

Effects of inbreeding in the Glanville fritillary butterfly (*Melitaea cinxia*)

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Previous studies suggest that inbreeding depression may be strong enough to elevate the rate of extinction of local populations in a metapopulation of the Glanville fritillary butterfly. Laboratory experiments were conducted to assess the consequences of inbreeding for fitness components through two generations of inbreeding. Clutch size, egg hatching rate, survival of the progeny until diapause and larval weight at diapause were measured in crosses between individuals from different small populations and in within-family crosses. The magnitude of inbreeding depression did not differ significantly between the two generations, with the exception that there was a marked increase in the proportion of pairs that failed to produce any offspring in the second generation of full-sib mating. As expected, the effect of inbreeding on clutch size was evident only in the second generation, where the parents themselves were highly inbred. Inbreeding depression of the magnitude reported here can explain the increased extinction risk of natural small populations of the Glanville fritillary butterfly.

Introduction

Inbreeding has adverse effects on fitness-related traits in various taxa (Frankel & Soulé 1981: pp. 59–77, Charlesworth & Charlesworth 1987, Crnokrak & Roff 1999, Hedrick & Kalinowski 2000, Keller & Waller 2002, for a review), including many insect species (e.g. Frankham *et al.* 1993, García *et al.* 1994, Roff 1998, Higashiura *et al.* 1999, Armbruster *et al.* 2000, Saito *et al.* 2000, van Oosterhout *et al.* 2000, Bijlsma *et al.* 2000). Habitat loss and fragmentation reduce the sizes and increase the level of isolation of populations in many species, which exposes them to elevated levels of inbreeding (Frankham 1995). Accordingly, the effects of

inbreeding in species living in fragmented landscapes have been the focus of several studies in past years (Saccheri *et al.* 1998, Richards 2000, Haag *et al.* 2002, *see also* Ingvarsson 2001). The dynamics of genetic load and inbreeding pose especially challenging questions in the case of metapopulations consisting of many small, extinction-prone local populations.

The Glanville fritillary butterfly (*Melitaea cinxia*) is an endangered species in Finland, where it is restricted to the Åland Islands in the northern Baltic (Marttila *et al.* 1990, Hanski 1999: pp. 207–232). The species has a one-year life cycle in Finland. Adult butterflies fly in June (Kuussaari 1998), females typically mate only once (Kuussaari *et al.* 1998), and they oviposit in

large clusters of 100–200 eggs. The larvae hatch in July and live in sib-groups until the last larval instar in the following spring. For winter, the larvae spin a conspicuous winter web in which they diapause (Kuussaari 1998). In the Åland Islands, *M. cinxia* forms a large metapopulation of several hundred local populations living in small discrete patches of habitat (Hanski *et al.* 1995, Hanski 1999: pp. 207–232). Local populations are typically small and often isolated; many of them consist of only one larval family, such that all individuals in the given habitat patch are siblings (Hanski *et al.* 1995, Hanski 1999: pp. 207–232). Therefore, inbreeding is likely to occur regularly in this metapopulation, especially because females do not discriminate against siblings as mates (S. Haikola *et al.* unpubl. data). Given the very small size of most local populations and their frequent turnover (Hanski *et al.* 1995), one could assume that selection has purged most of the deleterious alleles causing inbreeding depression (e.g. Frankel & Soulé 1981: pp. 59–77, Hedrick 1994, Lacy & Ballou 1998). However, this is not the case: prior work shows that substantial genetic load remains in this metapopulation (Haikola *et al.* 2001).

Previous laboratory experiments on *M. cinxia* have demonstrated that one generation of within-family mating substantially lowers egg hatching rate and that larval survival over diapause is negatively affected by inbreeding (Haikola *et al.* 2001, Nieminen *et al.* 2001). Laboratory tests indicated lowered mating success in inbred populations (Haikola *et al.* 2001), and field observations suggested that inbred individuals have a shorter life-span than outbred individuals (Saccheri *et al.* 1998). The effects of inbreeding appear to be severe in natural populations, as Saccheri *et al.* (1998) found that the extinction risk of local populations in the Åland Islands is significantly correlated with reduced heterozygosity in small populations. The increased extinction risk due to inbreeding has also been confirmed experimentally (Nieminen *et al.* 2001).

