OUTBREEDING DEPRESSION, BUT NO INBREEDING DEPRESSION IN HAPLODIPLOID AMBROSIA BEETLES WITH REGULAR SIBLING MATING

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Abstract.—In sexual reproduction the genetic similarity or dissimilarity between mates strongly affects offspring fitness. When mating partners are too closely related, increased homozygosity generally causes inbreeding depression, whereas crossing between too distantly related individuals may disrupt local adaptations or coadaptations within the genome and result in outbreeding depression. The optimal degree of inbreeding or outbreeding depends on population structure. A long history of inbreeding is expected to reduce inbreeding depression due to purging of deleterious alleles, and to promote outbreeding depression because of increased genetic variation between lineages. Ambrosia beetles (Xyleborini) are bark beetles with haplodiploid sex determination, strong local mate competition due to regular sibling mating within the natal chamber, and heavily biased sex ratios. We experimentally mated females of *Xylosandrus germanus* to brothers and unrelated males and measured offspring fitness. Inbred matings did not produce offspring with reduced fitness in any of the examined life-history traits. In contrast, outcrossed offspring suffered from reduced hatching rates. Reduction in inbreeding depression is usually attributed to purging of deleterious alleles, and the absence of inbreeding depression in *X. germanus* may represent the highest degree of purging of all examined species so far. Outbreeding depression within the same population has previously only been reported from plants. The causes and consequences of our findings are discussed with respect to mating strategies, sex ratios, and speciation in this unusual system.

Key words.—Coleoptera, outcrossing, population structure, purging, reproductive isolation, Xyleborini.

Inbreeding results in increased levels of homozygosity, which usually lead to inbreeding depression (Keller and Waller 2002). The main mechanism that has been proposed to explain the fitness reduction associated with inbreeding depends on the genetic load of recessive deleterious alleles (partial dominance hypothesis; Charlesworth and Charlesworth 1999; Roff 2002). Increased homozygosity through inbreeding results in increased expression of deleterious alleles, and thus inbreeding depression. Anything that would reduce the genetic load would therefore also reduce inbreeding depression, which results in two predictions. First, prolonged inbreeding should lead to purging of the genetic load due to increased exposure of deleterious mutations in homozygotes (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Waller 1993). Second, haplodiploid species, in which deleterious mutations are regularly exposed to selection in haploid males, should have a lower genetic load than diploid species (Bruckner 1978; Crozier 1985; Werren 1993).

The first prediction has been confirmed by comparative (Husband and Schemske 1996) and experimental studies (Latta and Ritland 1994; McCall et al. 1994; Crnokrak and Barrett 2002). However, in a variety of species and taxa with regular inbreeding or selfing, inbreeding depression is still observed (Ritland 1990; Demeester 1993; Doums et al. 1996; Wedekind et al. 1998; Weeks et al. 1999; Haag et al. 2002). The second prediction has received little attention until recently, when comparative studies showed that haplodiploid organisms experience less inbreeding depression than diploids, although it may still be substantial (Antolin 1999; Henter 2003). Since both haplodiploidy and regular sibling mating should reduce deleterious load, one could conclude that it should be lowest in haplodiploid species with chronic in-

breeding. However, theory predicts that haplodiploid species benefit less from prolonged inbreeding than diploid species (Werren 1993). In fact, genetic load of these females may even increase slightly with continuous sibling mating. In agreement with theoretical predictions, no difference was found in the extent of inbreeding depression between presumably outcrossing and sib-mating species of haplodiploid hymenoptera (Henter 2003). Therefore, inbreeding depression may be expected in haplodiploid organisms even in the case of no male dispersal and exclusive sibling mating.

Although outbreeding is generally associated with elevated fitness compared to inbreeding, this is only true up to certain levels of parental dissimilarity. Above this level, a decrease in fitness known as outbreeding depression can occur (e.g., Price and Waser 1979; Mitton 1993). Mechanisms responsible for this effect may be either physiological, such as underdominance (heterozygote disadvantage), breaking up of coadapted gene complexes and epistatic interactions between alleles, or environmental, such as disruption of local adaptations (Price and Waser 1979; Waser and Williams 2001; Edmands 2002). In a number of plants, there is evidence for both inbreeding and outbreeding depression (Waser 1993a; Barrett and Harder 1996). Many studies have reported a negative relationship between parental divergence and offspring fitness (see Edmands 2002), and there are species in which fitness achieves an optimum at intermediate levels of similarity (Waser 1993a; Waser et al. 2000). However, the critical level of dissimilarity is still poorly understood (Edmands 2002). In sessile organisms such as plants, outbreeding depression can occur over relatively small spatial scales, which is probably related to restricted gene flow (Waser and Price 1994; Waser et al. 2000). Thus, when examining negative effects of inbreeding versus outbreeding, one has to consider

the potential reduction in fitness above a certain level of divergence, especially in highly structured populations with limited dispersal.

