are easy to find in the field in early autumn. Larvae resume feeding in early April, and they remain gregarious until

the last instar. Based on 3 yr of data, the average size of postdiapause larval groups has been 22–42 larvae per group with a maximum of 183 larvae in one group (Kuussaari 1998). Larval survival increases with group size, and there is no reduction in survival in the very largest groups (Kuussaari 1998). The postdiapause larvae are relatively mobile, especially in the final instar when a large proportion of the growth takes place, and they can, therefore, find new host individuals within a distance of several meters. Females mate soon after emergence and typically only once in their lifetime (Kuussaari 1998). Though a substantial fraction of butterflies migrate during their lifetimes (Hanski et al. 1994; Kuussaari et al. 1996), most matings occur among butterflies in the natal population.

Laboratory Studies

A laboratory stock of *M. cinxia* was established by collecting mated females from two large metapopulations (Kumlinge and Sälis) in Åland in 1995 (fig. 1; table 1). In 1995, the maximum and median local population sizes were 20 and 2 in the Kumlinge metapopulation and 26 and 2.5 in the Sälis metapopulation. From 1993 to 1999, none of the habitat patches in Kumlinge were continuously occupied, whereas during this same period, two patches in Sälis have had a local population. One of these two patches, with six to 30 larval groups per year, is the source

Table 1: Numbers of individuals in different treatments

Treatment ^a	Grandmothers	Mothers	Mothers with offspring released to Föglö	Mothers with offspring released to Vårdö
Crossbred:				
Kumlinge × Sälis	12 ^b	19	7	13
Inbred:				
Kumlinge	5	13	6	7
Sälis	4	13	3	10

Note: Number of grandmothers (P-generation) and number of mothers (F₁-generation) gives the number of females used in rearings to produce the experimental material.

^a Individuals from Kumlinge originated from two source populations (no. 1286 and no. 1309) and those from Sälis originated from one source population (no. 22).

^b Six grandmothers from Kumlinge and six from Sälis.

population for this study. The numbers of larval groups in the two patches from which the Kumlinge sample was collected were three each in spring 1995. We started the laboratory rearings with two families from Kumlinge and six from Sälis, numbers that are within the normal range of population sizes in these populations. The first laboratory generation was reared by mating butterflies with individuals from their original population (grandmothers in table 1). In the next generation, female butterflies were mated either to brothers (inbred lines; table 1) or to males from the other population (crossbred lines; table 1).

The field sites available for experimental populations were so limited that we could not include within-population but between-family crosses in this experiment, which would have been helpful for the interpretation of the results. Other material was collected from the same two metapopulations as used here and from two other metapopulations in Åland in 1995. This material was used in a laboratory experiment on inbreeding depression in *M. cinxia* (S. Haikola, W. Fortelius, B. O'Hara, M. Kuussaari, N. Wahlberg, I. J. Saccheri, M. C. Singer, and I. Hanski, unpublished data). The results of these experiments showed no difference in egg-hatching rate between within-population, between-family crosses, and between-population crosses (Kruskal-Wallis test: $H = 1.89$, $N = 55$, $P = .170$), which differed from within-family crosses (Kruskal-Wallis test with within-population, between-family crosses and between-population crosses combined: $H = 16.4$, $N = 129$, $P = .0001$). Therefore, our results are unlikely to be due to any added positive effect of between-population outbreeding.

Crossbred and inbred offspring were reared following the same procedure. Mated females laid eggs on potted host plants (*P. lanceolata*), and egg batches were incubated under standard conditions (20°–24°C, 60%–80% rH). The egg-hatching rate of each egg batch was recorded. The larval families were regularly moved onto fresh potted host plants, thus providing unlimited food resources. When the larvae reached the overwintering instar and made the win-

ter nest for diapause, they were moved outside to an unheated shed and were thus exposed to seminatural diapause conditions during autumn and winter. At this point, the condition of the winter nest was examined, and the larval families were grouped according to the experimental design (see "Field Studies"). At the end of diapause in the following spring, all larvae were counted and weighed, and the dead ones were removed.