The aim of this study was to carry out an inbreeding experiment on *M. cinxia* for two consecutive generations of within-family mating, and to clarify the life-history stages at which the adverse effects of inbreeding are manifested. Theoretically, if the value of a trait showing

inbreeding depression is due to additive effects of loci (i.e. no epistasis), the mean value of the trait should decrease in direct proportion to the inbreeding coefficient (Falconer & Mackay 1996: pp. 247–262). There is also the fundamental difference between the first two generations of inbreeding in that only in the second generation are the parents inbred (although in the present case there has probably been some background inbreeding in the populations before collection of the material). The question of the increase in inbreeding depression with increasing inbreeding is relevant for the dynamics of the Åland metapopulation of *M. cinxia*, where newly-founded populations often remain very small for a couple of generations. In the present study I examined the effect of inbreeding on clutch size and egg hatching rate (which have been the main focus of previous laboratory studies on *M. cinxia*), and early larval survival and growth (which have not been examined before in this context) during two generations of within-family crosses. The expectation was that the effect of inbreeding would be even more severe in the second than in the first generation as purging of the genetic load is unlikely to be effective during one generation of inbreeding under favourable laboratory conditions.

Material and methods

Laboratory experiments

Post-diapause *Melitaea cinxia* larvae were collected from natural populations in the Åland Islands in the spring of 1999. Five larvae were taken from one larval group from each of several small populations widely across the Åland Islands (distances between crossed small populations ranged from less than a kilometre to almost 40 kilometres). The populations consisted of one or two larval families. Some of these populations were newly-founded, some had been small for at least two generations, and for a few small populations the population history was unknown. In addition, five groups of larvae were collected from five large control populations. These samples consisted of 40 larvae from many families. Larvae were reared to butterflies in the laboratory, at Tvärminne Zoological Station, Finland.

Butterflies were mated in cylindrical net cages (diameter 41 cm, height 47 cm) under artificial light. Three types of crosses were made: (1) crosses within families from small populations, (2) crosses between individuals from different small populations, and (3) crosses between individuals from different large control populations (the sample sizes in each type of cross are shown in Table 1). Every butterfly mated only once, with two exceptions (two males mating twice). The progeny of the crosses within families and the crosses between different populations were used to establish the inbred and outbred lines, respectively.

Five types of crosses were conducted in the second generation: (1) the second generation of crosses within families, (2) the second generation of crosses between populations (i.e. individuals from crosses between small populations were crossed with each other), (3) crosses between inbred sons from sib-matings and outbred females (i.e. females from crosses between small populations), (4) crosses between outbred males (i.e. males from crosses between small populations) and inbred daughters from sib-matings, and (5) crosses between inbred individuals from different populations (the sample sizes in each type of cross are shown in Table 2). In cross types 2 to 5 there were no common populations in the background of the paired individuals. Every butterfly mated only once.

Mated females were placed to lay eggs in small net cages with a host plant, *Plantago lanceolata* or *Veronica spicata*. Plants were checked every second day for eggs and each egg batch was removed to a Petri dish. The dishes were kept at 20–25 °C and 70%–80% relative humidity. After a couple of days eggs were spread out and counted from an enlarged photocopy (Haikola *et al.* 2001). In the first generation, the larvae were counted with the same technique as the eggs three days after hatching. Egg hatching rate was calculated as proportion of eggs that produced a larva. The newly-hatched larvae were fed with cut *Plantago* leaves on a Petri dish. At the age of about one week the groups of larvae were moved to feed on potted host plants. In the second generation, larvae were counted while moving them to potted host plants soon after hatching. Larvae were reared at 20–30 °C and 20%–80% relative humidity. Large variation in temperature and humidity was due to the impact of outdoor weather conditions on the conditions in the rearing room. As soon as the larvae were ready for diapause, they were counted and each larval family was weighted. The weight of each family was divided by the larval number to obtain the average larval weight. The larvae diapaused at +2 °C and 80%–100% relative humidity. In the first generation all clutches were raised to diapause, whereas in the second generation only the first two clutches for each female were reared until diapause.