The close link between inbreeding depression and the evolution of mating patterns and sex ratios can be illustrated particularly well in the haplodiploid ambrosia beetles (Xyleborini, Scolytinae). The Xyleborini are the only other haplodiploid lineage in holometabolic insects apart from the Hymenoptera (Mable and Otto 1998), and probably all of the approximately 1200 xyleborine species show high levels of inbreeding (Kirkendall 1993). Males are unable to fly, sex ratios are highly female biased, and mating was believed to take place exclusively among siblings within the natal gallery (Kirkendall 1993). However, recently it has been shown that males may disperse short distances, and females may have the opportunity to mate with unrelated males instead of their brothers (Peer and Taborsky 2004). This may substantially alter population structure, fitness consequences of inbreeding, and optimal sex allocation (Greeff and Taylor 1997). The extent of inbreeding depression is likely to influence female mating decisions. If continuous sibling mating leads to a reduction of the genetic load through purging, then gene flow across lineages through males may counteract this process (but see Whitlock et al. 2000).

In Xyleborini, populations can be assumed to be highly structured due to their life history with limited male dispersal ability and local mating. Therefore, both inbreeding and outbreeding depression may be observed on a relatively small spatial scale. The aim of this study was to determine the fitness consequences of mating with brothers versus unrelated males with different genetic distances. We experimentally mated females to brothers, males from the same or different populations, and measured fitness traits of the resulting diploid female offspring. We expected to find moderate inbreeding depression in offspring produced by sib-mated females compared to outcrosses, and outbreeding depression that is visible in between-population crosses.

MATERIALS AND METHODS

Study Population

Xylosandrus germanus is one of nine xyleborine species in Europe. It is an Asian species with a body length of about 2.2 mm, which has been introduced throughout the Holarctic and was described in Europe for the first time in the 1950s (Gauss 1960). Dispersal flights last from May to June, when females colonize freshly fallen trees and start excavating a brood chamber or gallery. They carry the spores of fungi (ambrosia, *Ambrosiella hartigii*) in special structures (mycangia) and cultivate these fungi on the surface of their gallery walls. After the ambrosia has started to grow, the females oviposit and broods develop until the end of August. Although mating generally takes place among siblings, some males leave their natal gallery and search for other galleries on the same log. The mated females overwinter in their gallery and disperse the next spring (Gauss 1960; Heidenreich 1960, 1964). Female dispersal distance probably ranges up to 100 m (estimate based on our own trapping data; Heidenreich 1964).
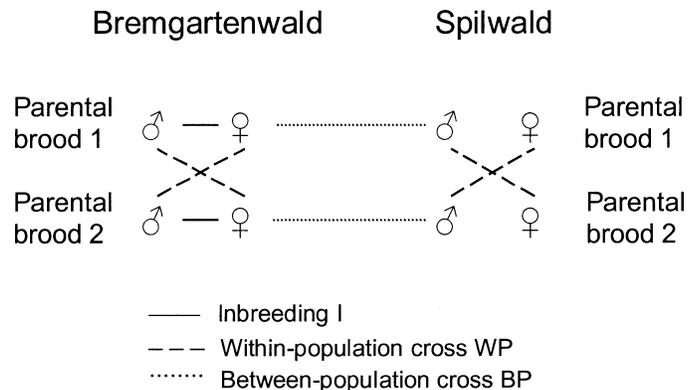

FIG. 1. Design of experimental crosses. From each parental brood from Bremgartenwald, one female per cross (inbreeding, within-population, between-populations) was used. The males for the inbreeding and within-population crosses originated from the same broods, while those for the between-population crosses came from Spilwald broods. From each Spilwald parental brood, one female and one male were used for within-population crosses.

