Cytoplasmic Incompatibility in *Drosophila simulans*: Dynamics and Parameter Estimates from Natural Populations

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ABSTRACT

In *Drosophila simulans*, cytoplasmically transmitted Wolbachia microbes cause reduced egg hatch when infected males mate with uninfected females. A Wolbachia infection and an associated mtDNA variant have spread northward through California since 1986. PCR assays show that Wolbachia infection is prevalent throughout the continental US and Central and South America, but some lines from Florida and Ecuador that are PCR-positive for Wolbachia do not cause incompatibility. We estimate from natural populations infection frequencies and the transmission and incompatibility parameter values that affect the spread of the infection. On average, infected females from nature produce 3–4% uninfected ova. Infected females with relatively low fidelity of maternal transmission show partial incompatibility with very young infected laboratory males. Nevertheless, crosses between infected flies in nature produce egg-hatch rates indistinguishable from those produced by crosses between uninfected individuals. Incompatible crosses in nature produce hatch rates 30–70% as high as those from compatible crosses. Wild-caught infected and uninfected females are equally fecund in the laboratory. Incompatibility decreases with male age, and age-specific incompatibility levels suggest that males mating in nature may often be 2 or 3 weeks old. Our parameter estimates accurately predict the frequency of Wolbachia infection in California populations.

MANY insect species harbor microbes related to *Wolbachia pipientis* that make infected males reproductively incompatible with uninfected females (BREEUWER *et al.* 1992; O'NEILL *et al.* 1992; ROUSSET *et al.* 1992; STOUTHAMER *et al.* 1993; SOLIGNAC *et al.* 1994). Egg hatch is significantly reduced in these crosses relative to the reciprocal cross, crosses between uninfected individuals or crosses between individuals carrying the same infection (FRENCH 1978; WADE and STEVENS 1985; HOFFMANN 1988; BREEUWER and WERREN 1990; MONTCHAMP-MOREAU *et al.* 1991).

In *Drosophila simulans*, Wolbachia are predominantly maternally transmitted (HOFFMANN *et al.* 1986; HOFFMANN and TURELLI 1988) as in other species. Infected females therefore tend to have a reproductive advantage in polymorphic populations, and the infection tends to spread both within and among populations (CASPARI and WATSON 1959; TURELLI and HOFFMANN 1991). We have documented this spread in California *D. simulans* populations (TURELLI and HOFFMANN 1991; TURELLI *et al.* 1992). The within-population dynamics and equilibrium frequency of the infection can be well approximated in terms of two central parameters: the relative hatch rate from incompatible crosses (uninfected female with infected male) and the efficiency of maternal transmission of the Wolbachia infection. Both of these parameters as well as the fecundity of infected *vs.* uninfected females can be estimated directly from wild-caught flies, thereby providing an opportunity to test predictions concerning the dynamics of this vertically transmitted infection in nature (HOFFMANN *et al.* 1990).

In our analyses of cytoplasmic incompatibility (CI) in *D. simulans*, we have found only one type of infection in California, known as type R (HOFFMANN *et al.* 1986). Uninfected strains are referred to as incompatibility type W. All California strains we have examined can be categorized as either R or W. R strains can be characterized either by the presence of Wolbachia [detected by electron microscopy (BINNINGTON and HOFFMANN 1989), DAPI staining (O'NEILL and KARR 1990) or a PCR assay (O'NEILL *et al.* 1992)] or by the incompatibility of R males with uninfected (W) females as determined by progeny testing. All infected California strains have incompatibility type R; whereas infected strains from Hawaii and various other Pacific and Indian Ocean islands carry Wolbachia that produce incompatibility type S, which is unidirectionally incompatible with W, but also bidirectionally incompatible with R (O'NEILL and KARR 1990; MONTCHAMP-MOREAU *et al.* 1991). We have previously shown that maternal transmission of Wolbachia appears to be perfect in long-established laboratory stocks, and paternal infection transmission occurs rarely (1–2%) in these stocks (HOFFMANN and TURELLI 1988; HOFFMANN *et al.* 1990). Perfect maternal

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transmission and rare paternal transmission were also inferred by NIGRO and PROUT (1990) from an analysis of the joint frequency dynamics of mtDNA variants and incompatibility types in laboratory cage populations. In contrast, we have shown that maternal transmission is imperfect, and paternal transmission is absent or extremely rare, in natural California populations of *D. simulans* (HOFFMANN *et al.* 1990; TURELLI *et al.* 1992). This was demonstrated directly by examining the progeny of wild-caught females and indirectly by following the joint frequencies of mtDNA variants and incompatibility types in nature.

We have also examined host fitness effects and incompatibility levels associated with the infection. HOFFMANN *et al.* (1990) analyzed several fitness-related traits in laboratory populations of infected and uninfected flies. Female fecundity was the only trait repeatably affected, with infected females 10–20% less fecund (see also NIGRO and PROUT 1990). In contrast, wild-caught females showed no fecundity deficit in three tests with flies from an orchard population. HOFFMANN *et al.* (1990) also determined that the relative egg hatch from incompatible crosses in this orchard was reduced by ~45% compared with compatible crosses. This incompatibility drives the rapid spread of Wolbachia within and among California *simulans* populations.

In this paper, we provide data, based on PCR analyses of thousands of flies, that refine our previous estimates of maternal transmission efficiency, relative fecundity, and levels of incompatibility in nature. The PCR assay, which can be reliably applied to single individuals, greatly facilitates surveys of infection frequencies and makes new classes of experiments possible. Our experiments address the following questions.

Are PCR results consistent with other methods for ascertaining Wolbachia infection? We provide data from several hundred lines demonstrating cross-validation with progeny tests and DAPI staining methods.

Has the infection continued to spread in California and how widespread is it elsewhere? We provide a longitudinal and geographical survey of infection frequencies in California and other North American locations and describe infection frequencies in samples from Central and South America, Africa, Hawaii and New Caledonia. These data show that some strains from southern Florida and Ecuador are apparently infected with Wolbachia (or a closely related species, as determined by PCR), but behave in progeny tests as if they are uninfected (compatible with W females and incompatible with R males). We also follow the frequency of a mitochondrial DNA variant associated with the infection in California (TURELLI *et al.* 1992).

What is the efficiency of maternal infection transmission in wild-caught females and how does it compare with laboratory stocks? HOFFMANN *et al.* (1990) found no examples of imperfect maternal transmission after examining several hundred sublines produced by long-established laboratory R stocks. In contrast, several wild-caught females produced both infected and uninfected progeny. To test the generality of this observation, we have undertaken a much larger survey of wild-caught females to determine the efficiency of maternal transmission.

What levels of incompatibility exist between infected males and uninfected females in nature? The ability to assay the infection status of individual males with PCR allows us to estimate this more accurately. Previous estimates (HOFFMANN *et al.* 1990) depended on estimating the fraction of incompatible crosses undertaken by uninfected females. These incompatibility estimates have very large confidence intervals, because they confound estimates of egg-hatch frequencies with the binomial variance associated with estimates of infection frequency. By combining the PCR assay of individual males with collections of virgin females from field-collected rotting fruits, we can now estimate directly the egg-hatch frequencies from all four crosses between infected and uninfected flies in nature.