Table 1. Clutch size, average egg hatching rate (%), survival (%) until diapause, and larval weight (mg) at diapause in the first generation. All females and clutches are included. Means in parentheses are for females that produced > 1 larvae (the values are means for females, not weighted by the number of clutches; survival rates and weights include only the first two clutches of each female). “Zeros” gives the number of females that produced ≤ 1 larvae over the total number of females.

Trait	Within-family crosses ¹			Crosses between small populations ²			Crosses between control populations ³		
	Mean	SD	N	Mean	SD	N	Mean	SD	N
Clutch size	156 (186)	81	62	124 (143)	80	49	128 (131)	61	7
Egg hatching rate	34 (43)	29	62	44 (58)	34	49	59 (66)	31	7
Survival	38 (48)	25	48	51 (59)	31	37	63 (71)	23	6
Larval weight	7.6 (7.6)	1.4	43	8.6 (8.9)	1.2	31	8.6 (8.8)	2.0	6
Zeros			4/25			7/22			0/4

¹ 25 females, 25 different populations (families).

² 22 females, 22 different population combinations, 32 different populations.

³ 4 females, 4 different population combinations, 5 different populations.

Table 2. Clutch size, average egg hatching rate (%), survival (%) until diapause, and larval weight (mg) at diapause in the second generation. All females and clutches are included. Means in parentheses are for females that produced > 1 larvae (the values are means for females, not weighted by the number of clutches). "Zeros" gives the number of females that produced ≤ 1 larvae over the total number of females.

Trait	Within-family crosses/ 2nd gen ¹		Crosses between small populations/2nd gen ²		Inbred male × Outbred female ³		Outbred male × Inbred female ⁴		Inbred male × Inbred female ⁵					
	Mean	SD	N	Mean	SD	N	Mean	SD	Mean	SD	N			
Clutch size ⁶	97 (103)	58	12	128 (134)	75	60	140 (155)	49	131 (125)	61	8	53	32	12
Egg hatching rate	26 (47)	31	12	45 (47)	36	61	40 (60)	37	19 (28)	25	9	0	0	12
Survival	42 (50)	32	5	57 (57)	30	36	65 (76)	31	65 (69)	13	6	6	6	6
Larval weight	6.2 (6.4)	1.1	5	6.4 (6.3)	1.2	30	6.7 (6.7)	1.2	6.0 (5.8)	1.4	6	6	1.4	6
Zeros			3/7			4/25					0/4			3/3

¹ 7 females, 5 different families.

² 25 females, 21 different crosses.

³ 5 females, 5 different crosses.

⁴ 4 females, 4 different crosses.

⁵ 3 females (all from the same family), 3 different crosses.

⁶ Two egg batches of 423 and 452 eggs, most probably consisting of two separate clutches each, are not included.

Statistical analyses

Rearing of *M. cinxia* in the laboratory is hampered by several problems. Larval mortality during diapause is high and it is difficult to get butterflies mated and the mated females to lay eggs without bright natural light. There was a tendency for the clutch size to become smaller and the egg hatching rate and larval survival to become lower with increasing time between mating and oviposition. For this reason, the time from mating to oviposition (oviposition delay) was included as an explanatory factor in all analyses comparing the two generations. Females that produced ≤ 1 hatched larvae were excluded, since fertilisation may have been unsuccessful in these cases (females may lay clutches of reasonable size even if they are not fertilised). As only the first two clutches for each female were raised to diapause in the second generation, any comparison of larval survival and weight between the generations is restricted to the first two clutches, although results for more clutches were available for several females in the first generation. Two egg clutches in the data set were exceptionally large, 423 and 452 eggs, and probably contained two separate clutches each (in a large data set of 250 *M. cinxia* egg clutches the biggest clutch had 280 eggs; M. Saastamoinen & I. Hanski, unpubl. data). These two clutches were excluded from all analyses concerning clutch size.

The effect of the oviposition delay

The effect of the oviposition delay on clutch size, egg hatching rate and larval survival in the within-family and between-population crosses were analysed with linear regression. To meet the assumption of normality, the arcsin square root transformed egg hatching rate and larval survival were used. In the analysis of clutch size and egg hatching rate the oviposition delay was used as an independent variable, in the analysis of survival rate both the larval number and the oviposition delay were used as independent variables. The larval number was included as a variable in the model as a previous study by Kuussaari (1998) shows that early larval survival increases with larval group size. Both treatment types were analysed separately in each generation.