Experimental Females

We collected mated females during dispersal flight in June 2003 with ethanol-baited traps in two different forests near Berne (Bremgartenwald and Spilwald, about 6 km apart). These females were transferred to the laboratory and allowed to excavate brood chambers and cultivate fungus in artificial medium in test tubes (see Peer and Taborsky 2004). After four weeks, when about half of the offspring had reached the pupal or adult stage, we dissected the galleries, and pupae and adults were removed. Since mating takes place within the natal gallery soon after eclosion, this step was necessary to obtain unmated females, which could then be paired according to experimental protocol.

Experimental Procedure

From each brood, we placed two female pupae per replicate into separate small plastic boxes with moist filter paper, and added a male or a male pupa. For females from Bremgartenwald, the male was either from the same brood (inbreeding), from a different brood from Bremgartenwald (within-population cross), or from a brood from Spilwald (between-population cross; Fig. 1). The males for the within-population crosses originated from the same broods as those for the inbreeding treatment to control for genetic differences between broods. This reciprocal design was not possible for between-population crosses. Because of heavily biased sex ratios, only two males were available for experimental crossings for most broods, and therefore crosses between males from Bremgartenwald and females from Spilwald could not be conducted. However, we also performed within-population crosses for females from Spilwald, and compared their offspring fitness to that of within-population crosses from Bremgartenwald females to avoid the possibility that potential population differences would be interpreted erroneously as effects of the crossing treatment.

Since it is not possible to observe the development of offspring in intact galleries, eggs were extracted for monitoring early life-history traits. For this reason, we used two

females per brood in each replicate. One (randomly assigned) was used to obtain data on early life-history traits of offspring (destructive method) and the other was used to measure total brood sizes produced in the presence of maternal care. In total, we used 30 broods per treatment and population, which resulted in 240 experimental matings.

Following eclosion of experimental males and females, these were kept together in the experimental boxes for at least two days to ensure successful copulation. Females were then allowed to tunnel in artificial medium in test tubes. Because they had been removed from their brood chambers prematurely, the mycangia of these females were devoid of fungal spores from their natal gallery. Therefore, we artificially inoculated the medium with ambrosia fungus from already established laboratory galleries. In consequence, broods with fungal contamination or unsuccessful experimental inoculation with ambrosia fungus had to be excluded from the experiment, so that final sample sizes differed between treatments.

Early Life-History Traits of Offspring

On day 11 after gallery initiation, we dissected half of the experimental galleries and extracted the eggs. They were placed on a surface of artificial medium contained in small transparent plastic boxes. The eggs carried fungal mycelium on their surface, so that ambrosia started to grow on which the larvae could feed after hatching. Because of the absence of brood care, there often was excessive ambrosia growth or fungal contamination of the medium and accumulation of debris. Therefore, offspring development and survival was obviously reduced compared to undisturbed galleries. All treatments suffered equally from this effect and thus could be compared. We recorded the following data: number of eggs extracted, number of eggs and larvae present on day 7, number of larvae present on day 12, and number of pupae present on day 21 after extraction. The timing of data recording was determined during pilot observations of the durations of egg, larval, and pupal stages. Between treatments, we compared the proportions of (1) eggs hatched on day 7, (2) larvae surviving from days 7 to 12, and (3) larvae undergoing pupation from days 12 to 21. For a more integral measure of fitness we multiplied hatching rate, larval survival, and pupation rate for the broods where all three measures were available to obtain total fitness.

Total Number of Offspring and Offspring Fecundity

The other half of the experimental galleries was left untouched for eight weeks, when brood development was complete. We then dissected the galleries and counted the number of male and female offspring. To estimate the environmental quality within the brood chambers, ambrosia growth (nonexistent, bad, good) and occurrence of contamination with other fungus than ambrosia (none, present) were recorded. To measure F_1 fecundity, three female offspring from each brood were randomly selected and again transferred to artificial medium in tubes. After 14 days, we dissected the resulting galleries and counted the eggs. For analyses, the number of eggs was averaged over the three offspring females from each brood. An additional fitness trait that may differ

between inbred and outbred offspring is the capability to transfer ambrosia successfully and prevent contamination of the brood with harmful fungi. Thus, we also scored ambrosia growth and contamination for these offspring galleries, and calculated the probability of successful ambrosia propagation and of contamination of offspring galleries.