Is the transmission efficiency of individual wild-caught R females associated with their compatibility with young laboratory R males? If R females with a relatively low transmission efficiency carry a relatively low density of Wolbachia, these females may show partial incompatibility with young R males, known to exhibit extreme incompatibility with W females. In their analyses of maternal transmission with wild-caught females, HOFFMANN *et al.* (1990) found that one female produced an isofemale line of indeterminate compatibility type (*i.e.*, partially compatible with both R and W) and also produced both R and W F_1 sublines. In contrast, all other infected females from nature produced only one or 0 W sublines out of 10. These data suggest that some infected females may harbor relatively few microbes, making them partially incompatible with both our reference infected (R_R) and uninfected (W_W) stocks (HOFFMANN *et al.* 1990) and relatively likely to produce uninfected progeny. Data from other species also suggest an association between incompatibility and infection levels (BOYLE *et al.* 1993; BREEUWER and WERREN 1993; SOLIGNAC *et al.* 1994). To test the generality of this observation, we have surveyed wild-caught females to determine the relationship between compatibility with young R_R males and the reliability of maternal transmission.

Is the decline of incompatibility with male age similar under laboratory and field conditions? Previous experiments indicated that as infected males from laboratory stocks age, they become increasingly compatible with uninfected females, while the age of uninfected females has no effect on compatibility (HOFFMANN *et al.* 1986, 1990). To understand how this relates to levels of incompatibility in nature, we have monitored changes in incompatibility levels with male age under field conditions.

We incorporate our new parameter estimates into simple models that help elucidate the distribution, dynamics and evolution of the alternative incompatibility types of *D. simulans*.

MATERIALS AND METHODS

Stocks and field collections: Collections in California were generally made in fruit orchards by the methods described in HOFFMANN *et al.* (1990). The collection sites, dates, and numbers of isofemale lines are listed in Tables 1 and 2 in RESULTS. Progeny tests were performed using our reference infected (R_R) and uninfected (W_W) stocks (HOFFMANN *et al.* 1990).

Collections from other locations in the US, Central and South America, Pacific Islands, and Africa were made by colleagues. The flies were either sent to us as isofemale lines or as adults just collected from the field. The collection sites, dates, and numbers of isofemale lines are listed in Table 3 in RESULTS. All flies were cultured on standard Drosophila medium at 20–23°.

Assays for infection status and incompatibility type: Three assays were used to determine infection status: progeny tests of isofemale lines (HOFFMANN *et al.* 1990), a PCR assay (O'NEILL *et al.* 1992), and a microscopic examination of DAPI-stained embryos (O'NEILL and KARR 1990). We cross-validated the methods with blind tests, in which alternative assays were performed in different laboratories and/or by different investigators.

Progeny tests for incompatibility type: Females from the field were set up individually in vials, and the resulting isofemale lines were tested as in HOFFMANN *et al.* (1990). At least three replicate crosses were made to our R_R and W_W stocks for each line. Crosses that produced ambiguous results were repeated. Some of the stocks that were incompatible with both R and W were crossed to reference S strains (O'NEILL and KARR 1990; MONTCHAMP-MOREAU *et al.* 1991), infected with Wolbachia that produce bidirectional incompatibility with R and unidirectional incompatibility with W.

PCR assay: To test for the presence of Wolbachia in an individual fly, we performed PCR, using two pairs of primers simultaneously, on a crude DNA extract obtained from an individual male. We obtained a higher success rate for the PCR reaction using single males *vs.* females, so all of our PCR results are from males. Our protocol is based on O'NEILL *et al.* (1992). To extract DNA, we ground a fly in an Eppendorf tube with 30 μl of STE (10 mM Tris, 1 mM EDTA, 25 mM NaCl) and 1 μl of proteinase K (20 μg/μl in dH_2O), incubated for 30 min at 37°, then for 10 min at 95°. The extract was spun for 3–5 min at full speed in a microfuge, and 1 μl of the supernatant was added to a 25 μl PCR reaction mix. The PCR conditions included 2.5 mM $MgCl_2$ and ~400 nM concentration of each primer. Our temperature profile began with 1 min at 95°, then 26–30 rounds of: 30 sec at 95°, 30 sec at 52°, and 1 min at 75°.

We used the primers "76–99 forward" (5′ TTGTAGCCTGCTATGGTATAACT 3′) and "1012–994 reverse" (5′ GAATAGGTATGATTTTCATGT 3′) of O'NEILL *et al.* (1992), which amplify a fragment of ~0.9 kb of rDNA from Wolbachia. As a positive control for the DNA extraction and PCR reaction, we simultaneously used primers in each reaction to amplify segments of single-copy Drosophila nuclear genes, either *suppressor of sable, su(s)* (VOELKER *et al.* 1991) *(su(s)* forward, 724–753, 5′ AACTGGCGAAAAATTCGACG 3′; and *su(s)* reverse, 1113–1092, 5′ TCTTAGCGTGACTGCTTTATGC 3′) or *suppressor of forked, su(f)* (LANGLEY *et al.* 1993) (*su(f)* forward, 5812–5829, 5′ ATTGACTACCTGTCCCAT 3′ and *su(f)* reverse, 6192–6174, 5′ ATAAGTAAATTCGGCGGTT 3′), which yield fragments of ~400 bp. After 26–30 rounds of PCR amplification, a crude DNA extract from infected flies produces a large quantity of amplified Wolbachia DNA that can be easily seen as a band of ~0.9 kb in an ethidium-stained agarose gel and is readily distinguishable from the much smaller control band. This technique detects both the R and S infections of *D. simulans* (O'NEILL *et al.* 1992), but it does not distinguish between them.

For our 1992 survey work, we determined the infection status of a line by performing the PCR assay on an individual male. For later experiments (as indicated below), we designated a line as uninfected only if four separate males from the line were shown to be uninfected by PCR. This greatly reduces the probability that a line carrying the infection is scored as uninfected because of imperfect maternal transmission by wild-caught females. Imperfect maternal transmission was probably not a major problem for our 1992 survey work, because we tested isofemale lines held in the laboratory for some months and laboratory culture reduces the incidence of imperfect transmission (see below).

DAPI assay: We used a modification of the method of O'NEILL and KARR (1990) described in HOFFMANN *et al.* (1994).

Cross-validation experiments: For all tests, different assays were performed by different people. We used both PCR and progeny testing on 165 lines collected from various California populations in August and October 1992: 75 from Winters (26 were R and 49 were W from progeny testing); 23 from Gridley (11 R, 12 W), 12 from Ivanhoe (all R), 9 from Piru (all R), 16 from Paradise (12 R, 4 W), and 30 from Manton (15 R, 15 W). We used the PCR and DAPI assays on 323 California lines collected in October 1992: 99 from Manton (53 R, 46 W from PCR), 60 from Orland (29 R, 31 W), 77 from Satiety (44 R, 33 W), and 87 from Piru (85 R, 2 W). The PCR and DAPI results were compared only after all tests were complete. We found very few disagreements and only seven lines were rechecked.

To test the reliability of the PCR assay for non-California stocks, we undertook progeny tests on subsamples from various locations, including those with incompatibility type S.

Geographical and longitudinal surveys of infection frequencies: Almost all of our estimates of infection frequencies are based on PCR assays of isofemale lines. The alternative infected incompatibility types were distinguished by progeny tests.

Assays of mtDNA restriction enzyme genotypes: We used the methods described in TURELLI *et al.* (1992) to determine restriction-enzyme haplotypes of 112 California isofemale lines collected in October 1992: 82 were type W and 30 were type R. Our assays determined the relative frequency of variants A and B described by HALE and HOFFMANN (1990).

Maternal transmission: Three experiments were performed to determine the frequency with which R females produce W offspring. A fourth estimate of maternal transmission was obtained from the experiment described in the next section.