I separately assessed whether there was any difference in the effect of the oviposition delay on clutch size and egg hatching rate between the within-family and between-population crosses in the first generation. The effect on clutch size was analysed with a linear mixed model and the effect on egg hatching rate with a generalised linear mixed model assuming binomial distribution and using logit link function (S-PLUS 6.1.2, McCullagh & Nelder 1989, Venables & Ripley 2002). The oviposition delay, treatment (cross type) and their interaction were used as fixed factors and female identity as a random factor in the models.

Comparison of the effects of inbreeding between the two generations

Differences in clutch size and larval weight between the within-family and between-population crosses and between the first and second generations were analysed in the combined data set with a linear mixed model. Differences in egg hatching rate and larval survival to diapause were analysed using a generalised linear mixed model assuming binomial distribution and using logit link function. The oviposition delay, generation, treatment (within-family versus between-population cross) and generation \times treatment interaction were used as fixed factors and female identity as a random factor in all models.

The importance of the inbreeding history of the two sexes

To find out whether the inbreeding history of the male or the female had an effect on the traits studied, I analysed possible differences in clutch size, egg hatching rate, larval survival and weight between the cross types 3 and 4, in which only the male or the female was inbred (*see* section Laboratory experiments above). The clutch size and larval weight were analysed with a linear mixed model and the egg hatching rate and larval survival with a generalised linear (mixed) model in the same way as the differences between the generations above. The treatment (cross type) was used as a fixed factor and female identity as a random factor in the models. In the survival

rate analysis, approximate 95% confidence intervals for the female effect (SD) ranged from 0 to infinite, hence female identity was not included as a factor in the final analysis of this trait. To take into account overdispersion, quasi distribution family with variance defined as $\mu(1 - \mu)$ (μ = the expected response) was used, and the significance of the cross term was assessed with an F -test. To keep the model relatively simple and because there were no significant effects of the oviposition delay on the trait values in this data set, the oviposition delay was not included as a factor in these analyses.

Results

The effect of the oviposition delay

In the first generation, the decrease in clutch size and egg hatching rate with increasing time since mating were significant both in the within-family ($t_{50} \approx -3.10$, $p \approx 0.003$ and $t_{50} \approx -5.72$, $p < 0.001$ for clutch size and egg hatching rate, respectively) and between-population ($t_{35} \approx -3.26$, $p \approx 0.003$ and $t_{35} \approx -3.12$, $p \approx 0.004$) crosses (Fig. 1). In the second generation, the oviposition delay had no effect on clutch size nor egg hatching rate in the within-family matings, but here the sample size was small (Fig. 1). In the between-population crosses, the patterns were the same as in the first generation ($t_{53} \approx -4.96$, $p < 0.001$ and $t_{54} \approx -2.96$, $p \approx 0.005$).

In the first generation, though larval survival decreased with increasing time from mating to egg laying in both treatments, the effect was significant only in the within-family crosses ($t_{44} \approx -3.32$, $p \approx 0.002$). The number of larvae in the clutch had a small positive effect on larval survival in the crosses between populations in both the first ($t_{32} \approx 3.03$, $p \approx 0.005$) and the second ($t_{32} \approx 3.05$, $p \approx 0.005$) generation. No effect of larval number on survival was found in the within-family matings. In the second generation, the oviposition delay did not significantly affect larval survival in either treatment.

The effect of the oviposition delay on clutch size or egg hatching rate did not differ between the within-family and between-population crosses (non-significant oviposition delay \times treatment

