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INBREEDING AND OUTBREEDING DEPRESSION IN *CAENORHABDITIS* NEMATODES

Elie S. Dolgin,[1,2] Brian Charlesworth,[1,3] Scott E. Baird,[4] and Asher D. Cutter[1,5]

[1]*Institute of Evolutionary Biology, University of Edinburgh, King's Buildings, Edinburgh, EH9 3JT, United Kingdom*

[2]*E-mail: elie.dolgin@ed.ac.uk*

[3]*E-mail: brian.charlesworth@ed.ac.uk*

[4]*Department of Biological Sciences, Wright State University, Dayton, Ohio 45435*

E-mail: scott.baird@wright.edu

[5]*Department of Ecology and Evolutionary Biology, University of Toronto, 25 Harbord Street, Toronto, Ontario M5S 3G5, Canada*

E-mail: asher.cutter@utoronto.ca

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The nematode *Caenorhabditis elegans* reproduces primarily by self-fertilization of hermaphrodites, yet males are present at low frequencies in natural populations (androdioecy). The ancestral state of *C. elegans* was probably gonochorism (separate males and females), as in its relative *C. remanei.* Males may be maintained in *C. elegans* because outcrossed individuals escape inbreeding depression. The level of inbreeding depression is, however, expected to be low in such a highly selfing species, compared with an outcrosser like *C. remanei.* To investigate these issues, we measured life-history traits in the progeny of inbred versus outcrossed *C. elegans* and *C. remanei* individuals derived from recently isolated natural populations. In addition, we maintained inbred lines of *C. remanei* through 13 generations of full-sibling mating. Highly inbred *C. remanei* showed dramatic reductions in brood size and relative fitness compared to outcrossed individuals, with evidence of both direct genetic and maternal-effect inbreeding depression. This decline in fitness accumulated over time, causing extinction of nearly 90% of inbred lines, with no evidence of purging of deleterious mutations from the remaining lines. In contrast, pure strains of *C. elegans* performed better than crosses between strains, indicating outbreeding depression. The results are discussed in relation to the evolution of androdioecy and the effect of mating system on the level of inbreeding depression.

KEY WORDS: **Androdioecy, *Caenorhabditis elegans*, *Caenorhabditis remanei*, hermaphrodite, inbreeding depression, mating system, outcrossing, selfing.**

Mating between close relatives causes an increase in homozygosity, which usually results in a decline in fitness known as inbreeding depression. Inbreeding depression is a central factor in the evolution of mating systems, particularly in relation to the evolution of the rate of self-fertilization in hermaphroditic organisms (Jarne and Charlesworth 1993; Uyenoyama et al. 1993). Some models that incorporate inbreeding depression predict the evolution of either complete selfing or complete outcrossing (e.g., Lande and Schemske 1985). Nevertheless, mixed mating systems abound, and other theoretical studies have found conditions under which partial selfing is evolutionarily stable (see review by Goodwillie et al. 2005).

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The nematode *Caenorhabditis elegans* exhibits a mating system in which hermaphrodites are self-fertile, but can outcross only with males. This androdioecious reproductive mode makes *C. elegans* a useful system for studying the evolution of outcrossing versus selfing. Under both laboratory and natural conditions, however, males are rare (Hodgkin and Doniach 1997; Barrière and Félix 2005, 2007). Studies of molecular variation in wild-caught strains of *C. elegans* indicate that selfing is the primary mode of reproduction, with low but detectable rates of outcrossing (Denver et al. 2003; Barrière and Félix 2005, 2007; Sivasundar and Hey 2005; Cutter 2006).

There is an ongoing debate about the evolutionary significance of males in *C. elegans*. Although males are present only at extremely low population frequencies, a significant portion of the genome is dedicated to male-specific functions (Jiang et al. 2001), which seems to be maintained by selection (Cutter and Ward 2005). One obvious selective advantage to males is their escape from any inbreeding depression caused by the selfing of hermaphrodites, which is thought to be important in other androdioecious species (Lloyd 1975; Charlesworth 1984; Otto et al. 1993; Rieseberg et al. 1993; Weeks et al. 1999; Stewart and Phillips 2002; Cutter et al. 2003). However, inbreeding depression has not been observed in *C. elegans* for life span (Johnson and Wood 1982; Johnson and Hutchinson 1993), brood size (Chasnov and Chow 2002), and various other life-history traits (Johnson and Hutchinson 1993).

This is consistent with theoretical analyses, which show that very high levels of inbreeding are associated with large reductions in the frequencies of deleterious recessive or partially recessive mutations, leading to reduced inbreeding depression (Charlesworth and Charlesworth 1998). The restriction of gene flow by a predominance of selfing may also lead to greater levels of population subdivision, which could further reduce inbreeding depression (Waller 1993; Theodorou and Couvet 2002; Whitlock 2002; Glémin et al. 2003). In addition, restricted recombination, resulting from low rates of outcrossing and migration (Barrière and Félix 2005, 2007; Cutter 2006), could lead to the accumulation of different favorable combinations of alleles in different local populations, resulting in hybrid breakdown (outbreeding depression) following outcrossing (Templeton 1986).

Phylogenetic evidence indicates that the ancestor of *C. elegans* was gonochoristic (separate males and females)—changes from gonochorism to hermaphroditism have independently occurred at least 10 times in rhabditid nematodes (Kiontke et al. 2004; Kiontke and Fitch 2005). To understand the evolution and regulation of mating systems, comparative studies of inbreeding depression in conspecific and congeneric populations with different modes of reproduction have been undertaken in other groups (e.g., Holtsford and Ellstrand 1990; Demeester 1993; Johnston and Schoen 1996). Given the prominence of *Caenorhabditis* species as model organisms in biological research, it is surprising that little is known about inbreeding depression levels in the genus. Even in *C. elegans*, all the studies mentioned above involved the N2 strain, which has been maintained in the laboratory for thousands of generations. Natural isolates that have not adapted to laboratory conditions might exhibit different levels of inbreeding depression (Stewart and Phillips 2002), especially in view of the observed variation among strains in male production and persistence within laboratory populations (Teotónio et al. 2006).

The purpose of this study was to examine the relationship between breeding system and inbreeding depression, using two *Caenorhabditis* species with contrasting mating systems, *C. elegans* and *C. remanei*. To this end, we used worms recently derived from the wild, and assayed fitness-related traits using similar methodologies for the two species, to obtain estimates of the levels of inbreeding depression. We found that the outcrossing species, *C. remanei*, suffered strong multigenerational inbreeding depression, with the majority of inbred lines going extinct. In contrast, the selfing species, *C. elegans*, mostly exhibited outbreeding depression. We discuss the implications of these results for patterns of genetic diversity and the evolution of mating systems.