The first experiment used females collected from a citrus orchard in Ivanhoe, California in April 1993. It was aimed at determining whether most infected females produced some uninfected progeny or whether a minority of infected females was responsible for most of the imperfect maternal transmission. To test this, we used 59 isofemale lines set up from wild-caught females. F_1 progeny were used to set up isofemale sublines for each isofemale line. We set up 49 sets of 10 sublines (*i.e.*, 10 sublines each derived from a single F_1 daughter from a wild-caught female), and 10 sets of 50 sublines. The infection status of each subline was determined by applying

the PCR assay to individual males. Only one male was analyzed per line. Because of imperfect maternal transmission, some lines scored as W may have been infected. We provide a correction for this in the RESULTS. For the subsequent experiments, a line was scored as W only after four separate males were scored as uninfected.

The second experiment used isofemale lines established in April 1993 when the first experiment was set up. These isofemale lines were maintained in the laboratory with discrete generations for 6 months. From each of 29 isofemale lines, we set up 10 isofemale sublines. This experiment compared maternal transmission rates for stocks maintained in the laboratory with those for females directly from the field.

The third experiment used 50 sets of 10 F_1 isofemale sublines, established from females caught at Ivanhoe in November 1993. The aim of this experiment was to provide another estimate of field maternal transmission efficiency.

Maternal transmission *vs.* incompatibility: To determine whether the fidelity of maternal infection transmission was associated with variation in the level of compatibility with young R_R males, rotting fruit with larvae was collected in a citrus orchard at the University of California, Riverside in April 1993. The fruit was held in gauze-covered cages in a lathe house on the University of California, Davis campus. Virgin females were collected directly from the rotting fruit. The females were held for 1–3 days, then placed with two R_R laboratory males (1–2 days old) in vials with spoons holding dark-colored medium, covered with a thin layer of live yeast. Egg number and egg hatch were scored over 2 days to estimate incompatibility. After 2 days with the R_R males, the females were transferred to fresh yeasted vials, held for 2 days, then transferred to fresh vials for 2 more days. Females held in yeasted vials will lay a large number of eggs and thereby utilize some of their stored sperm, increasing their likelihood of remating. Females were finally placed individually in fresh vials with two W_W males. The vials were monitored continuously for several hours for remating. To determine maternal transmission rates, we wanted to ensure that females had mated with uninfected males to reduce the probability that progeny from uninfected ova produced by infected females would die because of incompatibility with R sperm. Isofemale lines were established only from females observed to mate with W_W males; from each isofemale line, we tried to establish 11 isofemale sublines to determine the fidelity of maternal transmission. We assayed the infection status of these sublines by PCR.

Incompatibility and fecundity in the field: Three different assays were used to estimate the relative egg hatch rates from incompatible *vs.* compatible crosses in nature: one based on wild-caught females, one based on wild-caught males, and one based on mating wild-caught males to virgin females collected from rotting fruit. The two assays that use wild-caught females also provide estimates of the relative fecundity of infected *vs.* uninfected females.

Female assay: This assay is described in detail in HOFFMANN *et al.* (1990): we place wild-caught females individually in vials with spoons containing dark medium on which eggs can be easily seen, score the number of eggs they produce over 1 or 2 days and the fraction of eggs that hatch, then set up isofemale lines to determine infection status. Egg hatch is scored only for females that produced ≥ 10 eggs. We applied this assay to four samples collected within 20 km of Davis, California: two obtained from the Satiety Vineyard, 3 km from Davis, in November 1992, and two obtained from the Wolfskill Experimental Orchard in Winters in March and April 1993.

Following HOFFMANN *et al.* (1990), H, the relative egg-hatch rate from incompatible crosses was estimated as $H \equiv H_I/H_C = [\bar{H}_W - (1-p)\bar{H}_R]/p\bar{H}_R$, where H_I denotes the hatch rate from incompatible crosses (*i.e.*, W♀ × R♂), H_C denotes the hatch rate from compatible crosses (*i.e.*, R × R, W × W, or R♀ × W♂), $\bar{H}_W$ and $\bar{H}_R$ denote the average hatch rates from field-collected W and R females, and p denotes the frequency of type R adults in the population. For H and other parameter combinations with unknown sampling properties, bootstrap confidence intervals were obtained using the bias-corrected and accelerated method of EFRON and TIBSHIRANI (1993, Ch. 14) [the acceleration constant for H was computed with a trivariate extension of their Eq. (15.36)]. For each experiment, we used all data available to estimate p. For instance, for the Satiety samples from November 11 and 12, we pooled the data on infection status of the females collected on both days to estimate p more accurately. The samples that yielded our estimates of p, $\bar{H}_W$, and $\bar{H}_R$ were bootstrapped separately for each bootstrap replicate. Our estimator of H ignores the possibility that sperm competition may favor sperm from R males (WADE and CHANG 1995), because this does not occur in *D. simulans* (HOFFMANN *et al.* 1990).

Male assay: We mated wild-caught males to virgin W_W females, scored egg hatch over 2 days, then determined the infection status of the males with PCR. We analyzed a sample of 152 males collected in Ivanhoe, California in November 1993. Because we expected R to be at a high frequency, we simultaneously set up 20 crosses with W_W laboratory males to obtain an estimate of the hatch rate for crosses with uninfected males. H was estimated as the ratio of the hatch rates from crosses to R *vs.* W males (the six wild-caught W males were pooled with the W_W males). Bootstrap confidence intervals were obtained.

Combined male and female assay: This assay combines the previous two. We collected rotting fruit with larvae from a citrus orchard in Winters, CA, in March 1993. The fruit was held in small gauze-covered cages in an unprotected lathe house. We collected virgin females directly from the rotting fruit. When they were 1–3 days old, virgin females were mated singly to wild-caught males collected over two successive days from the same orchard as the rotting fruit. We scored egg number and egg hatch. The individual wild-caught males were tested by PCR for their infection status. The females were remated to W_W males to set up isofemale lines whose infection status was determined by PCR. This design allowed us to estimate the fecundity and egg-hatch rates from all four possible crosses between R and W for flies reared under natural conditions. Bootstrap confidence intervals were obtained for the relative hatch rate from incompatible crosses. To avoid misidentifying females because of imperfect maternal transmission, we repeated the PCR test on four separate males for 51 of the 55 lines that initially tested as W.

Effect of male age on incompatibility: Two experiments were performed at different times of year based on samples from different populations and using different experimental designs. In the first experiment, we collected rotting fruit from both Riverside and Winters, CA, in April 1993. The rotting fruit was held in gauze-covered cages in a lathe house and newly emerging males were collected each day. The males were held in vials in the lathe house until they were used for crosses in the laboratory. Each day we established cohorts of 14–18 newly eclosed males that we held together in a vial for aging. Over a period of 4 weeks, we mated males 1-to-16-days-old to W_W females and scored egg hatch. The infection status of individual males was determined by PCR.

In the second experiment, we collected fruit in November 1993 from a citrus orchard in Ivanhoe, CA. As before, the fruit was held outdoors and we collected newly emerging males daily. We simultaneously collected males from laboratory R_R and W_W cultures. Unlike the previous experiment, all of the Ivanhoe males were aged in cohorts on rotting fruit

(collected at Ivanhoe) in outdoor cages, rather than in vials. To protect against contamination, citrus with unbroken skin was held in gauze-covered buckets before being sliced and added to the cages for male aging. The R_R males were split into three groups, one held in the laboratory, the other two in replicate field cages with rotting fruit from the same batch used for the Ivanhoe male cages. Each treatment was replicated with cohorts of same-age males. Males from one day to five weeks old were mated to W_W females, and egg hatch scored. The infection status of all of the Ivanhoe males and many control males was determined by PCR.