Some of the postdiapause larvae could not be used for the field experiment because equal numbers were required for each experimental population. These larvae were reared to adult butterflies in the laboratory to record their longevity. When butterflies hatched, they were individually marked, and their hatching date was recorded. Marked butterflies were placed in cages (diameter 41 cm, height 47 cm) in indoor conditions, protected from direct sunlight, and fed with an approximately 10% honey solution. Cages were monitored twice a day for dead butterflies.

Field Studies

The 12 habitat patches (dry meadows) used for the field experiment are located on large islands in the eastern Åland archipelago where no *M. cinxia* populations are currently present (fig. 1, Vårdö and southern Föglö). In a survey conducted in 1993, there was one local population in the middle of the study area in Föglö, and in 1993–1994, there was one population in the southwestern part of Vårdö. There was no difference in the average patch quality measures between these two study areas and those parts of Åland that are currently populated by *M. cinxia*. Therefore, we suggest that the current absence of *M. cinxia* from these areas is caused by geographical isolation and sparse habitat patch networks, which makes entire metapopulations vulnerable to extinction (Hanski 1999), rather than to unsuitable habitat for *M. cinxia*. Out of the suitable habitat patches on the study islands, we selected the habitat patches with the highest quality, taking into consideration

the patch area, the amount of host plants, and an index of overgrowth.

The experimental populations were randomly assigned to the 12 habitat patches (four patches with crossbred and four patches with inbred experimental populations in Vårdö, two patches with crossbred and two patches with inbred experimental populations in Föglö; fig. 1; table 1). The experimental populations each consisted of three larval families of originally 100 larvae each, which gave a total of 300 larvae per experimental population before larvae were exposed to diapause mortality. Larval families were unrelated in crossbred experimental populations. In the inbred experimental populations, larval families were highly related, usually with sib-mothers all mated to their brothers (in some groups the sib-mated mothers were first cousins). After considerable mortality during diapause in some of the families, the larval number in each experimental population was standardized to the lowest number then possible, which was 178 larvae. Because of mortality, two of the original families were very small (or nonexistent) after diapause and so were combined with one of the larger larval groups in the same family. Therefore, the average larval group sizes in the experimental populations were 59 (three groups per experimental population; 10 populations) or 89 (two groups per experimental population; two populations) larvae. These figures are higher than the average postdiapause larval group sizes but much lower than the maximum group sizes observed in the field (see "Life Cycle of *Melitaea cinxia*").

The experimental populations were introduced to the habitat patches on April 11 and 12, 1998. Host plants onto which the larvae were introduced were selected because they were within dense stands and surrounded by low vegetation, a microhabitat that egg-laying females prefer (M. Kuussaari, unpublished data; M. Nieminen, M. C. Singer, W. Fortelius, K. Schöps, and I. Hanski, personal observations). The larvae were counted in the field on either April 27 or April 28.

Adult butterflies were captured and marked in the eight populations on the island of Vårdö between June 7 and June 25 (fig. 1). Each patch was visited on every sunny day with constant searching effort per unit space (1 min/100 m²). Because of the exceptionally cloudy and rainy weather in June 1998, there were only 7 d on which butterflies could be captured.

The breeding success in the 12 experimental populations in the first field generation was examined in the beginning of September 1998, when the surviving larval groups had built the conspicuous winter nest and are easiest to survey (M. Nieminen, J. Pöyry, and I. Hanski, unpublished data). The experimental patches and their surroundings within a 1-km radius were searched thoroughly. The overwintering success in the field was recorded in late April 1999.

The surviving larvae were collected to restore the pre-experimental unoccupied status of the habitats.

Statistical Analyses

We used parametric ANOVA when data were approximately normally distributed for testing categorical data, and if this expectation was not fulfilled, we used the Kruskal-Wallis nonparametric test. For testing differences in two-by-two tables, we used Fisher's exact test. Logistic regression was used to test for differences between proportions. We tested the causes of mortality during diapause with generalized linear modeling (accumulated analysis of deviance; see McCullagh and Nelder 1983), which is analogous to log-linear models. We only analyzed the full model. A generalized linear model was performed with GENSTAT 5 (version 3.2; GENSTAT 5 Committee 1987); two-factor ANOVAs, with SYSTAT 7 (Wilkinson 1993); and all other statistical tests, with Statistix 4.1 (Analytical Software, Tallahassee, Fla.).