Analyses

Before analyzing the treatment effect, all variables were tested for differences between populations, only using data from within-population crosses. Whenever there was a tendency for a population difference in a trait ($P < 0.1$), we excluded between-population crosses from the final analyses to prevent confounding effects of intrinsic differences between populations (the traits concerned were total brood sizes and offspring fecundity). Only broods from Bremgartenwald females were used for between-treatment comparisons.

We used a generalized linear models approach to analyze fecundity (number of eggs) and brood sizes (number of female pupae and adult offspring) with Poisson regressions. Hatching rates, larval survival, and pupation rates were analyzed with a weighted logistic regression, which uses broods as individual datapoints and assumes a binomial error distribution of the number of successes (e.g., hatched eggs) per brood. Both Poisson and logistic regressions employ logit link functions to linearize the data. The significance of effects was tested by stepwise deletion from the final model. Whenever the final model was overdispersed, we used F statistics instead of χ^2 statistics to test for significance (Crawley 1993). For the analysis of total fitness, the percentage data were arcsine square-root transformed to obtain a normal distribution of the residuals, and analyzed using an ANOVA. All data were analyzed with R 1.8.0 (The R Developing Core Team, 1993, Vienna).

Matriline (brood from which the parental female originated) was always included in the analyses to control for potential genetic differences between broods. This accounts for the differences between residual degrees of freedom and sample size. Because we found that ambrosia growth and fungal contamination have strong effects on brood development and individual offspring fitness, we also included these factors and removed them from the final model if they were not significant.

RESULTS

Differentiation among Populations and Broods

Parental females, whose clutches were used for the early life-history trait measurements, laid on average 15.1 eggs ($n = 102$). We found a highly significant effect of matriline (Table 1), but not of population on the number of eggs laid ($F_{1,37} = 2.8$, $P = 0.22$; $n = 102$ clutches). None of the early life-history traits of within-population crosses differed between the two populations used in the experiment (hatching rates: $F_{1,28} = 0.005$, $n = 30$, $P = 0.94$; larval survival: $F_{1,20} = 0.17$, $P = 0.68$, $n = 22$; pupation rate: $F_{1,14} = 1.604$, $P = 0.21$, $n = 16$ clutches). Average brood size across all treatments and populations was 24.3 ($n = 73$ broods). When comparing only within-population crosses, total brood sizes

TABLE 1. Influence of female lineage and treatment (inbreeding, within-population cross, between-population cross) on fitness measurements. All significant sources of variation are shown in bold. Ambrosia growth and fungal contamination of the natal gallery were retained in the final model as covariables if their effect was significant.

| | | Matriline | | | Crossing | | | Natal gallery | | | | | |
| | | | | | | | | Ambrosia growth | | | Fungal contamination | | |
	n	df	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P	df	F/χ^2	P
Parental fecundity[1]	102	63	9.13	**<0.001**									
Hatching rate (%)[2]	42	26	59.06	**<0.001**	2	8.05	**0.018**						
Larval survival (%)[1]	33	23	1.02	0.44	2	0.47	0.63						
Pupation rate (%)[1]	30	21	4.69	**<0.001**	2	0.55	0.58						
Brood size[1,3]	37	26	6.61	**<0.001**	1	1.95	0.16	1	9.32	**<0.001**	1	22.63	**<0.001**
Postdispersal:													
Offspring fecundity[2,3]	33	26	85.02	**<0.001**	1	2.43	0.12	1	4.86	**0.03**			
Ambrosia growth[1]	40	23	1.86	**0.007**	2	0.31	0.73	1	13.69	**<0.001**	1	7.39	**<0.001**
Fungal contamination[1]	40	23	1.72	**0.018**	2	0.38	0.62	1	5.12	**0.02**			

[1] Using F-test because of overdispersion.
[2] Using χ^2 test.
[3] Between-population crosses were excluded due to differences between populations.

from Spilwald tended to be larger than those from Bremgartenwald (population: $F_{1,28} = 2.90$, $P < 0.1$; ambrosia growth: $F_{1,28} = 12.45$, $P < 0.001$; contamination: $F_{18,25} = 103.84$, $P < 0.001$, $n = 32$ broods), and fecundity of F_1 offspring differed significantly between populations (population: $F_{1,19} = 11.73$, $P < 0.001$; contamination: $F_{1,19} = 13.89$, $P < 0.001$, $n = 22$ broods). The probability of contamination of F_1 galleries ($F_{1,20} = 1.08$, $P = 0.3$.) and successful ambrosia transfer ($F_{1,20} = 0.83$, $P = 0.36$) did not differ between populations. Thus, for all traits except total brood sizes and offspring fecundity the between-population crosses were included in the subsequent analyses of treatment effects.