RESULTS

Cross-validation of assays for infection status: Three assays were used to determine infection status: progeny tests of isofemale lines, a PCR assay (O'NEILL *et al.* 1992) and a DAPI assay (O'NEILL and KARR 1990). For all of the California lines tested, the PCR results match closely those from progeny testing and the DAPI assay. In blind tests, we assigned 165 California lines to R and W incompatibility types with both PCR and progeny testing, and found agreement for 164 of the 165 lines. Progeny tests indicated 70 R and 65 W lines, whereas one of the progeny-tested R lines from Gridley was identified as a W line using PCR. There were no cases in which progeny-tested W lines were PCR positive. We also found initial agreement between 316 out of 323 California lines whose infection status was determined by DAPI and PCR. Initially, we found disagreement between four lines from Satiety (three R and one W from PCR) and three lines from Piru (all R from PCR). The DAPI assay was repeated on these seven lines, and three of the discrepancies were resolved, leaving only four (two from Satiety and two from Piru) that were R from the PCR assay and W from the DAPI assay. Occasional disagreements between the methods are expected because of imperfect maternal transmission, whereby infected females from the field produce uninfected progeny at a low rate (see HOFFMANN *et al.* 1990 and the data presented below). We are confident therefore that both PCR and DAPI can be used to assay infection frequencies in natural populations of *D. simulans*, but PCR is easier for large-scale surveys. We performed additional progeny tests to determine the incompatibility types of lines that test positive for Wolbachia by PCR only for geographical areas not previously assayed. To control for imperfect maternal transmission, we scored lines as W in later experiments only if four separate individuals were PCR-negative for Wolbachia.

Geographical surveys for infection frequency and type: We will report in turn our data from California, other continental US locations, and populations outside of North America.

California: Figures 1 and 2 and Tables 1 and 2 show that the "wave of advance" of type R described in TURELLI and HOFFMANN (1991) and TURELLI *et al.* (1992) has continued northward in California. However, as discussed below, the spatial distribution of frequencies in northern California is no longer consistent with our initial description as a "Bartonian wave," *i.e.*, a diffusion-like wave associated with an unstable equilibrium (BARTON 1979; TURELLI *et al.* 1992). In particular, in October 1992, all 10 samples north of Escalon, which span ~380 km, have statistically homogeneous infection frequencies, which average 0.53 (see Table 1). We had attempted to assess the impact of dispersal mediated by fruit transport by comparing the infection frequencies in two samples separated by ~10 km in Paradise: "Nobel," a long-established apple orchard, and "Heinke," a commercial juicing yard that, when we sampled, was filled with fruit just shipped from Camino, ~130 km to the south. No differences were found, as expected from the homogeneity of contemporaneous samples spanning 380 km.

Our October 1992 samples from Piru, Arvin, Shandon and Watsonville (spanning ~400 km) were statistically homogeneous and provide a pooled frequency of 0.94, which is consistent with the long-term apparent equilibrium observed in Riverside (Figure 2). Similarly, our temporal data (Figure 2) from various California populations indicate that the infection frequencies continue to increase within populations more or less as expected from the data and analysis of TURELLI and HOFFMANN (1991). Our samples collected north of Escalon in August 1992 are not significantly heterogeneous, and neither are our northern samples collected in October 1992 (see Table 1). However, the pooled samples from August and October are significantly different (average frequencies 0.37 *vs.* 0.53, $G = 13.06$, d.f. $= 1$, $P < 0.001$), again indicating that the infection frequency is increasing as expected within these populations.

Although the frequency of R in a population appears to be stable once it reaches a high frequency, the Ivanhoe population provides an exception. The estimated frequencies of R during October 1992 and November 1993 are statistically homogeneous ($G = 0.79$, d.f. $= 1$, $P > 0.3$) and consistent with the long-term apparent equilibrium frequency observed in Riverside (see Figure 2). However, the estimated frequency in April 1993 is significantly lower than each of these (overall, $G = 10.07$, d.f. $= 2$, $P < 0.01$). This temporary drop in the frequency of R suggests temporal fluctuation in parameters influencing the frequency of the infection, such as a decreased level of incompatibility between infected males and uninfected females or a decreased fidelity of maternal transmission. The nominal drop in Escalon/Crow's Landing between August 1992 and April 1993 is not statistically significant: $G = 1.90$, d.f. $= 1$, $P > 0.1$. The fact that a similar drop was not observed in April 1993 at Riverside suggests that the Ivanhoe result was a local event rather than a general seasonal effect.

Samples (1993, 1994) from across the continental US: Wolbachia infection is now prevalent in all US popula-

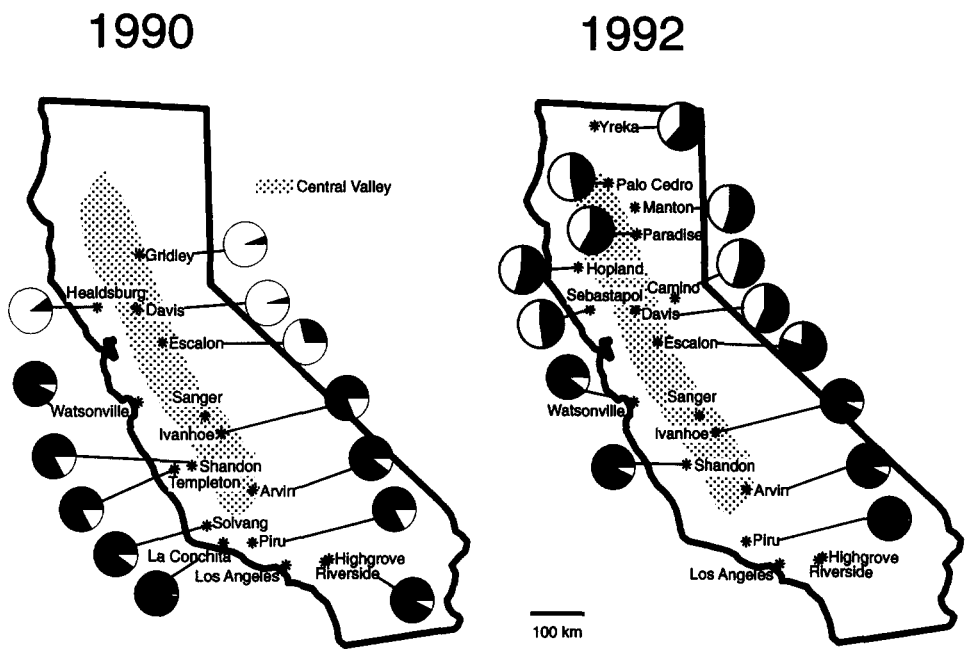

FIGURE 1.—Approximate location of California collection sites in October 1990 and October 1992 and estimates of infection frequency. Shaded portions in the pie diagrams represent infection frequencies. The 1990 data are from TURELLI and HOFFMANN (1991). The 1992 sample sizes, estimated frequencies and confidence intervals are given in Table 1.

tions surveyed (Table 2). Progeny tests on 52 infected lines (five from Fayetteville, AR; eight from Beltsville, MD; 11 from Hampton, VA; four from Austin, TX; 11 from Columbia, SC; and 13 from Tampa, FL; data not shown) show that they are all incompatibility type R, except for the lines from Tampa, FL, which will be discussed below. We had previously analyzed very few lines from outside of California (HOFFMANN and TURELLI 1988). Nevertheless, the 1993 sample of six R lines from Raleigh indicates a statistically significant increase in the frequency of R over a sample of six W lines from Frisco, NC (~275 km away) collected in 1985 ($P < 0.01$, Fisher's exact test). This suggests a relatively recent increase of R in the US Southeast.