Materials and Methods

NEMATODE POPULATIONS

Caenorhabditis elegans strains were recently isolated from three localities in France: Franconville (48°98′N, 2°23′E), Hermanville (49°28′N, 0°32′W) and Merlet (44°45′N, 4°42′E) (Barrière and Félix 2005). These strains were selfed for a few generations to reduce any within-strain heterozygosity before freezing, and were only thawed shortly before initiating the present experiment. Five crosses were established, which represent a variety of within and between population comparisons: three crosses between strains from different populations (JU318 × JU370; JU322 × JU399; JU342 × JU466), one cross between strains isolated within 10 cm of each other in Franconville (JU364 × JU368), and one cross between strains isolated 15 m apart in Merlet (JU314 × JU323). All crossed strains were selected on the criterion of being divergent at a number of amplified fragment length polymorphism (AFLP) markers found among a larger dataset, as described in Barrière and Félix (2005), so as to ensure the crosses were between genetically distinct strains (Table 1). We also present two other measures of genetic divergence from two subsequent studies that used these strains, the number of silent-site pairwise single nucleotide polymorphisms (SNPs) (Cutter 2006) and the mean squared difference in microsatellite repeat length (Barrière and Félix 2007), and test whether genetic distance affects the magnitude of the observed effect.

Table 1. Strains used in crosses, with geographic source in parentheses, and molecular divergence data. The number of differing AFLP markers is from a total of 149 measured and 31 found polymorphic. The number of silent site SNPs is from six regions on two chromosomes spanning a total of 3372.4 silent sites across all loci, with the diversity measure π_{si} shown in parentheses. The mean squared difference in microsatellite repeat length ($\delta\mu^2$) is from six microsatellite loci across five chromosomes.

Strain 1	Strain 2	Geographic distance	Number of differing AFLP markers[1]	Number of silent-site pairwise SNPs (π_{si})[2]	Mean squared difference in microsatellite repeat length[3]
JU364 (Franconville)	JU368 (Franconville)	<10 cm	9	0 (0.0)	0
JU314 (Merlet1)	JU323 (Merlet2)	~15 m	14	21 (0.00614)	28.3
JU318 (Merlet1)	JU370 (Franconville)	570 km	15	5 (0.00144)	3.5
JU342 (Merlet2)	JU366 (Franconville)	570 km	8	21 (0.00610)	21.7
JU322 (Merlet2)	JU399 (Hermanville)	655 km	9	17 (0.00494)	17.8

[1]Barrière and Félix 2005, [2]Cutter 2006, [3]Barrière and Félix 2007.

Males of *C. elegans* were generated in each strain by heat-shock at 26–28°C for ~6 h, and the worms were then returned to 20°C to allow selfing. The resulting male offspring were then crossed in abundance to hermaphrodites, in order to maintain populations with a ~50:50 sex ratio. Each line was then maintained at 20°C for three generations as both a mixed-sex and a pure hermaphrodite population.

Crosses were carried out in four separate experimental blocks at different time points, with two to three genetically distinct crosses per block, and each particular cross was performed twice. It is of interest to note that another cross was also attempted, JU393 × JU407, but the male mating performance of JU393 was so poor that mating frequency was extremely low and these data were discarded. The particularly poor male mating ability of the JU393 strain was also independently observed by H. Teotónio (pers. comm. 2006).

Caenorhabditis remanei populations were established from dauer larvae found living in association with terrestrial isopods in the Wright State University (WSU) Biological Preserve in Southwestern Ohio (39°47′N, 84°03′W). Sampling was done following the protocol of Baird (1999). Briefly, *Trachilipus rathkii* isopods were collected during a single day from several locations within a 0.1 km radius in a wooded area of the WSU Biological Preserve, and sacrificed on 60-mm NGM-lite agar plates seeded with *Escherischia coli* OP50. Nematodes obtained in these collections were considered to be from a single genetically diverse, random mating population, consistent with current knowledge of *C. remanei* from this area (Cutter et al. 2006).

Plates were periodically monitored for ~two days for the appearance of nematodes, and any worms obtained were transferred to a different plate. In total, 30 females and 24 males were isolated, allowed to develop to adulthood, and to mate randomly for ~one day, allowing the possibility of multiple matings, to initiate the study population. The males were then killed and the 30 gravid adult females were subdivided onto three fresh plates, with 10 females per plate, to establish three subpopulations. These plates were then maintained as large outbred populations for ~six generations before initiating the experiment, and concurrently frozen for storage at –80°C. Except where specifically noted, worms of both *C. elegans* and *C. remanei* were maintained at 20°C on 35-mm NGM-lite agar Petri plates seeded with *E. coli* OP50, using standard techniques (Sulston and Hodgkin 1988).

EXPERIMENTAL CROSSES AND LIFE-HISTORY ASSAYS

In *C. elegans*, for each cross we measured fecundity and longevity in four classes of hermaphrodite: pure-strain F1s from each strain, and the two reciprocal hybrid F1s. Individuals of the same class were not independent of each other, as they often came from the same family, considering multiple hermaphrodite offspring of a cross involving a single mother and five to six fathers to belong to the same family. Altogether, 767 worms were assayed, composed of an average of 38 worms per class in each cross, with a mean family size of 5.85, yielding a total number of 178,861 offspring. The general features of the protocol are presented in Figure 1.

We first synchronized populations and cleaned plates of any bacterial contamination by using alkaline hypochlorite (Sulston and Hodgkin 1988). To ensure that the hermaphrodites assayed were the result of crossing rather than selfing, we placed five to six larval stage L4 males from the same strain with one L4 hermaphrodite. After two to three days of mating, each hermaphrodite was moved to a fresh plate to lay eggs for 5 h. After two to three days, we verified that the F1 offspring that developed from these eggs had a male to hermaphrodite ratio that did not differ significantly from 1:1, using a χ^2-test with one degree of freedom. These F1 hermaphrodites were then used for fecundity and longevity assays.

In *C. remanei*, multiple plates from each subpopulation were set up with a single female and a single male at the L4 stage