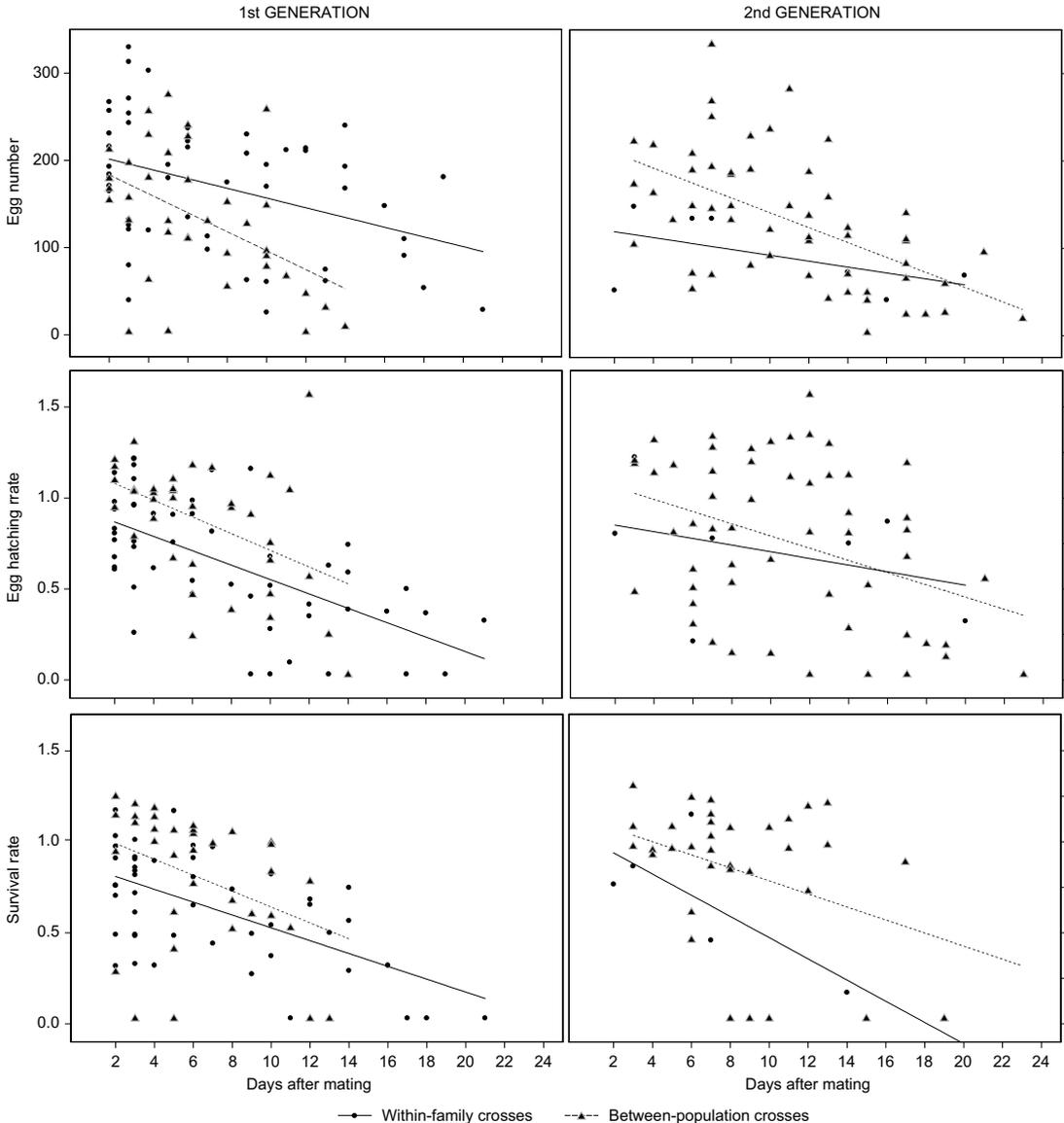


Fig. 1. The relationship between the time from mating to oviposition and egg number, arcsin square root transformed egg hatching rate and larval survival in the within-family and between-population crosses in the first (left panels) and second (right panels) generations. Females that produced ≤ 1 hatched larvae are excluded.

interactions, $F_{1,51} \approx 3.00$, $p \approx 0.09$ and $F_{1,51} \approx 0.774$, $p \approx 0.383$, in the analyses of clutch size and egg hatching rate, respectively).