We obtained 1994 samples from South Carolina and Florida to test the prediction that these locations, which were highly polymorphic in 1993, would become nearly monomorphic for the infection in 1994. Both locations produced unexpected results.

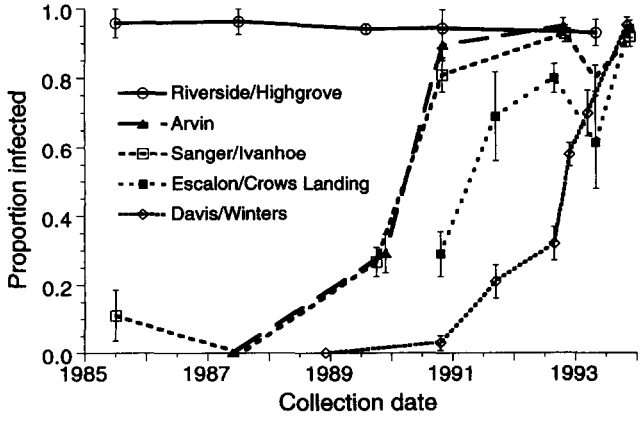

FIGURE 2.—Infection frequency at five sites in California over time. See Figure 1 for approximate locations.

TABLE 1

Frequency of infected (R) flies in 1992 California collections (south to north)

Location	Date	N^a	freq(R)	95% CI[b]
Piru	Oct.	87	0.98	(0.92, 0.997)
Arvin	Oct.	92	0.95	(0.88, 0.98)
Shandon	Oct.	103	0.96	(0.90, 0.99)
Ivanhoe	Oct.	101	0.93	(0.86, 0.97)
Watsonville	Oct.	98	0.90	(0.82, 0.95)
Pool the above[c]		480	0.94	(0.92, 0.96)
Escalon	Aug.	93	0.80	(0.70, 0.87)
Sebastopol	Oct.	100	0.48	(0.38, 0.58)
Winters	Aug.	91	0.32	(0.22, 0.42)
Davis (Satiety)	Oct.	88	0.57	(0.46, 0.67)
Davis (Satiety)	Nov.	209	0.57	(0.50, 0.64)
Camino	Oct.	64	0.55	(0.42, 0.67)
Hopland	Oct.	100	0.55	(0.45, 0.65)
Gridley	Aug.	96	0.42	(0.32, 0.52)
Orland	Oct.	64	0.47	(0.34, 0.60)
Paradise (Heinke)	Oct.	98	0.53	(0.43, 0.63)
Paradise (Noble)	Oct.	102	0.64	(0.52, 0.71)
Manton	Oct.	100	0.54	(0.43, 0.64)
Palo Cedro	Oct.	103	0.47	(0.37, 0.57)
Yreka	Oct.	13	0.62	(0.32, 0.86)
Pool "north" Aug.[d]		187	0.37	(0.30, 0.44)
Pool "north" Oct.[e]		832	0.53	(0.50, 0.57)

[a] Number of isofemale lines tested.

[b] "Exact" confidence intervals with probability 0.025 in each tail were computed numerically from the binomial distribution.

[c] G test for homogeneity gives 7.44 with 4 d.f., $P > 0.1$.

[d] August samples from Gridley and Winters: G test for homogeneity gives 1.93 with 1 d.f., $P > 0.15$.

[e] All October samples north of Escalon: G test for homogeneity gives 7.99 with 9 d.f., $P > 0.5$.

TABLE 2

Frequency of infected (R or A) flies in 1993–1994 continental US collections

Location	Date	N^a	freq(R)	95% CI[b]
San Diego, CA	6/93	16	0.81	(0.54, 0.96)
Death Valley, CA	4/93	79	0.82	(0.72, 0.90)
Riverside, CA	4/93	44	0.93	(0.81, 0.99)
Crows Landing, CA	4/93	13	0.62	(0.32, 0.86)
	11/93	88	0.94	(0.87, 0.98)
Ivanhoe, CA	4/93	110	0.80	(0.71, 0.87)
	11/93	140	0.97	(0.93, 0.99)
Davis, CA	10/93	90	0.96	(0.89, 0.99)
Orland, CA	4/93	12	0.58	(0.28, 0.85)
Eugene, OR	11/93	115	0.62	(0.52, 0.71)
Seattle, WA	9–10/93	16	0.81	(0.54, 0.96)
Tempe, AZ	3–4/93[c]	39	0.82	(0.66, 0.92)
Austin, TX	5–6/93	21	0.81	(0.58, 0.95)
Fayetteville, AR	10/93	29	0.97	(0.82, 0.999)
Valparaiso, IN	5/93	4	0.50	(0.07, 0.93)
Columbus, OH	10/93	5	0.60	(0.15, 0.95)
Beltsville, MD	11/93	41	0.90	(0.77, 0.97)
Hampton, VA	9/93	20	0.95	(0.75, 0.999)
Raleigh, NC	10/93	6	1.00	0.61
Columbia, SC	10/93	11	0.55	(0.23, 0.83)
	6/94	7	0.00	0.35
	7/94	33	0.97	(0.84, 0.999)
Tampa Bay, FL	4/93	23	0.39	(0.19, 0.61)
	8/94	6	0.83	(0.36, 0.996)
Miami, FL[d]	6/94	125	0.40	(0.31, 0.39)

[a] Number of isofemale lines tested.
[b] As in Table 1 for polymorphic samples; for monomorphic samples, we provide 95% upper bounds or lower bounds.
[c] Pooled two statistically homogenous samples from different sites and months [freq(R) = 22/26 and 10/13].
[d] PCR-based data from BILL BALLARD (personal communication).

Because *D. simulans* is often rarer than *D. melanogaster* early in the year, a June 1994 collection from Columbia, SC, provided only seven *simulans* lines out of 100 lines collected. Nevertheless, all seven were type W (Table 2), suggesting that the frequency of R may have dropped between October 1993 and June 1994 ($P < 0.05$, Fisher's exact test). This is reminiscent of the "dip" seen at Ivanhoe in early 1992. However, a sample collected only one month later indicated an R frequency of 0.97 [95% CI = (0.84, 0.999)], a value consistent with our expectations from the 1993 sample and temporal dynamics displayed in Figure 2.

We obtained only six lines from Tampa, FL, in 1994, but data from a much larger sample from Miami were provided by BILL BALLARD (personal communication). Neither sample indicates a statistically significant increase in the frequency of infected flies. Moreover, unlike all other infected lines from the continental US on which we have done progeny tests, the infected lines from Tampa behaved like incompatibility type W; males from these lines failed to cause incompatibility when they were mated with W females, while incompatibility was evident when females from these lines were mated with R infected males (data not shown). This was discovered after we had found infected lines from Ecuador that also behaved like uninfected stocks in progeny tests (see below).

TABLE 3

Frequency of infected flies (I) from collections outside the continental US

Location	Date	N^a	freq(I)	95% CI[b]
San Carlos, Mexico	3/93	27	0.93	(0.76, 0.99)
Texcoco, Mexico	10/93	94	0.86	(0.78, 0.92)
La Selva, Costa Rica	3/94	10	1.00	0.74
Atacames, Ecuador	10/93	18	0.72	(0.46, 0.90)
Montevideo, Uruguay	5/93	30	1.00	0.90
Capetown, South Africa	7/93	16	0.87	(0.62, 0.98)
Zimbabwe	5/93	76	0.96	(0.89, 0.99)
Volcano, Hawaii	1991	53	0.98	(0.90, 0.999)
Noumea, New Caledonia	7/93	40	0.96	(0.87, 0.999)

[a] Number of isofemale lines tested.
[b] See Table 1.