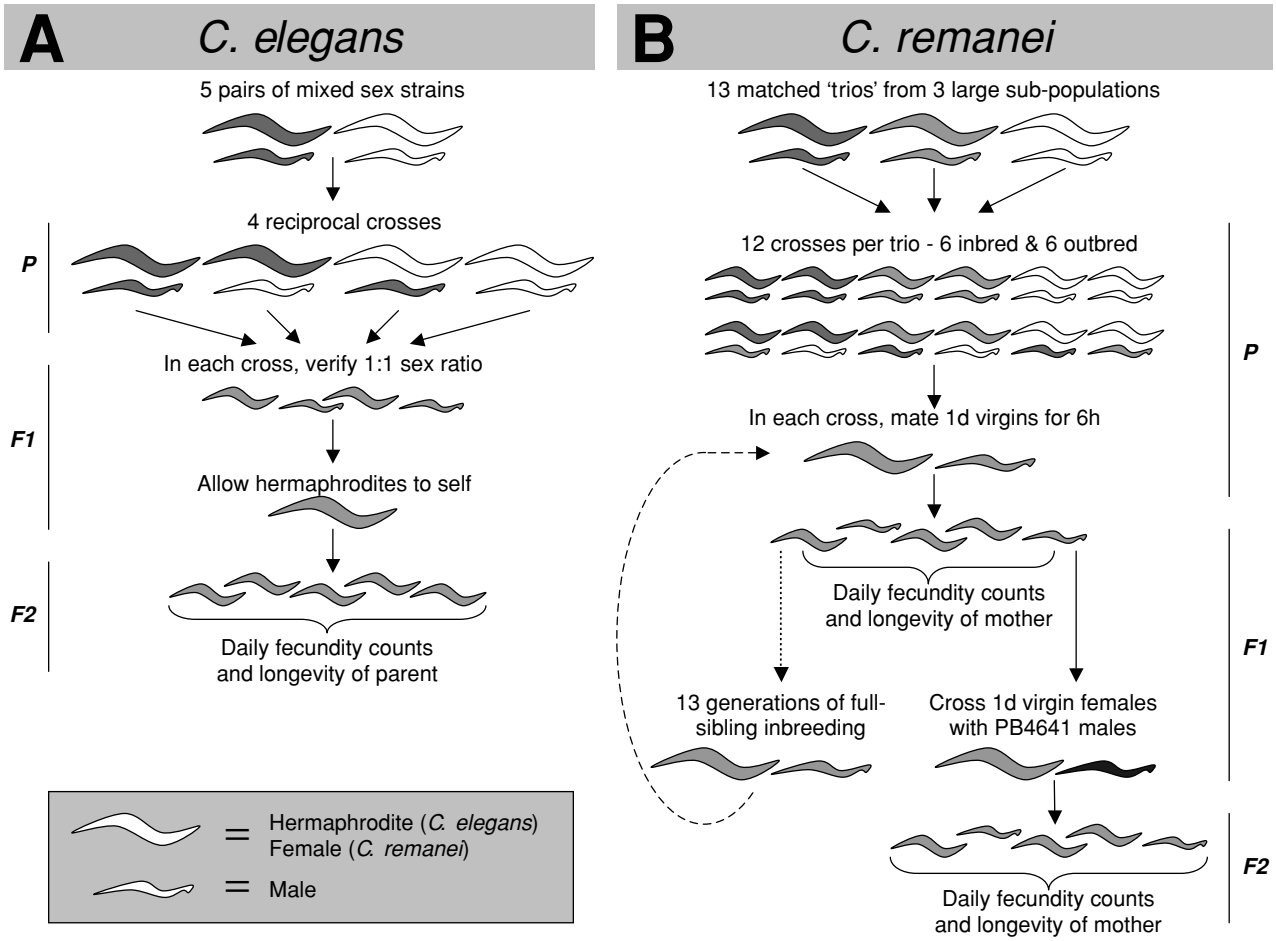

Figure 1. Experimental protocol for *C. elegans* (A) and *C. remanei* (B). The fitness-related traits were daily fecundity, giving a measure of total brood size and relative fitness (w), and longevity. The experimental protocol was designed to measure similar components of fitness for the different breeding systems.

in order to obtain full sibling offspring. Only plates with many offspring (> 300) were subsequently used, to guarantee healthy outbred starting populations. To ensure that the individuals being crossed were as unrelated as possible, we only used crosses between subpopulations for the outbred crosses. This is because individuals within subpopulations may have mated during the ~six generations between the field collection and establishment of the subpopulations, and initiating the experiment. To this end, we randomly chose one plate from each of the three subpopulations to create 13 "trios."

Within each trio of subpopulations, we set up six inbred crosses, two from each subpopulation, and all six possible outbred crosses between each subpopulation, including both the reciprocal crosses (Fig. 1B). We then measured productivity and longevity in the resulting outbred and full-sibling inbred females, and in the F1 progeny of these individuals crossed to males of the standard strain, PB4641 (selected because it is a highly inbred strain, which is being used for genomic sequencing). Using PB4641 males allows us to standardize the male contribution in all treatments and isolate the genetic effects of inbreeding depression on the F1 females. A total of 215 worms were assayed, 71 inbred and 63 outbred individuals, of which 46 inbred F1 and 35 outbred F1 progeny were crossed with PB4641. A total of 60,750 offspring were counted.

In addition, we maintained a number of inbred lines for 13 generations of full-sibling mating. We attempted to propagate 39 randomly selected lines, representing all three subpopulations taken from inbred F1 progeny, by placing full-sibling single males with single females, isolated as L4s. In establishing our inbred lines, we set up two mating plates of each inbred line in each generation, to allow for failure to mate. If no progeny were found on either plate, the line was deemed to have gone extinct. Otherwise, one of the plates with progeny was selected at random to propagate the inbred line. After 13 generations, we measured the fecundities of six classes of individuals from the extant inbred lines: (1) full-sibling inbred females, (2) inbred females crossbred with males from different inbred lines, (3) outbred females thawed from the three subpopulations frozen before initiation of

the experiment, (4) inbred females crossed to PB4641 males, (5) crossbred F1 females whose parents were from different inbred lines crossed to PB4641 males, and (6) outbred females crossed to PB4641 males. At this stage, a further 170 worms were assayed and 38,106 offspring counted. We also froze the extant inbred lines at –80°C and repeated these productivity measures again in a second block.

Fecundity and longevity assay methods were similar between the two species, with a few notable exceptions. In *C. elegans,* L4 hermaphrodites were isolated onto individual plates (there was no discernible difference in the timing of development between pure-strain and hybrid worms). Once they had developed into adults, they were allowed to lay eggs, and were transferred to fresh plates every 24 h for three days, and then to new plates to measure late fecundity and longevity. In *C. remanei*, male and female virgins were isolated at the L4 stage and kept separate for 40–44 h until they had become ~ one-day-old adults. Although serially inbred lines of *C. remanei* had delayed growth rates, we ensured that they had molted to adults many hours before the time of mating. Female reproduction in *C. remanei* is limited by the number of sperm transferred upon mating. Consequently, we placed a single male and female together on a fresh plate and permitted them to mate for 6 h to mirror the sperm-limitation experienced by selfing protandrous *C. elegans* hermaphrodites, where spermatogenesis precedes oogenesis, and once egg maturation begins, the hermaphrodite possesses its full complement of sperm. The male was then killed and the female moved to a new plate for 18 h, transferred every 24 h for two more days, and then moved to new plates to measure late fecundity and longevity. Plates without any offspring were observed in 13 of the 156 crosses in the parental generation, with no difference between inbred and outbred crosses ($\chi_1^2 = 0.08$, $P = 0.78$). It was assumed that mating had not been successful on these plates, and they were omitted from further analyses.

For both species, after the timed plate transfers for days 1–3, the worms were transferred after two days, and subsequently only if offspring or bacterial contamination was observed, although these were both rare events. Worms were then checked every one to two days, and were deemed to have died if they did not respond to gentle agitation with a platinum pick. Eggs on the laying plates were allowed to hatch and develop for three days, and were then counted to give daily egg-to-late-larval fecundities for days 1–3. For *C. remanei*, day 1 was divided between the first 6 h mating period and the subsequent 18 h, which was denoted as day 1.5 for the calculation of w (see below). Late fecundity beyond day 3 was summed and included in measures of total brood size.

Daily fecundities were used to generate two fitness components: brood size (the unweighted sum of all progeny over the entire life span including late productivity) and relative fitness, w. This second measure is proportional to the expected fitness of an age-structured population, defined as $w = \sum_x e^{-rx} l_x m_x$ (Charlesworth 1994), where $l_x m_x$ is the product of survivorship to age x and fecundity at day x, and r is a constant scaling term, equal to the growth rate of the population as a whole. Due to the short development time of these worms and the sperm-limited fecundity of *C. elegans* hermaphrodites or singly mated *C. remanei* females, the timing of egg laying is the principal factor affecting w. For *C. elegans*, r was calculated by defining the grand mean fitness of the pure strains as $\bar{w}_{\text{pure-strain}} = 1$, obtained using $l_x m_x$ pooled across all pure-strain worms. This r value was used to weight the productivity by a negative exponential function of age. For *C. remanei*, r was calculated by defining the mean fitness of the outbred individuals in the parental cross as $\bar{w}_{P-\text{outbred}} = 1$. This value of r was then used to calculate w for the crosses with PB4641 males and for the crosses after 13 generations of inbreeding.