Inbreeding depression in the two generations

The mean egg hatching rate and larval survival

were lower for the within-family than between-population crosses in both generations (Tables 1, 2 and Figs. 2, 3). Including all females and all clutches, the percentage inbreeding depression (inbred compared with outbred individuals in the same generation) in egg hatching rate was 24% in the first and 43% in the second generation. The reduction in larval survival due to inbreeding was the same (27% and 25%) in the first and

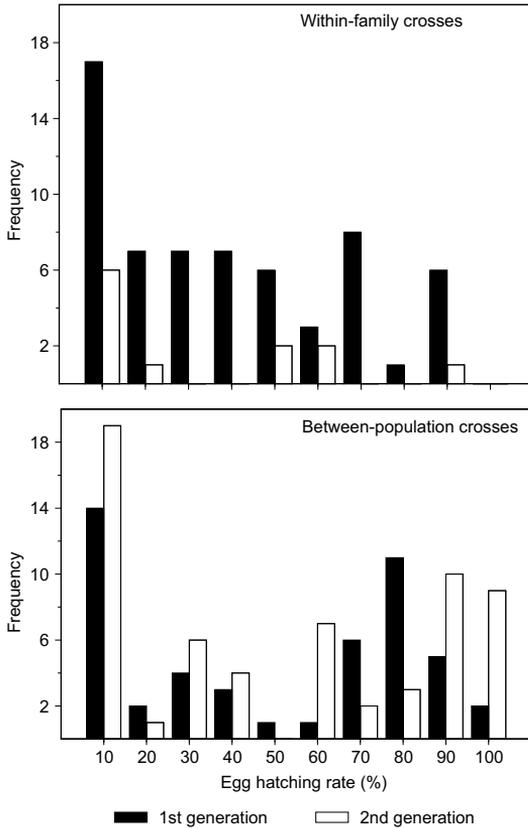


Fig. 2. Egg hatching rate in the within-family crosses in the first and second generations, and in the between-population crosses in the first and second generations. All females and clutches are included.

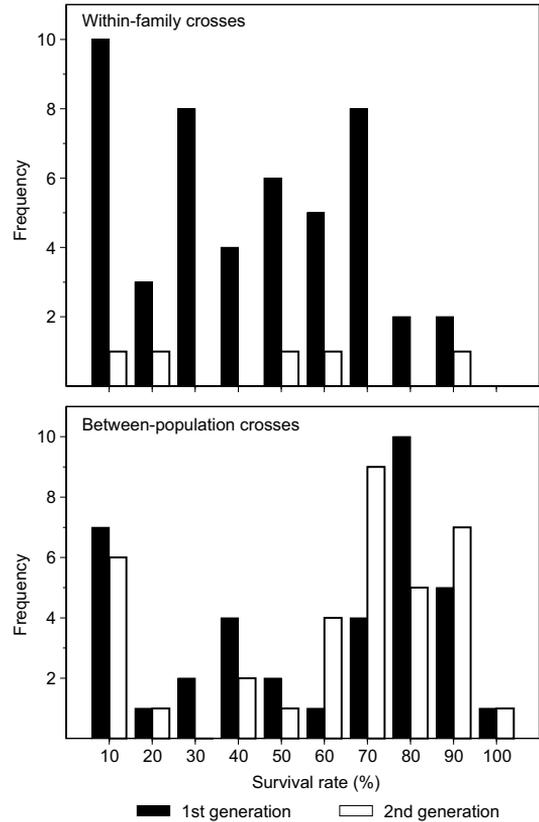


Fig. 3. Larval survival until diapause in the within-family crosses in the first and second generations, and in the between-population crosses in the first and second generations. All females and clutches are included.

the second generation. Inbreeding decreased average larval weight by 11% in the first and 3% in the second generation (the coefficients of inbreeding depression = 1 - (inbred trait value/outbred trait value) (Lande & Schemske 1985) are shown in Table 3). Unfortunately, the estimates for the second generation are based on small numbers of inbred crosses (Table 2), partly due to a large fraction of females that produced no eggs that would hatch.

In the comparison of clutch size between the two generations, the interaction between the generation and treatment terms was significant, as the mean clutch size was larger in the within-family crosses than in the between-population crosses in the first generation, but smaller in the second generation (Table 4; note that females that produced ≤ 1 hatched larvae were excluded from the analysis, *see* means in brackets in

Tables 1 and 2). The average clutch size was particularly small for cross type 5, in which both parents were inbred, in the second generation (Table 2). The clutch size did not differ between cross types 3 and 4 in the second generation ($F_{1,4} \approx 0.557, p \approx 0.497$).

Table 3. Coefficient of inbreeding depression ($\delta \pm SE$) in clutch size, egg hatching rate, survival and larval weight in the first and second generations. The standard errors of the coefficients have been estimated by bootstrapping the data 10 000 times.