Samples from Central and South America, Pacific Islands and Africa: The infection frequencies are given in Table 3. All the populations sampled show a preponderance of infected lines. We progeny tested nine infected lines from San Carlos, Mexico, eight infected lines from Costa Rica, and two infected lines from Uruguay. All 19 were incompatibility type R.

Similarly, all eight infected lines that we progeny tested from southern Africa (four from South Africa and four from Zimbabwe) displayed incompatibility type R. From Table 3, we see that the infection is near fixation in both samples from southern Africa. The observed frequencies are consistent with an equilibrium between imperfect maternal transmission and unidirectional incompatibility very similar to the one observed in southern California.

The infected lines from Ecuador behaved inconsistently. As shown in Table 3, 13 of the 18 lines tested from Ecuador were infected with Wolbachia, as determined by PCR. As expected, all of the uninfected lines displayed incompatibility type W in progeny tests. However, eight of the 13 infected lines also displayed incompatibility type W in progeny tests, while the remaining five displayed the expected incompatibility type R. The aberrant (infected, but compatible with type W) lines will be denoted type A, because similar lines were first discovered in Australia (A. A. HOFFMANN, unpublished data). We crossed these lines among themselves to determine their compatibility. The data from a factorial crossing experiment involving two isofemale lines of each type (R, W and A) from our Ecuador sample are displayed in Table 4. Egg hatch was scored only for females observed to mate. The data show that the Ecuador R lines are unidirectionally incompatible with both the Ecuador W and A lines, as expected from progeny

TABLE 4

Fraction of eggs hatched from reciprocal crosses between three types of lines from Ecuador

	♂				♂		
♀	R_2	A_2	W_2	♀	R_1	A_1	W_1
R_1	0.79 ± 0.22 (18)	0.90 ± 0.07 (21)	0.94 ± 0.06 (8)	R_2	0.95 ± 0.03 (17)	0.86 ± 0.22 (19)	0.65 ± 0.48 (15)
A_1	0.01 ± 0.03 (19)	0.86 ± 0.21 (21)	0.95 ± 0.06 (8)	A_2	0.08 ± 0.10 (17)	0.94 ± 0.03 (22)	0.79 ± 0.37 (12)
W_1	0.01 ± 0.01 (12)	0.90 ± 0.08 (18)	0.85 ± 0.17 (8)	W_2	0.17 ± 0.07 (8)	0.93 ± 0.04 (8)	0.91 ± 0.08 (5)

Although type A is PCR positive for Wolbachia, it behaves like W in progeny tests. Values are means ± SD; parenthetical data are number of replicates.

tests with R_R and W_W. Moreover, the A and W lines are mutually compatible, as expected from their compatibility with W_W.

In Hawaii and New Caledonia, the type S infection, which is unidirectionally incompatible with W and bidirectionally incompatible with R, is near fixation (Table 3). In 1991, progeny tests were performed on 53 Hawaiian lines; 52 were type S (in agreement with the results of O'NEILL and KARR 1990) and one was type W. A 1993 sample of 40 lines from New Caledonia produced 38 infected and two uninfected lines, as determined by PCR. Progeny tests of four of the infected New Caledonia lines showed that they were of incompatibility type S, as expected from the results of MONTCHAMP-MOREAU *et al.* (1991). These findings suggest that the Wolbachia associated with type S infection are not fixed in natural populations, and uninfected individuals may persist because of imperfect maternal transmission as in populations that are predominantly type R.

mtDNA analyses: We sampled three California populations with intermediate R frequencies (Davis, Orland and Manton) and one nearly monomorphic for R (Piru; Table 5). We estimated the frequency of two mtDNA variants, A and B, described by HALE and HOFFMANN (1990). As expected from the data and analyses of TURELLI *et al.* (1992), the frequency of variant A, which was prevalent in northern California before the advance of type R, is at intermediate frequencies among the uninfected individuals in Davis, Orland and Manton. As noted above, the October 1992 infection frequencies in these three populations were statistically homogeneous ($G = 1.02$, d.f. $= 2$, $P > 0.5$); the weighted average is 0.52. Similarly, the frequencies of variant A among the W lines in these populations are statistically homogeneous ($G = 1.06$, d.f. $= 2$, $P > 0.5$), with average frequency 0.49. Unlike our previous analyses, in which all 92 R lines examined carried variant B, we have found variant A in one infected line from Orland.

Maternal transmission: A weakness of our analysis of the April 1993 Ivanhoe sample is that only one male was assayed by PCR from each subline. If the male was infected, the subline must have been founded by an infected female. However, if the male was uninfected, his mother may or may not have been infected. Thus, we can estimate the transmission frequency only indirectly. Before presenting the model used for this estimate, we will first summarize the results in terms of the number of infected and uninfected males. For five of the 49 groups of 10 isofemale sublines and one of the 10 groups of 50 isofemale sublines, all of the males assayed were uninfected. Thus, six of the 59 females collected from nature were uninfected. Data on the fraction of uninfected F_2 male progeny from the 53 infected females are displayed in Figure 3, which separates the results for the wild-caught females with 50 sublines from the females with 10 sublines. The data are incomplete. Ten of the 490 sublines were lost from wild females producing 10 sublines. Nevertheless, at least eight sublines were scored from each of the 44 infected females. Out of these females, 21 produced only infected males (201 sublines) and 23 produced

TABLE 5

Frequency of mtDNA variant A in California

		W lines			R lines	
Location	freq(R)[a]	N	freq(A)	95% CI[b]	N	freq(A)
Piru	0.98	3	0		1	0
Davis (Satiety)	0.57	33	0.52	(0.34, 0.69)	3	0
Orland	0.47	18	0.39	(0.17, 0.64)	5	0.2
Manton	0.54	28	0.54	(0.34, 0.72)	21	0

Frequency measured in October 1992 samples of California populations with different frequencies of type R flies.

[a] Estimates from Table 1.

[b] As in Table 1.

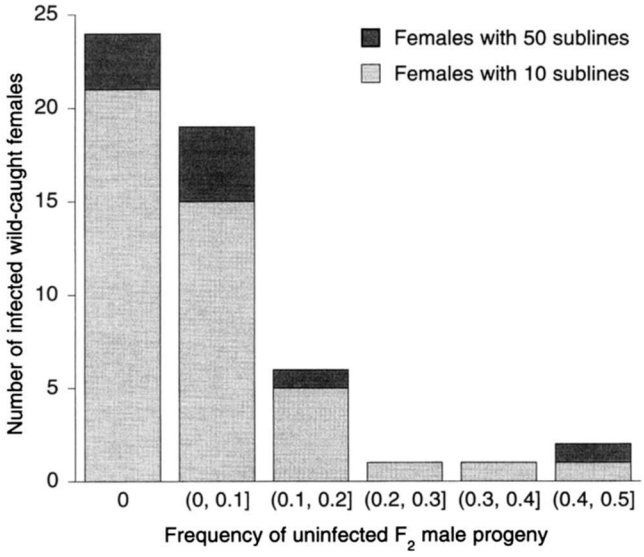

FIGURE 3.—Histogram showing the number of infected females from nature that yielded different frequencies of uninfected F_2 males. The graph presents separately the results from 44 infected females used to establish 10 sublines *vs.* nine infected females used to establish 50 sublines.

both infected and uninfected males (36 uninfected males out of 219 sublines). Overall, 36 males of the 430 assayed were uninfected.