STATISTICAL ANALYSIS

Longevity was analyzed by Kruskal–Wallis tests, and brood size and w were analyzed by general linear mixed models, using the JMP statistical package, version 5.1 (SAS Institute, Cary, NC). Residuals were normally distributed for brood size and w in *C. elegans*, and for w in *C. remanei*, but brood size data for *C. remanei* had to be square-root-transformed to normalize the residuals, because of the large differences between inbred and outbred worms. For *C. elegans*, fixed factors were breeding class (i.e., hybrid vs. pure strain), cross identity, block, block × breeding class, block × cross identity, and breeding class × cross identity. A random effect in the analysis was family, with Z-statistics used to test significance. Heterosis was also measured within each cross. Heterosis is usually defined as the relative increase in fitness of hybrids between strains due to increased heterozygosity. We measure it as the excess of the mean fitness of the hybrid F1s, relative to the mean of the pure-strain values (Falconer and Mackay 1996, p. 253), defined here as

$$H_j = \left[\frac{\bar{X}_j(\text{hybrid}) - \bar{X}_j(\text{purestrain})}{\bar{X}_j(\text{purestrain})} \right],$$

where $\bar{X}_j$(hybrid) and $\bar{X}_j$(purestrain) represent the means for the particular trait under investigation, X, of hybrid and pure-strain F1s within the jth cross. Thus, a positive value of H denotes inbreeding depression, and a negative value of H indicates outbreeding depression. The means within the same cross were compared using t-tests.

For *C. remanei*, inbreeding level and trios of matched subpopulations were included as fixed factors in the analysis of brood size and w for the assays of the first parental (P) generation of inbreeding and the F1 cross with PB4641 males. For the P generation cross, the inbreeding levels were sibling-mated and randomly mated, and for the F1 × PB4641 cross the inbreeding levels were inbred and outbred. For the assays after

13 generations of inbreeding, brood size and w were measured, but not longevity. Two general linear models were analyzed, with block and inbreeding class included as fixed factors. In the first analysis, the inbreeding classes were inbred (within lines), crossbred (the crosses between different inbred lines) and outbred, while in the second, the F1s of these classes crossed to PB4641 males were considered. The means were compared between the fully inbred, crossbred, and outbred females and the F1s crossed with PB4641 males, using Tukey tests. To analyze the rate of extinction of inbred lines over the course of 13 generations of serial inbreeding, we performed random permutation tests for a least-squares linear regression model, in order to determine appropriate significance thresholds that take into account the correlations among extinction rates between successive generations. The permutation tests were performed by holding the generation numbers constant and permuting the per generation rates of extinction 10^7 times, and then comparing the resulting r^2 values.

Results

OUTBREEDING DEPRESSION IN *C. ELEGANS*

For both total brood size and relative fitness (w), pure-strain F1s performed significantly better than hybrids ($F_{1,631} = 16.4$, $P < 0.0001$ for brood size; $F_{1,631} = 22.7$, $P < 0.0001$ for w). Both of these traits were affected by the cross identity ($F_{3,631} = 10.9$, $P < 0.0001$ for brood size; $F_{3,631} = 8.3$, $P < 0.0001$ for w), and the magnitude of outbreeding depression also varied significantly between different crosses (as indicated by breeding class × cross identity: $F_{4,631} = 8.5$, $P < 0.0001$ for brood size; $F_{4,631} = 8.3$, $P < 0.0001$ for w). The overall trend was significantly towards outbreeding depression with mean heterosis values of –0.064 and –0.093 for brood size and w, respectively (Fig. 2). When examining each cross independently, however, outbreeding depression was only significant in some crosses (three of five for brood size; two of five for w), and one cross showed a significant positive heterosis value for brood size (Fig. 2).

The block in which assays were carried out was also a significant factor for brood size ($F_{2,631} = 11.1$, $P < 0.0001$), but not for w ($F_{4,631} = 2.81$, $P = 0.06$). Interactions between block and genetic factors for brood size were not significant ($F_{3,626} = 0.64$, $P = 0.59$ for block × cross identity; $F_{3,626} = 2.1$, $P = 0.13$ for block × breeding class). This indicates that experimental noise was introduced into the data by undertaking the experiment over four blocks. However, the lack of significant interactions or strong block effects on w suggests that these effects were, for the most part, in the same direction for all genotypes.

Relative fitness, *w,* is measured from daily progeny counts and so is not independent of total brood size. These two measures were significantly positively correlated ($r = 0.475$; $t_{764} = 13.1$, $P < 0.0001$), with the magnitude and direction of outbreeding depression for both brood size and w in the same direction ($r = 0.88$), although this is limited by sample size ($t_3 = 1.31$, $P = 0.14$). Neither geographic proximity nor the number of polymorphic AFLP markers shared between the crossed strains affected the direction of outbreeding depression ($P > 0.59$ for all regression analyses). There was, however, a nonsignificant trend towards a positive relationship between the magnitude of outbreeding depression and both the number of pairwise single-nucleotide polymorphism differences ($F_{1,3} = 4.49$, $P = 0.12$ for brood size; $F_{1,3} = 2.06$, $P = 0.25$ for w) and the mean squared difference in microsatellite repeat length ($F_{1,3} = 12.46$, $P = 0.03$ for brood size; $F_{1,3} = 6.98$, $P = 0.07$ for w) (see Table 1). This suggests that more divergent strains may exhibit stronger outbreeding depression. More crosses would be needed to test this possibility.

Unlike for total brood size and w, hybrids and pure-strain worms showed no difference in longevity ($\chi^2_1 = 0.12$, $P = 0.73$). Although the five crosses exhibited different life spans($\chi^2_4 = 20.7$, $P = 0.0004$), there was no difference in the longevity of pure-strain versus hybrid F1s in any of the individual crosses (all $P > 0.05$; see Fig. 2C). Blocks also had significant effects on longevity ($\chi^2_3 = 35.1$, $P < 0.0001$), although breeding class remained nonsignificant when considering any particular block independently (all $P > 0.05$). Family was a significant effect for brood size ($Z = 4.8$, $P < 0.0001$), w ($Z = 5.2$, $P < 0.0001$), and longevity ($Z = 1.9$, $P = 0.03$), suggesting a considerable amount of variation among parents of the same genotype. This is probably not due to genetic diversity in the parental strains, since the strains used here were selfed for a few generations before freezing, and heterozygosity was found to be quite low in a subsample of fresh isolates collected from the same locations as the ones being used here (Barrière and Félix 2005). It is more likely that this is caused by variation in the ages of the mothers. Eggs laid by older worms tend to be more developed than those laid by younger worms. Therefore, even though we used a timed egg lay, the ages may not have been developmentally synchronized if the mothers were of different ages (Peters et al. 2003).