	1st generation	2nd generation
Clutch size	-0.26 (0.15)	0.24 (0.14)
Egg hatching rate	0.24 (0.12)	0.43 (0.20)
Survival	0.27 (0.10)	0.25 (0.24)
Larval weight	0.11 (0.03)	0.03 (0.08)

The egg hatching rate was higher in the between-population than in the within-family crosses (significant at the level of 0.1, non-significant interaction term (*see below*) excluded: $F_{1,58} \approx 3.90, p \approx 0.053$). The magnitude of inbreeding depression in this trait did not differ between the two generations (non-significant generation \times treatment interaction, Table 4). The egg hatching rate was substantially higher in cross type 3, in which the male was inbred, than in cross type 4, in which the female was inbred, but the difference was not statistically significant ($F_{1,5} \approx 1.97, p = 0.219$). The proportion of females that produced ≤ 1 larvae (sterile pairings) was very high (60%) in the inbred crosses (cross types 1 and 5) in the second generation (Tables 1 and 2). The generation \times treatment (cross types 1 and 5 versus cross type 2) \times sterility interaction was significant in a log-linear model ($p \approx 0.04$ for the next best model generation \times treatment + sterility, likelihood $\chi^2_3 = 8.36$).

Larval survival rate was significantly higher in the between-population than in the within-family crosses ($F_{1,58} \approx 11.9, p = 0.001$, non-significant interaction term (*see below*) excluded). Again, however, the severity of inbreeding depression did not differ between the generations (Table 4). There was no significant difference between cross types 3 and 4 ($F_{1,10} \approx 2.34, p \approx 0.157$).

Larval weight at diapause differed between the treatments (Table 4). The difference between the treatments (greater larval weight in between-population crosses, non-significant interaction

term (*see below*) excluded: $F_{1,55} \approx 6.17, p \approx 0.016$) is due to a difference in the first generation, whereas in the second generation the treatments did not differ from each other. The generation \times treatment interaction is not quite statistically significant (Table 4). This analysis is hampered by the small number of observations in the within-family crosses in the second generation. The larval weights did not differ between cross types 3 and 4 ($F_{1,5} \approx 0.811, p = 0.409$).

Discussion

In the first generation of full-sib crosses and crosses between small populations, the adult individuals themselves did not differ systematically between the treatments in terms of their presumed level of inbreeding. This means that any differences in the reproductive success between the treatments due to parental inbreeding (van Noordwijk & Scharloo 1981, Keller 1998, Su *et al.* 1996) would only be seen in the second generation. One could assume that possible inbreeding depression in early zygote viability due to zygotes' own properties would also be more severe in the second generation of within-family crosses, where the zygotes are more inbred. In the present results, the mean clutch size was much lower in the within-family than between-population crosses in the second generation. This is what we expect if clutch size is to a large extent determined by the parental genotypes.

Table 4. Analyses of clutch size (number of eggs), egg hatching rate, larval survival rate to diapause and larval weight in the within-family and between-population crosses (Treatment) in the first and second generations (Generation). The female identity was included in the models as a random factor (approximate 95% confidence intervals for the female effect (SD), clutch size: lower 16.8, est. 29.4, upper 51.5, egg hatching rate: lower 0.725, est. 0.956, upper 1.26, larval survival: lower 0.194, est. 0.394, upper 0.799, larval weight: lower 0.488⁻³, est. 0.792⁻³, upper 1.29⁻³).

	Clutch size			Egg hatching rate			Larval survival			Weight		
	d.f.	F	p	d.f.	F	p	d.f.	F	p	d.f.	F	p
Intercept	1, 89	536.036	< 0.001	1, 90	0.066	0.797	1, 57	18.476	< 0.001	1, 54	2573.825	< 0.001
Oviposition delay	1, 89	52.237	< 0.001	1, 90	51.875	< 0.001	1, 40	0.247	0.622	1, 36	0.826	0.369
Generation	1, 57	0.112	0.739	1, 57	2.424	0.125	1, 57	7.594	0.008	1, 54	43.076	< 0.001
Treatment	1, 57	2.178	0.146	1, 57	3.874	0.054	1, 57	11.823	0.001	1, 54	6.169	0.016
Generation \times Treatment	1, 57	7.891	0.007	1, 57	0.001	0.977	1, 57	1.409	0.240	1, 54	2.520	0.118