Effect of Treatment on Offspring Fitness

Egg hatching rates were significantly influenced by inbreeding status (i.e., treatment) and matriline (see Table 1), such that eggs resulting from inbred matings had higher hatching success than eggs from either of the outbred matings (medians were 100%, 89.0%, and 88.9% for inbreeding, within-population crosses, and between population crosses, respectively; Fig. 2). Polynomial contrasts revealed that hatching success of within-population crosses was lower than of within-brood crosses ($P < 0.01$), but was not significantly further reduced in between-population crosses ($P = 0.44$). Both larval survival (medians 83.3%, 100%, and 87.5%) and pupation rate (medians 19.5%, 38.1%, and 50.0%) did not differ significantly between treatments, but matriline had a large effect on pupation rate (see Table 1). Also, when combining all three measures into total fitness, crossing had no significant effect (ANOVA: $F_{2,8} = 1.14$, $P = 0.37$, $n = 40$). Using helmert contrasts, no significant effect was found when comparing sibling versus (WP + BP) or WP versus BP ($P = 0.27$ and 0.57, respectively).

Brood sizes were largely influenced by matriline (see Table 1), ambrosia growth, and fungal contamination, but inbreeding status (i.e., treatment) had no effect (mean brood sizes were 22.35 and 21.0, respectively). Matriline and ambrosia growth in the natal gallery significantly affected fecundity of F_1 offspring. However, inbreeding status had no influence

(mean numbers of eggs laid were 18.2 and 13.1, respectively; see Table 1). Similarly, neither the proportion of F_1 offspring galleries with successful ambrosia growth nor the proportion of galleries with contamination was affected by inbreeding status, but matriline again had an effect on both (see Table 1).

DISCUSSION

Our results show that *X. germanus* does not suffer from inbreeding depression in any of the examined fitness traits. On the contrary, inbreeding resulted in higher offspring hatching rates than either of the outcrossing treatments. In haplodiploid insects and mites, inbreeding depression is lower than in diploids due to purging of recessive deleterious alleles in male hemizygotes (Antolin 1999; Henter 2003). This finding, which had been based mainly on data from haplodiploid Hymenoptera, is taken one step further by the complete absence of inbreeding depression in our study system, which represents a phylogenetically independent group. Ecologically imposed inbreeding may promote the evolution of haplodiploidy, because inbreeding depression will be reduced through purging of deleterious alleles in haploid males, and effective mutation rates are lower due to lower ploidy levels (Werren 1993). Inbreeding has been argued to select for longer haploid phases in a wide range of organisms and reproductive systems (Mable and Otto 1998). Nevertheless, in haplodiploid species, fitness of inbred individuals may also be reduced by 20–40% compared to outbred individuals (Antolin 1999; Henter 2003). Until now it could not be demonstrated that a history of inbreeding further enhances purging in haplodiploids due to the expression of deleterious alleles in homozygous diploid females. However, *X. germanus* is probably the species with the highest degree of continuous sibling mating in which inbreeding effects ever have been examined. Theoretical work has shown that purging may not occur until a critical threshold selfing/inbreeding rate is reached (Lande et al. 1994).