INBREEDING DEPRESSION IN *C. REMANEI*

Full-sibling mated females had a lower total brood size and w compared with randomly mated females, although this effect was not significant ($F_{1,120} = 1.43$, $P = 0.23$ for brood size; $F_{1,120} = 1.35$, $P = 0.25$ for w). However, when these females' F1 progeny were crossed with PB4641 males, inbreeding level was a significant factor for both brood size ($F_{1,67} = 4.02$, $P = 0.049$) and w ($F_{1,67} = 7.77$, $P = 0.007$) (Fig. 3). Our experimental design allows us to try to disentangle the sources of inbreeding depression. The poorer performance of sibling-mated females in the parental generation, although nonsignificant (probably because of high variance in the productivity-related traits), can possibly be attributed to embryonic or larval inviability. The difference in

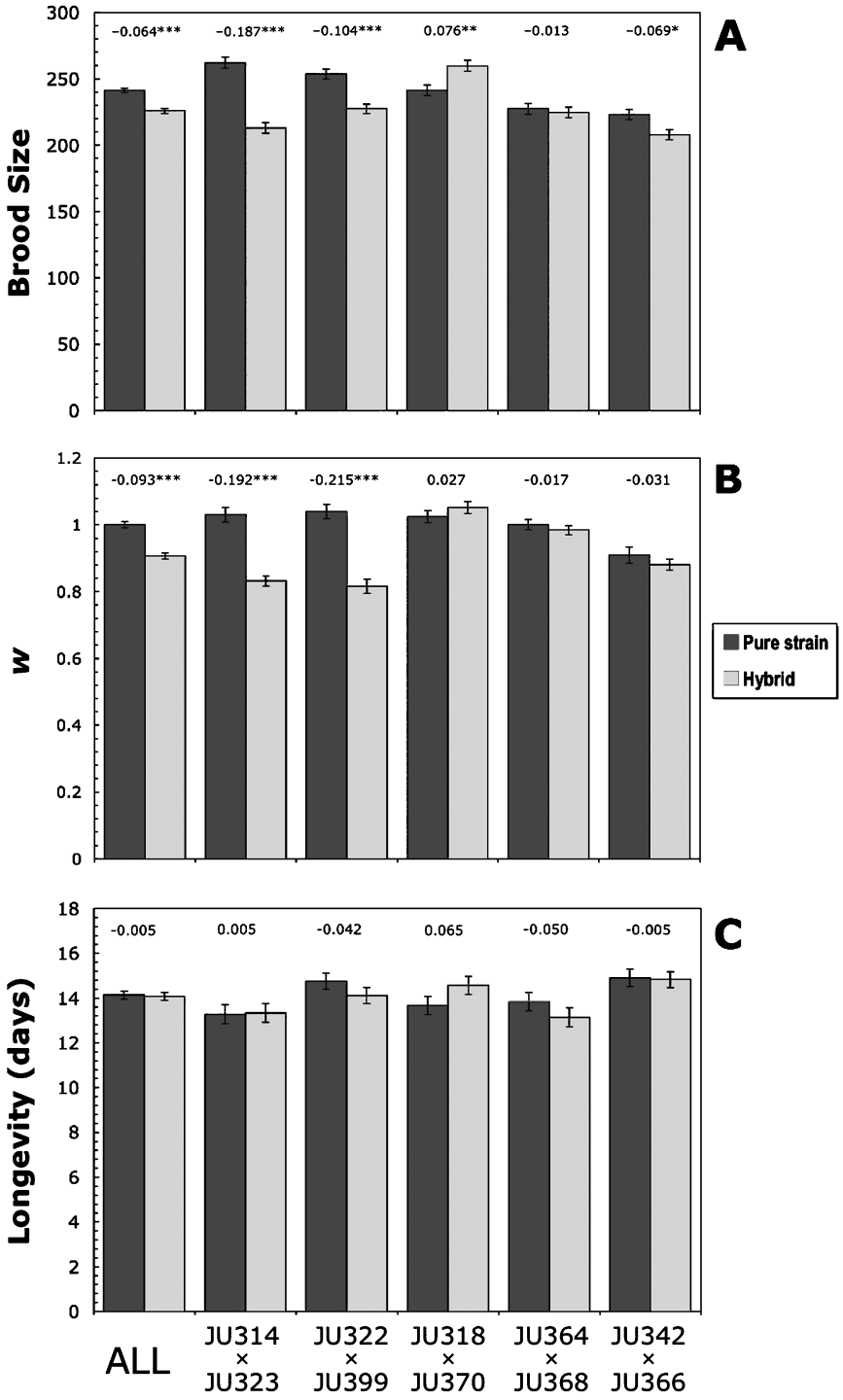

Figure 2. **Measurements of mean pure-strain (darkly shaded bars) and hybrid (lightly shaded bars) brood sizes (A), relative fitnesses (B), and longevities (C) by cross identity and overall in *C. elegans*. Numbers above the bars show heterosis values, calculated as (hybrid/pure strain)—1. Asterisks indicate significance of difference between pure-strains and hybrids in a Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate $\pm$ 1 SE.**

the F1s crossed to PB4641 males indicates reduced egg production and/or a reduction in the timing of egg laying in the inbred females, since the number of sperm and the mating performance of the PB4641 fathers are invariant. In both generations among crosses, inbreeding level had no effect on longevity ($\chi^2_1 = 0.01$, $P = 0.91$ for parentals; $\chi^2_1 = 0.09$, $P = 0.77$ for F1 $\times$ PB4641). There were no significant effects of the trios of randomly matched subpopulations on any of the measures in either of the crosses.