Results

Laboratory Studies

The egg-hatching rate was significantly lower in egg batches laid by inbred females (median = 70.7%) than it was in those laid by crossbred females (median = 89.8%; table 2), and it did not differ significantly between inbred lines from the two source populations (table 2). The integrity of the winter nest is important for the overwintering survival of *Melitaea cinxia* larvae, which promptly attempt to repair a broken nest (Kuussaari 1998). In our experiment, winter nests were perforated significantly more often in inbred larval groups than in crossbred larval groups, evidence of poor winter-nest construction in the inbred groups (table 2). In a more conservative analysis, in which larval groups were pooled within families, there was still a difference between inbred and crossbred larval groups (table 2). This result indicates reduced larval activity and capacity to construct a high-quality winter nest in inbred larval groups.

Larval survival through diapause did not differ significantly between inbred lines from the two source populations (table 2). The average percentage of dead larvae after diapause was higher in inbred than in crossbred families: 13% of larvae in the inbred groups (range: 0%–100%; SD = 25.1) and 0.5% in the crossbred groups (range: 0%–2.2%; SD = 0.812) died during the winter. The difference is highly significant (table 3). Independent of the association with inbreeding, the numbers of dead larvae following diapause significantly increased with perforation of winter nests (table 3). The variances of inbred and

Table 2: Results of statistical tests

Variable	Test	Inbred sample size			Crossbred sample size	df	Test statistic	P
		Kumlunge	Sälis	Total				
Laboratory studies:								
Egg-hatching rate between inbred origins ^a	Kruskal-Wallis	13	13	26	2.45	.118
Egg-hatching rate between inbred and crossbred	Kruskal-Wallis	13	13	26	19	...	16.7	<.001
Nests with hole(s) vs. nests without holes	Fisher's exact test	11	12	23	21006
Families with perforated nest(s) vs. families with intact nests(s) only	Fisher's exact test	9	9	18	18008
Mortality of larvae in diapause between inbred origins ^{a,b}	Logistic regression	9	9	18	...	16	.95	.342
Average dry weight of dead larvae in inbred vs. crossbred families ^b	ANOVA	5	9	14	5	1, 17	15.9	.001
Average dry weight of dead larvae between inbred origins ^{a,b}	Kruskal-Wallis	5	9	1402	.894
Average weight of surviving larvae in inbred vs. crossbred families ^b	ANOVA	9	9	18	18	1, 34	1.70	.202
Average weight of surviving larvae between inbred origins ^{a,b}	ANOVA	8	9	17	...	1, 16	.71	.412
Longevity of inbred vs. crossbred butterflies ^c	ANOVA	52	21	1, 70	2.19	.143
Longevity of female vs. male butterflies ^c	ANOVA	28/24	15/6	1, 70	15.7	<.001
Field study:								
Breeding success: inbred vs. crossbred experimental populations	Kruskal-Wallis	3	3	6	6	...	6.47	.011

Note: Parametric ANOVA was used with normally distributed data and Kruskal-Wallis nonparametric test with data with skewed distribution. Logistic regression was used for testing proportions.

^a Tests between inbred origins were performed to test for the possible difference between inbred lines originating from different source populations.

^b Sample size gives the number of larval groups.

^c The source populations of inbred butterflies were pooled in rearing.

crossbred treatments in table 3 differ significantly from each other (ratio of variances is 32.8; $P \ll .0001$, F -test with 31, 31 df), as the crossbred treatment survived so well. In our test, we used the average dispersion parameter, but the result is not dependent on this. If we had used the dispersion parameter estimated for crossbred treatment separately (0.644), all factors in table 3 would have been significant; and if we had used the dispersion parameter estimated for the inbred treatment separately (21.1), the three factors that are now significant still would have been significant. Furthermore, when we compare the deviances of the different factors, it is evident that factors "died" (which is not interesting in this context), "died \times holes," and "died \times inbred" have by far the largest deviances; that is, they are by far the most significant factors.