The lack of inbreeding depression also has implications for the mating system, which has been explored extensively in plants (e.g., Lande and Schemske 1985; Waller 1993; Barrett and Harder 1996), but not in animals. Local mate com-
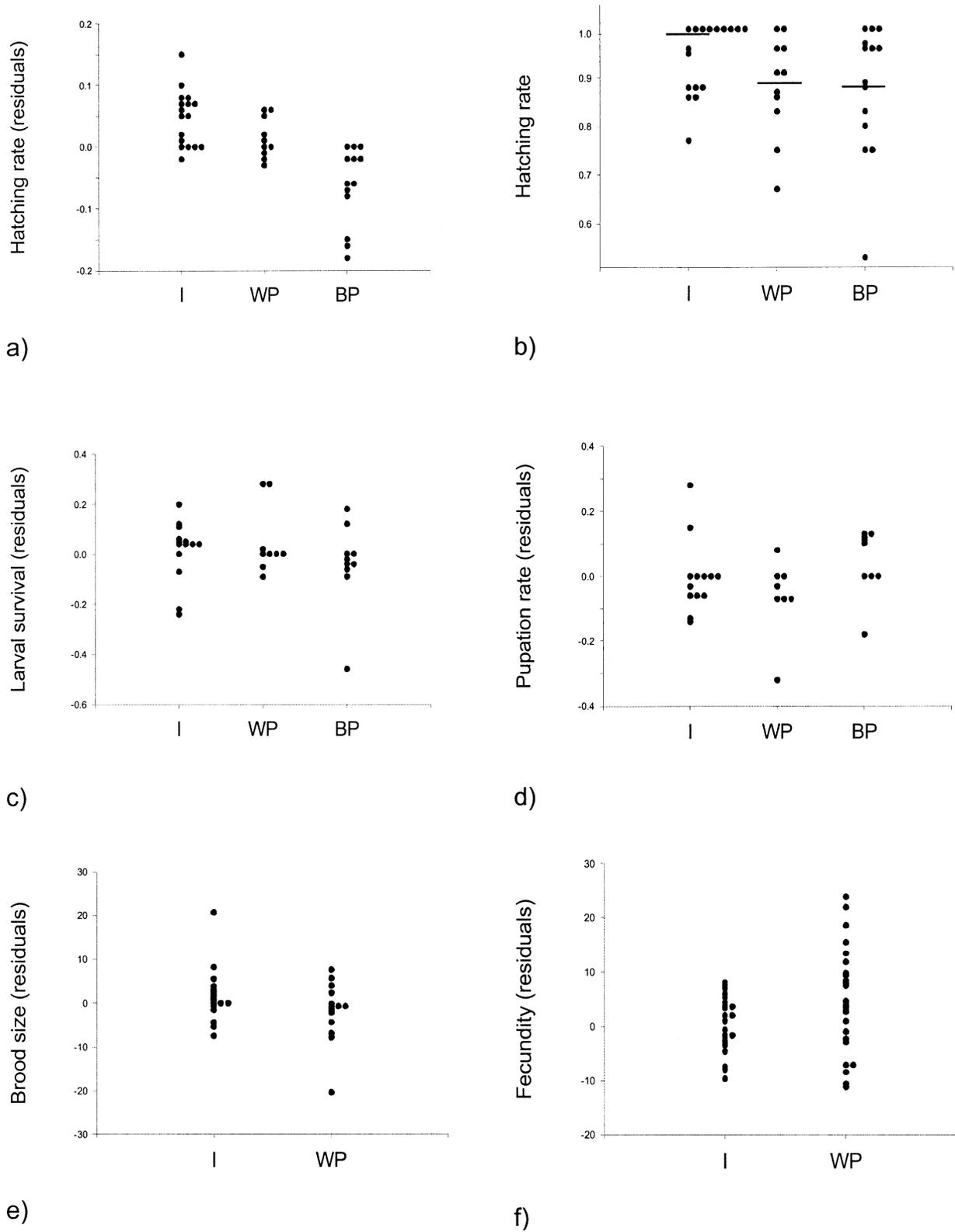
FIG. 2. Effect of treatment (I, inbreeding; WP, within-population cross; BP, between-population cross) on different fitness measures. Datapoints always represent individual broods. (a–d) Early life-history traits, (e) resulting brood sizes, and (f) F_1 fecundity. Datapoints aligned horizontally on the ordinate represent identical values. Except for (b), residual values are corrected for all other sources of variation besides treatment. In (b) the raw data without correcting for matriline and the corresponding medians are shown to visualize the effect of matriline. The lack of a significant difference in hatching rates between WP and BP may be due to small sizes of broods with low hatching rates in BP. As a result these broods have a lower weight in a weighted logistic regression.

petition leads to female-biased sex ratios, provided that the benefits of sibling mating outweigh any costs of inbreeding depression (Denver and Taylor 1995). The strength of the sex-ratio bias is predicted to depend on the extent of inbreeding depression, such that lower inbreeding depression should lead to lower production of males (Greeff and Taylor 1997; De Jong et al. 1999). However, there are hardly any data on the effects of inbreeding on individual fitness in haplodiploid species with local mate competition (Antolin 1999; Henter 2003). *Xylosandrus germanus* females produce only 5–10% males (Peer and Taborsky 2004), which is one of the most extreme sex-ratio biases found in animals and may be a result of low inbreeding costs.