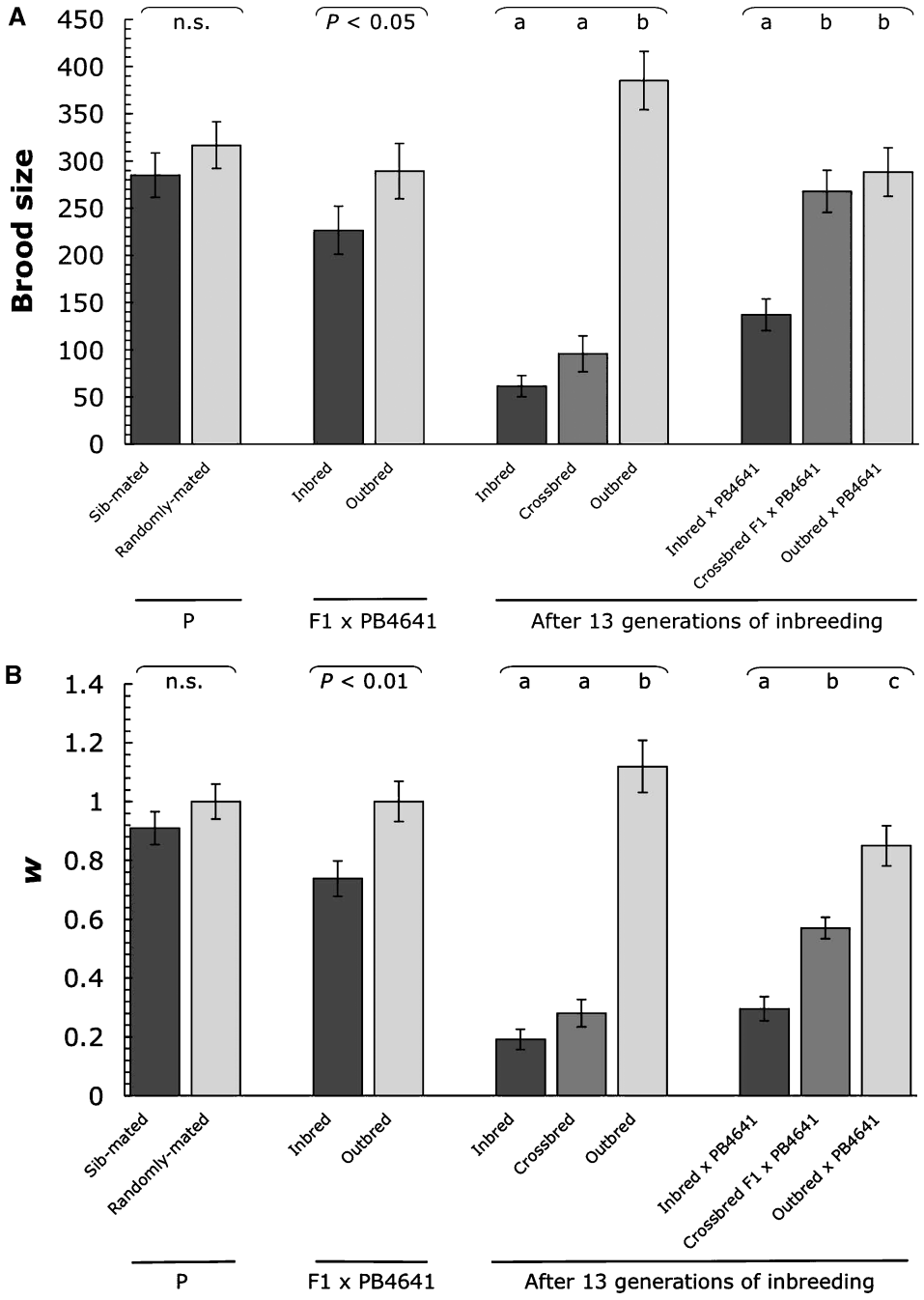

Figure 3. **Measurements of brood size (A) and relative fitness (B) in *C. remanei* for the parental generation (P), the F1 females mated to PB4641 males (F1 × PB4641), and crosses after 13 generations of full-sibling mating. Darkly shaded bars denote sibling-mated females and their inbred progeny, lightly shaded bars denote randomly mated females and their outbred progeny, and intermediate shaded bars denote inbred females crossbred with males from different inbred lines and their progeny. Post hoc significance tests were done separately for each of the four sets of crosses—Student's *t*-tests for *P* and F1 × PB4641, and two separate Tukey tests for crosses after 13 generations of inbreeding. Each comparison is represented by a curved line above the bars being evaluated with the significance denoted underneath. Identical letters within a comparison indicate nonsignificant differences, but letters across comparisons or across panels are unrelated. Error bars indicate ± 1 SE.**

For the assay of total brood size and w on the five extant lines after 13 generations of full-sibling inbreeding, the effects of inbreeding level were highly significant ($F_{2,72} = 51.3$, $P < 0.0001$ for brood size; $F_{2,72} = 56.4$, $P < 0.0001$ for w). Outbred females had greater brood size and relative fitness than inbred females mated to males from the same ("inbred") or different ("crossbred") inbred lines (Fig. 3). Crossbred females had ~ 50% greater mean performance than inbred females, although this effect was not

statistically significant due to large variances ($q_{72,3} = 1.67$, $P > 0.20$ for brood size; $q_{72,3} = 1.16$, $P > 0.50$ for w). The lower values for crossbred versus outbred females suggests that these inbred parents have reduced gamete production and/or mating ability, although the increased performance of crossbred versus inbred females suggests that larval inviability may play a role in the source of inbreeding depression as well.

Taking the F1 progeny females from the crosses between the strains surviving after 13 generations of full-sibling inbreeding and mating them to PB4641 males also yielded a highly significant effect of inbreeding level on fitness ($F_{2,92} = 12.3$, $P < 0.0001$ for brood size; $F_{2,92} = 29.5$, $P < 0.0001$ for w). Outbred females crossed to PB4641 males had greater trait values than inbred females crossed to PB4641 males (Fig. 3), with the reduced performance of the inbred females being significantly greater after 13 generations of inbreeding as compared to after 1 generation ($t_{70} = 2.91$, $P = 0.005$ for brood size; $t_{70} = 5.95$, $P < 0.0001$ for w).

Crossbred F1 females mated with PB4641 males performed significantly better than inbred females ($q_{92,3} = 6.00$, $P < 0.001$ for brood size; $q_{92,3} = 6.21$, $P < 0.001$ for w); however, despite no difference in the mean total brood sizes for crossbred F1 females and outbred females mated to PB4641 males ($q_{92,3} = 1.10$, $P > 0.50$), w was significantly lower for crossbred F1 females ($q_{92,3} = 5.92$, $P < 0.001$). Because crossbred F1 individuals are the progeny of unrelated inbred lines, they have an inbreeding coefficient of 0. The near equivalence of brood sizes indicates that crossbred F1 females had fecundity levels restored to outbred levels, but the reduction in w suggests a maternal effect on the timing of egg production, because the parental lines of the crossbred F1 female had been inbred for 13 generations. There was little evidence of purging of deleterious mutations during the process of inbreeding, because the crossbred F1 females did not perform significantly better than outbred females for either total brood size or w (Crnokrak and Barrett 2002). Block effects were not significant for any of the traits in *C. remanei*.

Over the course of serially inbreeding for 13 generations, 34 of the 39 lines went extinct. To avoid the possibility of lines failing to mate successfully, we set up two plates of each inbred line in each generation. From the first generation of inbreeding, we found that 8.3% of single male–female crosses failed to mate in a 6 h period. Because we observed no difference between inbred and outbred crosses in the frequency of failure to mate in both the parental cross ($\chi^2_1 = 0.08$, $P = 0.78$) and the F1 × PB4641 cross ($\chi^2_1 = 0.05$, $P = 0.83$), we assume that failure is a random event; therefore, < 1% of lines should fail to mate on both plates, although this is conservative because we allowed the inbred lines to mate indefinitely. Figure 4 shows the per generation and cumulative rates of extinction of inbred lines. The per generation extinction rate was consistently much greater than 1% ($t_{12} = 4.00$, $P = 0.0009$), with a mean of 13.9% (SE = 3.2%), and there was a positive association between the per generation rate of extinction and time, as indicated by a permutation test on a linear regression ($r^2 = 0.44$, $P = 0.015$). Because this significance value is nearly equivalent to that found for a parametric regression analysis ($t_{11} = 2.97$, slope = 0.020, $P = 0.013$), we used a parametric quadratic regression to test for purging. Purging of deleterious mutations over time would be expected to lead to a decreasing rate of extinction (Crnokrak and Barrett 2002); however, a quadratic regression did not provide a better fit to the data ($t_{10} = 0.51$, $P = 0.62$), indicating that fitness decline does not taper off. It is worth noting that the extant lines after 13 generations were derived from all three initial subpopulations. Preliminary data from sequencing of exonic nuclear DNA indicates a large number of single nucleotide