The average dry weight of larvae that died during the diapause was significantly lower in inbred than in crossbred families (inbred: 1.0–2.1 mg, mean 1.7 mg, SD = 0.31; crossbred: 2.1–2.6 mg, mean 2.4 mg, SD = 0.21; table 2). In contrast, the average weight of surviving larvae did not differ between the two groups (inbred: 3.2–5.9 mg, mean 4.3 mg, SD = 0.72; crossbred: 2.6–5.5 mg, mean

4.0 mg, SD = 0.66; table 2). The significant difference in the weights of dead larvae between the treatments suggests different causes of death in the two groups. Neither the dry weight of dead larvae nor the weight of surviving larvae differed between inbred lines from the two source populations (table 2).

The overall longevity of butterflies reared from the post-diapause larvae not used in the field experiment did not differ between inbred and crossbred butterflies, but females lived significantly longer than males (table 2). The interaction between treatment and sex was not significant.

Field Studies

The numbers of larval groups and the number of larvae per experimental population, which were counted 16 d after the release, did not differ between inbred and crossbred populations. Inbred and crossbred experimental populations did not differ significantly in the numbers of marked butterflies, the numbers of recaptured butterflies, nor in the longevity of butterflies. However, the sample size remained so small (95 marked and 21 recaptured but-

Table 3: Statistical tests for the causes of mortality during diapause (generalized linear model: accumulated analysis of deviance)

Source	df	Deviance	Mean deviance	Deviance ratio	<i>P</i>
Died	1	2912.291	2912.291	293.40	<.001
Holes	1	12.828	12.828	1.29	.260
Inbred	1	4.901	4.901	.49	.485
Died × holes	1	200.653	200.653	20.22	<.001
Died × inbred	1	85.720	85.720	8.64	.005
Holes × inbred	1	10.580	10.580	1.07	.306
Died × holes × inbred	1	15.587	15.587	1.57	.215
Residual	64	635.255	9.926
Total	71	3877.815	54.617

Note: Variables: “died” = died versus survived larvae, “holes” = perforated versus solid winter nests, and “inbred” = inbred versus crossbred treatments.

terflies), because of the exceptionally poor weather in June 1998, that the tests have very limited statistical power.

All six crossbred populations but only two inbred populations produced offspring that survived until late summer 1998 (fig. 1). The numbers of larval groups per population were significantly higher in crossbred populations (numbers of larval groups: 1, 1, 1, 2, 2, and 5) than in inbred populations (numbers of larval groups: 0, 0, 0, 0, 1, and 1; table 2). After diapause, larvae were found in four crossbred populations, but both larval groups in the inbred populations that had existed in the previous autumn had disappeared.

Discussion

The results show a significant difference in the performance of experimental inbred and crossbred populations, which supports the previous observational results on inbreeding-related extinctions in *Melitaea cinxia* (Saccheri et al. 1998) and highlights the potential importance of inbreeding depression in the dynamics of small and isolated local populations. Because many formerly continuous habitats are becoming increasingly fragmented (e.g., McNeely et al. 1995), thus forcing many species and populations to live in small and isolated habitat patches, inbreeding depression poses a potential threat to many taxa. Inbreeding depression may have particularly strong negative effects in populations with minimal inbreeding in the past, thus making populations currently experiencing fast habitat fragmentation especially vulnerable. Such strong effects of inbreeding depression occur frequently in artificial selection (Barrett and Charlesworth 1991). More research is needed to determine the frequency of substantial inbreeding depression in populations living in fragmented landscapes, but in the mean time, those involved

in conservation and habitat management should consider inbreeding depression to be a potentially serious threat.