Xylosandrus germanus suffers from outbreeding depression in hatching rates even when crossed within the same population. Genetic mechanisms of outbreeding depression (as opposed to local adaptation) over moderate distances are thought to occur mainly in highly inbred species (Waser 1993b). Restricted recombination can lead to intrinsic coadaptation of genes within a gene pool (Templeton 1986). Disruption of such gene complexes results in reduced fitness, which has been shown theoretically and experimentally with parthenogenetic strains of *Drosophila* (Templeton et al. 1976, 1986). A number of plant species with restricted gene flow have the highest fitness at intermediate levels of outbreeding (e.g., Waser et al. 2000). This effect may occur within very small spatial scales. In animals, there has been little evidence for outbreeding depression within a single population so far.

However, *X. germanus* is subjected to outbreeding depression at the smallest possible scale or genetic distance: sibling mating, which in a haplodiploid population with continuous inbreeding is comparable to selfing, resulted in the highest egg hatching rates. Similar to our results, in a population of a highly inbred fern selfing resulted in the highest fitness (Schneller 1996). Recently, a negative correlation of fitness with outbreeding (measured as d^2, the genetic distance between parental gametes) has been reported for an isolated, highly inbred population of lizards (LeBas 2002). In *X. germanus*, there is little dispersal by males, mating takes place almost exclusively within the natal galleries, and a population may consist of a large number of highly differentiated inbred lines, which was suggested by the large effect of maternal identity on offspring traits (Table 1). Intrinsic coadaptation may lead to outbreeding depression whenever two different genotypes cross.

Outbreeding depression is often only apparent in the F_2 generation and later (Burton 1990; Fenster and Galloway 2000) or in fecundity reduction of the F_1 generation (Templeton 1986), when coadapted gene complexes are broken up through recombination at meiosis. In contrast, our study revealed outbreeding depression already at very early stages of F_1 (hatching rates), but no effect of outbreeding on fertility of F_1 females. If maternal effects are important and dependent on offspring genotype, coadaptation may occur not only between genes within one individual, but also between mother-offspring genes (Wolf 2000). Maternal genes may often explain as much as half the variance in offspring characters early in life. Endosymbionts have been shown to play an important role in the fertilization process of another xyleborine species (*X. ferrugineus*; Peleg and Norris 1972). It is possible that cytoplasmatic components such as these endosymbionts have additional effects within the eggs, resulting in maternal effects. In the zygote, selection may act against genes generating incompatibility with endosymbionts. In such a scenario, reduced hatching rates of eggs produced from outcrosses could be explained by nuclear genes, which are not adapted to the zygotic environment. Finally, the presence of *Wolbachia* in this species (R. K. K. Koivisto and K. Peer, unpubl. data) may result in cytoplasmatic incompatibility caused by different *Wolbachia* strains present in the population, which could result in reduced egg-hatching rates.

It may seem surprising that outbreeding depression was not detectable in total brood sizes and total fitness, since reduced hatching rates should result in a lower number of offspring reaching maturity. However, differences in maternal care and environmental factors such as ambrosia growth and fungal contamination may be more important at later stages in the life cycle, so that they override the outbreeding effect on hatching rates.

The findings of this study are highly relevant to understand speciation in the Xyleborini. In this group of bark beetles, haplodiploidy has apparently evolved in concert with close inbreeding and was followed by rapid diversification, possibly enhanced by the habit of fungus (ambrosia) cultivation (Normark et al. 1999). The tribe now consists of over 1200 species, compared to 40 species of its diploid sister group, the Dryocoetini (Jordal et al. 2000). As a result of continued inbreeding and reduced gene flow between lineages in the Xyleborini, the lack of inbreeding depression and the presence of outbreeding depression may have enhanced behavior-induced reproductive isolation. Above a certain threshold, outbreeding depression can be seen as equivalent to reproductive isolation (see also Coyne and Orr 1997; Waser et al. 2000). Further studies of genetic population architecture in this group may shed more light on the evolution of mating systems and speciation in general.

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