This study confirmed the previous results of inbreeding reducing egg hatching rate in *M. cinxia* (Haikola *et al.* 2001). Unfortunately, due to the small number of sib-mated females in the second generation, no definitive conclusions about differences in the effects of inbreeding between the generations can be drawn. The apparently higher inbreeding depression in the second than in the first generation seen in Tables 1 and 2 is due to the especially high proportion of females with ≤ 1 hatched larvae in the second generation of within-family crosses. In contrast, there was no significant interaction between generation and inbreeding treatment in larval survival nor in larval weight at diapause.

Keller (1998) has reported that in song sparrows, *Melospiza melodia*, relatedness between parents or male inbreeding coefficient did not affect egg hatching, but female inbreeding reduced egg hatching rate. Although the present sample size was small, there was a tendency for female inbreeding to have more impact on egg hatching rate than male inbreeding. Possible differences between the effects of male and female inbreeding on offspring fitness have not been studied in many species, and it appears that the relative importance of inbreeding in the two sexes varies between taxa (van Noordwijk & Scharloo 1981, Pray & Goodnight 1995, Su *et al.* 1996, Keller 1998). I. J. Saccheri *et al.* (unpubl. data) found that in the butterfly *Bicyclus anynana* the effect of male inbreeding on egg hatching rate was greater than that of female inbreeding, and that this effect was solely expressed as complete sterility of many inbred males. Consistent with these findings, two of the five pairings between an inbred male and an outbred female produced no larvae, whereas there were no sterile pairings between outbred males and inbred females. Furthermore, all three pairings involving inbred males and females were completely sterile.

In a previous study on *M. cinxia*, inbreeding was shown to affect negatively not only egg hatching rate but also larval survival over diapause (Nieminen *et al.* 2001). In this study, I investigated the effect of inbreeding on early larval survival, from egg hatching until diapause. The mean survival rates in the between-population crosses (means for the first two clutches) were 56% and 57% in the first and second gener-

ations, respectively, suggesting that the environmental conditions were not very different, or, at least, any variation in environmental conditions was unimportant with respect to larval survival. Overall, inbreeding had a negative effect on the early larval survival and this effect remained constant across the two generations. The mean larval weight was slightly lower in the within-family than in between-population crosses (the effect only seen in the first generation), which is consistent with an earlier result showing lower larval weight after diapause in the progeny of less heterozygous and hence probably more inbred females (Haikola *et al.* 2001). In this study, the mean weights differed markedly between the generations, which may be due to a general decrease in overall viability of the individuals due to laboratory rearing.

Previous studies have reported negative effects of inbreeding also on yet other fitness traits in *M. cinxia*. Thus the offspring of less heterozygous females had a longer pupal period than the offspring of more heterozygous females (Haikola *et al.* 2001), which might increase the probability of parasitism by pupal parasitoids (Lei *et al.* 1997). Inbreeding appears to affect also the adult stage, as in a field study females sampled later in the flying season were more heterozygous than females sampled earlier, suggesting that more homozygous individuals had a shorter life-span (Saccheri *et al.* 1998, *see also* a study of van Oosterhout *et al.* 2000 showing a negative effect of inbreeding on longevity in female *Bicyclus anynana*). The life-span of the female will affect the total number of offspring that she may produce, hence inbreeding depression in reproductive success could also take place through an effect on longevity. Furthermore, in a laboratory experiment butterflies originating from small isolated populations (likely to be inbred) suffered from low mating success as compared with butterflies from large well-connected populations, possibly due to inbreeding (Haikola *et al.* 2001). In brief, inbreeding adversely affects several fitness traits during the entire life-cycle of *M. cinxia*.

The particular aim of this study was to assess the level of inbreeding depression in the second generation of inbreeding. The two significant effects were reduced clutch size in the inbred

treatment in the second but not in the first generation and the high proportion of inbred pairings that produced no hatched larvae in the second generation. Both results may reflect the effect of the inbreeding level of the female parent as opposed to the level of inbreeding in the zygote. For the other traits I found no increased inbreeding depression from the first to the second generation. The result must be interpreted cautiously because the sample size remained small.

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