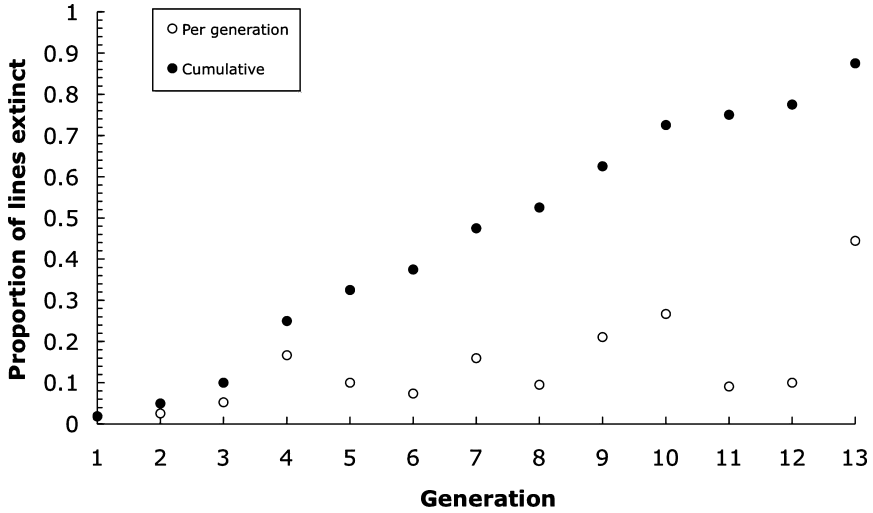

Figure 4. The rate of extinction of inbred lines in 13 generations of serial inbreeding. The open circles show the per generation proportion of lines going extinct; the closed circles show the cumulative proportion.

polymorphisms between the inbred lines, with some residual heterozygosity in at least one of the lines (results not shown). Thus, there appears to be no tendency to fix particular genotypes from a single subpopulations.

Discussion

OUTBREEDING DEPRESSION IN *C. ELEGANS*

Outbreeding depression is defined as the reduction in fitness following hybridization between divergent populations (Templeton 1986). When this occurs in first generation (F1) hybrids, as in our study of *C. elegans*, it could be caused by a disruption of beneficial interactions at three possible levels: between genes and the environment (local adaptation), within loci (underdominance), and between loci (coadapted gene complexes). The first two possibilities can probably be ruled out in our case. First, any effects of adaptation to local environmental conditions should be absent in the constant artificial laboratory environment, and there is no reason to suppose that outbred individuals should be any less adapted to this novel environment. Second, it is well known that underdominance does not lead to the maintenance of variability, even with high selfing rates (Kimura and Ohta 1971; Rocheleau and Lessard 2000), unless there is frequency-dependent selection under conditions similar to those involving local adaptation (Wilson and Turelli 1986). Therefore, the observed pattern of outbreeding depression on fecundity can probably be attributed to a breakdown of coadapted gene complexes, revealing deleterious allele combinations affecting the fitness of the F1 hybrids or the viability of the F2 recombinant progeny (Phillips and Johnson 1998). On the other hand, we did not find any inbreeding or outbreeding depression for longevity. This is consistent with earlier studies that found no effect of breeding class on life span (Johnson and Wood 1982; Johnson and Hutchinson 1993), and little evidence of directional effects of new mutations on longevity (Keightley and Caballero 1997; Vassilieva and Lynch 1999; Keightley et al. 2000; Halligan et al. 2003).

We studied a limited number of crosses between strains from France; however, because *C. elegans* shows little or no geographic structuring of molecular diversity on a large scale (Barrière and Félix 2005, 2007; Haber et al. 2005; Cutter 2006), outbreeding depression is probably not limited to strains from France. Indeed, outbreeding depression appears to be a global phenomenon in *C. elegans*. In crosses between the canonical N2 strain and a Hawaiian strain, CB4856, the progeny of F1 hybrids exhibit a significant increase in embryonic and early larval lethality, with similar hybrid incompatibilities observed between other wild strains (M. Rockman, H. Seidel, and L. Kruglyak, pers. comm. 2006; M. Ailion, pers. comm. 2006). In the related selfing species, *C. briggsae*, reproductive isolation was also found in crosses between strains AF16 and HK104, with approximately one third of F2 progeny exhibiting a delay in development and reduced intrinsic growth rates (S. Baird, unpubl. results).

Previous studies failed to show any effect of inbreeding level on fitness-related traits in *C. elegans* (Johnson and Wood 1982; Johnson and Hutchinson 1993; Chasnov and Chow 2002). However, as pointed out in the introduction to this paper, these all involved crosses where one of the strains was the long-maintained N2 strain, and the partner strains in the crosses had also been in culture for extended periods of time (Hodgkin and Doniach 1997). Here we attempted to overcome this problem by using strains recently caught in the wild. Using similar laboratory techniques to previous studies, we found a significant reduction in the performance of outcrossed individuals. Obtaining estimates under standardized optimal conditions is an important first step, but it is uncertain how this effect translates to performance under natural conditions. It is generally assumed that inbreeding depression is enhanced in harsher natural environments (see reviews by Crnokrak and Roff 1999; Keller and Waller 2002; Armbruster and Reed 2005). Whether this is also widely true of outbreeding depression remains to be seen, although in the selfing hermaphroditic snail, *Physa acuta*, outbreeding depression was observed only in the field but not in the lab (Henry et al. 2003). Potential evidence of outbreeding depression and selection against hybrids in wild populations of *C. elegans* comes from recent regular monitoring of haplotype frequencies in a population in France, where the frequency of a recombinant haplotype decreased significantly over the span of a year (Barrière and Félix 2007). Because the ecology of *C. elegans* is poorly understood, data from more natural conditions are needed.

IMPLICATIONS FOR *C. ELEGANS* DIVERSITY

It has long been recognized that selfing can promote the maintenance of linkage disequilibrium among loci whose alleles have epistatic effects on fitness ("coadaptation") (e.g., Stebbins 1957; Allard 1975), although selfing can also enhance randomly generated linkage disequilibrium between neutral variants, because it reduces the effective rate of recombination (Charlesworth 2003). In *C. elegans*, extremely low levels of diversity are found, with extensive linkage disequilibrium across the genome, both within and between chromosomes (Koch et al. 2000; Barrière and Félix 2005, 2007; Haber et al. 2005; Cutter 2006). This pattern is thought to be determined largely by self-fertilization and population subdivision, combined with rare but regular migration and outcrossing.

Our results suggest that outbreeding depression could be an additional important factor in shaping the genetic structure of *C. elegans*. Selection against hybrids would result in a reduction in the effective migration and outcrossing rates (Barton and Bengtsson 1986), thereby maintaining higher levels of linkage disequilibrium than in its absence. Outbreeding depression may also help to explain the large discrepancies between estimates of

outcrossing rates based on heterozygote frequencies at microsatellite loci (Barrière and Félix 2005, 2007; Sivasundar and Hey 2005), and measures from linkage disequilibrium, which are typically one to three orders of magnitude lower (Barrière and Félix 2005, 2007; Cutter 2006). If selection maintaining coadapted gene complexes caused hybrids between strains to have reduced contribution to the next generation, then the effective outcrossing rate will be greatly reduced.