We also lost some (25) of the sublines from the nine infected females producing 50 sublines. Of the nine groups of sublines, three contained only infected sublines (139 sublines); the remaining six produced 38 uninfected males out of 286 sublines. Overall, 38 males from a total of 425 sublines were uninfected. The groups of "10s" and "50s" are statistically homogeneous in their production of infected and uninfected males ($G = 0.09$, d.f. $= 1$, $P > 0.5$). Figure 3 shows clearly that individual females are extremely heterogeneous in their production of uninfected F_2 males [for the nine infected females with 50 sublines, $\chi^2_8 = 100.6$, $P < 0.001$]. A small fraction of females accounts for most of the imperfect transmission in both groups, whereas others show perfect maternal transmission even when 50 of their offspring were scored. We do not know if this heterogeneity is caused by environmental effects or by genetic variation among the females or their infections.

The significant among-female variation in transmission efficiency must be taken into account when estimating the overall efficiency of maternal transmission. For simplicity, we will assume that the F_1 females display the same degree of maternal transmission as their wild-caught mothers. Let v_i denote the fraction of uninfected progeny produced by an individual wild-caught female, known to be infected (because some of her grandsons are). The probability that one of her grandsons is uninfected is: $P(\text{uninfected } F_2) = P(\text{uninfected } F_2 | \text{infected mother})P(\text{infected mother}) + P(\text{uninfected } F_2 | \text{uninfected mother})P(\text{uninfected mother})$. For infected wild-caught females, this becomes $P(\text{uninfected } F_2) = v_i(1 - v_i) + v_i = v_i(2 - v_i)$. Hence, if f_i is the fraction of uninfected F_2 males assayed from the ith female, v_i can be estimated as

$$\hat{v}_i = 1 - \sqrt{1 - f_i}. \quad (1)$$

Because uninfected ova from wild-caught females may be preferentially eliminated by incompatibility with sperm from infected males, v_i, the frequency of uninfected progeny produced by infected mothers, provides a lower bound for μ_i, the frequency of uninfected ova produced by infected females. Assuming that uninfected ova from infected females are as incompatible with sperm from infected males as are ova from uninfected females, random mating implies that

$$v_i = \frac{\mu_i(1 - ps_h)}{1 - \mu_i ps_h} \leq \mu_i, \quad (2)$$

where p is the frequency of type R males and $H = 1 - s_h$ is the relative hatch rate from incompatible fertilizations. If, however, both infected and uninfected ova from infected females remain compatible with sperm from infected males, we would have $v_i = \mu_i$.

Applying Equation (1) to each group of sublines, then taking a weighted average of the v_i based on the number of F_2 examined, we obtain $\hat{v} = 0.047$ (applying Equation 1 to the pooled estimate $f = 74/855$ yields $\hat{v} = 0.044$). A 95% bootstrap confidence interval based on 5000 replicate bootstraps over sets of sublines is (0.029, 0.090).

Our second experiment examined the transmission fidelity of isofemale lines from Ivanhoe kept in the laboratory for 6 months before isofemale sublines were set up. These isofemale lines were established in April 1993 along with those that produced the data in Figure 3. A subline was identified as uninfected only after four separate males had been tested. If ≥70% of the progeny of an infected female are infected, which as indicated in Figure 3 holds for >90% of the infected females in nature, the probability of misidentifying such a female as uninfected with this procedure is <1%. Of the 29 females producing 10 isofemale lines, 20 were infected as indicated by the fact that at least some of their progeny were infected. Of the 200 sublines produced by these infected females, five were lost, 193 were R, and two were W (produced by different females from which 10 sublines were scored). This yields $\hat{v} = 0.010$. A 95% binomial confidence interval for this estimate is (0.001, 0.037). These data are consistent with the hypothesis that the fidelity of maternal Wolbachia transmission increases under laboratory conditions.

Our third experiment used isofemale sublines set up from wild-caught Ivanhoe females in November 1993. Out of 50 sets of 10 sublines, 49 produced R sublines. We scored 423 of the original 490 sublines, and all were found to be infected. This produces a 95% binomial upper bound for v of 0.007.

Finally we consider maternal transmission data from the experiment designed to assess the association between a female's transmission fidelity and her compatibility with young R_R males. This experiment more accurately estimates μ, the frequency of uninfected ova produced by infected females, because the females were held for 2 days on medium that induces egg laying, then mated to uninfected males before the sublines were established. We aimed to set up 11 isofemale sublines from each of 70 females, collected as virgins from rotting citrus from Riverside, then mated sequentially to R_R and W_W males. Of the 70 original females, 67 produced at least two sublines. Sixty of these produced at least one R subline, indicating that the female was infected. One of the 60 females produced 10 W sublines and one R subline. We believe that this subline represents a rare paternal transmission of Wolbachia from the R_R male to which the female was first mated rather than a case of extremely weak maternal transmission. We will therefore ignore the 11 sublines produced by this female in the analysis. From the remaining 59 females, 510 F_2 sublines were assayed and 24 of them were uninfected, as determined by PCR of at least four males. This yields $\hat{\mu} = 0.047$. A 95% bootstrap confidence interval based on 5000 bootstrap replicates over sets of sublines is (0.026, 0.080).

In this experiment, we kept track of the sequential PCR results for sublines from which the first male assayed was uninfected. Ten lines in which the first male was uninfected were found to be infected when three or more additional males were tested (this also occurred in the exceptional group of 11 sublines in which only one was found to be infected after multiple males were tested). Thus, the first male was uninfected from 34 of 510 sublines (corresponding to $f = 0.067$ in the notation of Equation 1). The model underlying Equation 1 implies that for $\mu = 0.047$, we expect $f = 0.092$. The 95% binomial confidence interval for the observed f is (0.047, 0.092). This provides at least qualitative support for our indirect estimate of v in the first experiment, but suggests that it may be a slight underestimate, as expected if the laboratory-reared F_1 females show more reliable maternal transmission than their wild-caught mothers. Although the estimate of μ from this experiment is consistent with that from our first experiment, G tests of homogeneity suggest that it is significantly greater than the estimate, $\hat{v} = 0.010$, from the Ivanhoe females kept in the laboratory for 6 months ($G = 6.27$, d.f. = 1, $P < 0.05$) and greater than the estimate, $\hat{v} = 0$, obtained from our November 1993 Ivanhoe sample ($G = 26.9$, d.f. = 1, $P < 0.0001$). These differences are too large to be explained by incompatibility between uninfected ova and sperm from infected males.

Maternal transmission *vs.* incompatibility: For individual females collected as virgins from rotting fruit, we determined the fraction of their eggs that hatch from crosses with young R_R males, the number of eggs laid over 2 days, and the fraction of their F_1 isofemale sublines that were infected. Figure 4 is a plot of egg hatch against fraction of infected sublines obtained from individual females. For simplicity, we can lump the females into three classes: those that produced only R sublines (denoted "R = 1"), those that produce both R and W sublines (denoted "0 < R < 1"), and those that produce only W sublines (denoted "W"). These classes produce the following average hatch rates: $\bar{H}_{(R=1)} = 0.889$ (SD = 0.171, $N = 43$), $\bar{H}_{(0<R<1)} = 0.696$ (SD = 0.316, $N = 14$), and $\bar{H}_W = 0.003$ (SD = 0.008, $N = 7$). As expected, uninfected females (those that produced all uninfected sublines) produce the lowest hatch rate. Among infected females, the hatch rate tends to increase with increasing fidelity of maternal transmission (Spearman's $r = 0.58$, $P < 0.001$; Kendall's $t = 0.249$, $P < 0.01$); and $\bar{H}_{(0<R<1)}$ is significantly lower than $\bar{H}_{(R=1)}$ (Mann-Whitney U = 255.5, $P < 0.001$). In contrast, there are no significant differences among the numbers of eggs laid by these groups: $\bar{F}_{(R=1)} = 44.8$ (SD = 18.4, $N = 43$), $\bar{F}_{(0<R<1)} = 40.6$ (SD = 16.4, $N = 14$), $\bar{F}_W = 54.0$ (SD = 24.6, $N = 7$) (ANOVA, $F_{2,61} = 1.19$, $P > 0.3$).