Inbreeding depression may be a particularly potent threat in insect species, such as *M. cinxia*, with gregarious larvae: because larvae in the same group are generally the offspring of one female, the effective population sizes tend to be extremely small, which may practically force breeding among close relatives in small populations. It was also evident that inbreeding depression strongly affected the integrity of winter nests, which are built cooperatively by the larvae, and that this reduction in winter-nest quality seriously affected the overwintering success of larvae (table 3). We are not aware of previous findings of this type, which suggests that inbreeding may have an especially strong impact in species with social cooperation.

Environmental stress often interacts with inbreeding depression, and when these factors act together, they may amplify the extinction rates of local populations (Gilpin and Soulé 1986; Keller et al. 1994; Bijlsma et al. 1997). In our study, there was a great difference between the inbred and crossbred treatments in larval survival during winter diapause, which may be one of the most stressful periods in the life cycle of *M. cinxia*. Furthermore, the very poor breeding success in the field of the inbred populations probably was caused by a low-egg-hatching rate, which is reduced by 25%–50% by just one generation of inbreeding in *M. cinxia* (Saccheri et al. 1998; S. Haikola, W. Fortelius, B. O'Hara, M. Kuussaari, N. Wahlberg, I. J. Saccheri, M. C. Singer, and I. Hanksi, unpublished manuscript). A large decrease in egg-hatching rate as a result of inbreeding has been previously detected in the butterfly *Bicyclus anynana* (Saccheri et al. 1996), which shows that inbreeding has similar effects in various butterflies. The weather was exceptionally rainy during the flight season of *M. cinxia* in 1998, which may have restricted mating and oviposition and perhaps adversely affected the already small larval groups in the inbred exper-

imental populations. The rainy weather may have decreased the breeding success of the experimental crossbred populations, too, even though it was significantly higher than the breeding success of the inbred populations. Furthermore, it is possible that laboratory-reared individuals do relatively poorly in nature on average, especially when the environmental conditions are unfavorable.

Saccheri et al. (1998) found that the larval weight in the spring was positively correlated with individual heterozygosity in *M. cinxia*. In this study, we found a difference in the weights of larvae that died during winter diapause: dead inbred larvae were lighter than dead crossbred larvae, although there was no difference among the living larvae. This result suggests that the growth rate of many inbred larvae was severely reduced, and thus the strongest effect of inbreeding on larval survival seems to occur during the winter diapause. The difference in the results of the two studies may have resulted from differences in the treatments of the larvae: we reared larvae in seminatural outdoor conditions during the winter, whereas Saccheri et al. (1998) kept diapausing larvae in constant laboratory conditions, which may have facilitated the survival of inbred larvae over diapause.

The increased extinction risk of inbred populations observed in this study has several implications for metapopulation dynamics. If all or most local populations in a metapopulation are small, the entire metapopulation might suffer from an increased extinction risk caused by inbreeding effects. Increased population extinctions caused by inbreeding would reduce the total number of migrants, the potential colonizers, within a metapopulation, which, in turn, would decrease the colonization probability of unoccupied habitat patches. Moreover, inbreeding may affect the colonization rate by making successful population establishment less likely: many new small populations remain short lived, unless they are rescued demographically and genetically by immigrants from other, genetically more diverse local populations. In urgent conservation situations, such genetic rescue could be achieved by judicious translocation of individuals in order to increase genetic variability and to reduce the adverse effects of inbreeding (Madsen et al. 1999). All metapopulation dynamical consequences of inbreeding increasing extinction risk would be especially critical in metapopulations close to the minimum viable metapopulation size (Hanski et al. 1996), where any even slightly negative factor could turn the population trajectory toward metapopulation extinction.

In summary, the most severe effects of inbreeding depression in small *M. cinxia* populations are manifested during the winter diapause and at reproduction, when low-egg-hatching rate in inbred populations result in small larval groups, which are known to have reduced survival (Kuussaari 1998). Consequently, inbreeding creates a neg-

ative cascade effect that is amplified by environmental stress: low-egg-hatching rate in inbred females leads to small larval groups, which leads to low survival of larvae, especially under unfavorable conditions. Therefore, genetic, demographic, and environmental causes of local extinction cannot be easily separated in *M. cinxia* nor, most likely, can they be in many other species with small local populations.

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