FREQUENCY OF MALES

Because males are nonessential for reproduction in *C. elegans*, their maintenance requires explanation. It is possible that androdioecy could simply be a byproduct of the sex-determination mechanism, which allows male production via nondisjunction of X-chromosomes in hermaphrodite meiosis (hermaphrodites and females are XX, males are XO). Chasnov and Chow (2002) argued that males are nonadaptive but persist because mating is sufficiently frequent to preclude degeneration of male-specific genes by deleterious mutation. Other arguments favor a selective advantage of outcrossing to explain the persistence of males, and it is conceivable that males and outcrossing are advantageous under different ecological settings (Schulenburg and Müller 2004), or particular demographic scenarios involving metapopulation dynamics (Pannell 2002; Weeks et al. 2006a). Our results, however, suggest that males and outcrossing will be selected against in natural populations, because the existence of outbreeding depression must reduce the frequency of males below that expected in its absence. This was verified by using equation (3a) of Cutter et al. (2003). For example, with the male reproductive efficiency calculated for the N2 strain (Cutter et al. 2003), which has a fairly typical male mating ability for the species (Teotónio et al. 2006), the observed level of outbreeding depression of 6–9% would reduce the expected equilibrium frequency of males by about one-third for any rate of X chromosome nondisjunction.

C. *REMANEI* INBREEDING DEPRESSION

Consistent with expectations (e.g., Lande and Schemske 1985; Charlesworth and Charlesworth 1998), the outcrossing species *C. remanei* exhibited much greater levels of inbreeding depression than the selfing *C. elegans*. This appears to reflect the effects of inbreeding on various components of fitness. *Caenorhabditis remanei* female reproduction is limited by the number of sperm transferred by their male partners, which is a product of sperm production and mating efficiency. We attempted to standardize the male contribution by allowing mating for a controlled period of time. We also ensured that mating had indeed occurred by discarding any crosses for which no progeny were observed, although this might underestimate inbreeding depression by ignoring crosses that were unsuccessful due to genetic incompatibilities rather than a failure to copulate. Also, by mating the F1 female progeny to PB4641 males, sperm limitation and male mating efficiency should be constant and independent of inbreeding level. This allowed us to compare directly the performance of inbred and outbred females. In these crosses, inbreeding depression will be solely due to females, whereas without the control PB4641 males, inbreeding depression can also be affected by any male component and by larval inviability of offspring. Comparisons of parental crosses with F1 × PB4641 crosses indicate that inbreeding depression in *C. remanei* is affected by both male and female performance, in addition to larval inviability (see Fig. 3).

EVIDENCE OF MATERNAL-EFFECT INBREEDING DEPRESSION

The result that crossbred F1 females mated to PB4641 males had lower w than outbred females, despite having nearly the same brood sizes, suggests that outcrossing does not completely eliminate the effect of prior inbreeding on fitness in *C. remanei*. This indicates a maternal effect on inbreeding depression. A few other studies have found that maternal inbreeding can influence outcrossed progeny fitness, although the magnitude of these effects is generally less than the direct genetic effects of inbreeding depression (e.g., Hauser and Loeschcke 1995; Lyons 1996; Vogler et al. 1999; Hayes et al. 2005). This highlights the need to separate the sources of inbreeding depression, in order to accurately estimate the magnitude of the direct genetic effects. Our initial F1 × PB4641 crosses should not have been influenced by maternal inbreeding depression, since the parents were outbred, so these should provide a good estimate of zygotic inbreeding depression.

The significantly lower w suggests that it is the timing of egg production and fertilization rather than the absolute brood size that is altered by maternal-effect inbreeding depression. In *C. elegans*, a number of maternal-effect genes have been identified that influence developmental and behavioral timing, known as Clk genes (Lakowski and Hekimi 1996). For example, *clk-1* mutants have a two- to six-fold reduction in their egg production rate, but display maternal rescue, indicative of maternal effects (Wong et al. 1995). It is possible that some of the inbred *C. remanei* lines in our experiment became homozygous for genes with similar effects, thereby causing maternal-effect inbreeding depression on physiological traits related to the timing of the reproductive schedule in the crossbred F1 progeny.

EVOLUTION OF ANDRODIOECY

Recent evidence suggests that the genetics of switching between different reproductive modes is relatively simple in *Caenorhabditis* species, requiring a very limited number of mutations (Nayak et al. 2005; Braendle and Félix 2006; Hill et al. 2006). This demonstrates the potential for frequent evolutionary transitions in mating systems in *Caenorhabditis*. Selfing could have many advantages in *Caenorhabditis*, most notably reproductive assurance in species

colonizing ephemeral habitats with fluctuating population dynamics and episodic low densities, although the natural history of the genus is poorly understood and it is unclear exactly how the habitats differ between the gonochoristic and androdioecious species (Kiontke and Sudhaus 2006; Weeks et al. 2006a). The potential benefits of selfing, however, must be weighed against the cost of overcoming the strong inbreeding depression found in *C. remanei*, which presumably reflects conditions experienced by a lineage upon first shifting to hermaphroditism.

Over the course of 13 generations of full-sibling mating in *C. remanei*, 87% of our inbred lines went extinct and the surviving lines were extremely sick, with very low fecundity, and exhibited both direct and maternal-effect inbreeding depression (see Figs. 3 and 4). Among the extant lines, there was little evidence of any rebound in fitness over time that would have indicated purging of deleterious alleles (Crnokrak and Barrett 2002), which suggests that the inbreeding depression is largely due to the cumulative effects of many deleterious alleles of small effect rather than a few segregating deleterious alleles of large effect (Willis 1999). Indeed, most inbred strains of *C. remanei* die out in the laboratory, and those that survive have much lower fecundity and longer generation times than noninbred strains, and appear to be less fit than recently established strains (S. Baird and E. Dolgin, unpubl. observations). Furthermore, newly arising hermaphroditic lineages would probably have suboptimal sperm numbers and brood sizes, thereby decreasing the rate of population expansion (Cutter 2004), lowering the effective population size, and increasing the likelihood of extinction.

In the long run, selfing lineages that survive are likely to be those that have purged much of their genetic loads; a higher degree of population subdivision in selfing lineages may facilitate such purging (Waller 1993; Theodorou and Couvet 2002; Whitlock 2002; Glémin et al. 2003). This makes it hard for transitions back to outcrossing to occur (Charlesworth and Charlesworth 1998). But are transitions to hermaphroditism in rhabditid nematodes evolutionary dead ends? Phylogenetic evidence suggests that hermaphroditism has evolved frequently in rhabditids, but these lineages rarely survive in the long run (Kiontke and Fitch 2005). This is consistent with the pattern found in several plant genera (Schoen et al. 1997; Goodwillie 1999; Truyens et al. 2005; but see Takebayashi and Morrell 2001). On the other hand, in the clam shrimp *Eulimnadia*, androdioecy appears to be the ancestral mode of reproduction in the genus, and this mating system has persisted for between 24 and 180 million years (Weeks et al. 2006b). However, the rate of selfing seems to be lower in *Eulimnadia*, and the level of inbreeding depression and maximal frequencies of males higher, than in *C. elegans* (Weeks et al. 1999; Weeks 2004). It is unknown how long *C. elegans* and *C. briggsae* have been selfing. Although the selfing species are separated by millions of years from their closest known relatives, the ecology and biodiversity of *Caenorhabditis* species is poorly understood and extensive sampling may yet identify new closely related outcrossing and self-fertile species, and narrow down the possible time since hermaphroditism evolved. Here, we have shown that natural isolates of *C. elegans* suffer from outbreeding rather than inbreeding depression. How the species arrived at this state, the time scale of this process, and whether the species will persist are intriguing problems.

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