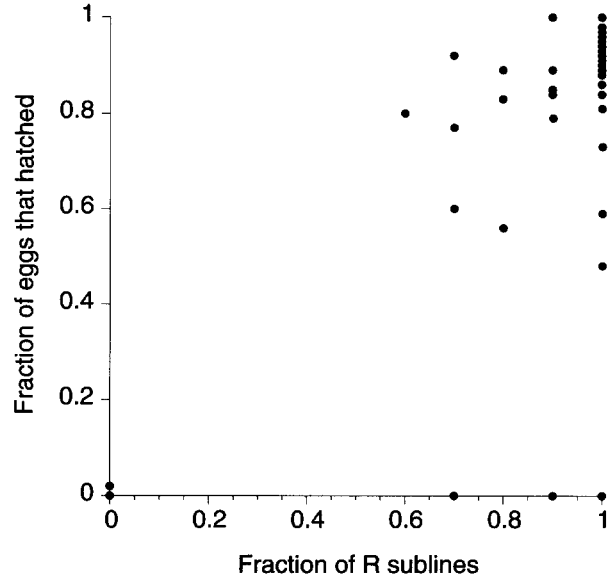

FIGURE 4.—Relationship between the fraction of infected sublines produced by a female and the fraction of her eggs that hatched in crosses to young R_R males. Each point represents the data from a single female.

Incompatibility and fecundity in the field: The results from the three experimental designs will be reported in turn.

Female assay: Table 6 presents the sample sizes, egg counts and hatch rates for each of the four collections. In the first two collections from Davis, we combined the PCR data for females collected on both days to estimate more accurately the frequency of infected flies. Hatch proportions were scored only for females that produced ≥10 eggs; hence the sample sizes for egg number (which were used to estimate the frequency of

TABLE 6

Egg hatch and number for field-collected females

Collection	R ♀		W ♀				
	N	$\bar{x}$	N	$\bar{x}$	P^a	$\hat{H}$	(95% CI[b])
Davis 11/11/92							
Number of eggs	30	30.8 ± 14.4	15	33.4 ± 15.5	>0.2		(0.70, 1.26)
Proportion hatched	28	0.56 ± 0.27	15	0.34 ± 0.28	<0.05	0.32[c]	(0.00, 0.88)
Davis 11/12/92							
Number of eggs	90	38.8 ± 17.5	74	35.9 ± 16.2	>0.1		(0.94, 1.24)
Proportion hatched	88	0.76 ± 0.24	72	0.63 ± 0.26	<0.001	0.69[c]	(0.51, 0.86)
Winters 3/93							
Number of eggs	63	54.8 ± 21.9	32	58.5 ± 18.2	>0.2		(0.80, 1.07)
Proportion hatched	62	0.89 ± 0.18	32	0.71 ± 0.32	<0.02	0.71	(0.48, 0.89)
Winters 4/93							
Number of eggs	83	42.7 ± 15.8	22	41.1 ± 23.6	>0.5		(0.83, 1.35)
Proportion hatched	80	0.91 ± 0.11	19	0.48 ± 0.31	<0.001	0.40	(0.21, 0.60)

$\bar{x}$ values are means ± SD.

[a] Probabilities are for t-tests (number of eggs) or Kruskal-Wallis tests (proportion hatched).

[b] 95% bootstrap confidence intervals for the relative number of eggs laid and H are based on 5000 bootstrap replicates (see MATERIALS AND METHODS).

[c] Based on an estimate of $p = 120/209$, obtained by pooling data from both days.

type R) tend to be slightly larger than those for egg hatch. As in the experiment reported above, which used very young females, the numbers of eggs produced in the lab by infected *vs.* uninfected wild-caught females are not statistically significantly different in any of these four experiments. In contrast to the small differences in egg number, infected females showed a significantly higher fraction of hatched eggs in each experiment, as expected in these populations that were highly polymorphic for R and W. We calculated average hatch rates for the females without weighting by the number of eggs they produced. As shown in Table 5 of HOFFMANN *et al.* (1990), these "unweighted" averages are very similar to weighted averages. Although the point estimates for H vary from 0.32 to 0.71, the wide confidence intervals make it difficult to infer systematic effects of environmental variation. The very low egg hatch for both R and W females on November 11, 1992 was associated with near-freezing temperatures, known to inactivate sperm (ASHBURNER 1989, p. 504).

Male assay: The PCR assay was successful in determining the infection status of 139 out of 152 wild-caught males. Of these, 133 were R and 6 were W. The data from the six wild-caught W males were combined with those from 19 W_W laboratory males (whose infection status was also verified by PCR) to produce a more stable estimate of H. The infected and uninfected males produced the following average hatch rates: $\bar{H}_R = 0.268$ (SD = 0.243, $N = 133$) and $\bar{H}_W = 0.626$ (SD = 0.304, $N = 25$). The hatch rates for both classes of flies were lower than usual because about half of the W_W females used were rather old (12 days old *vs.* 2 days) and they laid some unfertilized eggs. Nevertheless, the data from both the young and old females produced homogeneous estimates of $H = H_R / H_W$ ($\hat{H} = 0.402$ for old and $\hat{H} = 0.444$ for young), as expected if the additional source of unhatched eggs affected an equal fraction of the eggs produced by both types of females. The complete data set yields $\hat{H} = 0.428$, with a 95% bootstrap confidence interval of (0.35, 0.56), based on 5000 bootstrap replicates.

Combined male and female assay: We made 289 crosses between wild-caught males and virgin females collected from rotting fruit, and we obtained complete data (*i.e.*, egg number, egg hatch, and male and female infection status) from 198 pairs. Of the 91 pairs with incomplete data, five were discarded because <10 eggs were produced; two were W♀ × R♂ and three R × R. These crosses are included in our analysis of egg number. Of the 55 maternal lines that were initially scored as W with PCR, 51 were retested with three additional males. Of these, one proved to be infected in subsequent tests (the next three males were all infected). This is consistent with our data on imperfect Wolbachia transmission by wild-caught females.

The egg number and egg hatch results are summarized in Table 7. Consistent with our previous field assays, we find no significant differences among the egg numbers produced by the four crosses (ANOVA, $F_{3,199} = 0.60$, $P > 0.5$). From 1000 bootstrap replicates, a 95% bootstrap confidence interval for the relative number of eggs produced by R *vs.* W females is (0.93, 1.18). As expected from our laboratory crosses with R and W stocks, egg hatch rates are similar from the three crosses expected to be compatible. Although ANOVA indicates no significant differences among the average hatch rates ($F_{2,159} = 0.40$, $P > 0.5$), a more appropriate nonparametric test of these non-Gaussian observations indi-

TABLE 7

Results from crosses between wild-caught males and virgin females collected from rotting fruit

Cross	N^a	Egg number	Fraction hatched
W♀ × R♂	38	35.7 ± 16.1	0.482 ± 0.332
W × W	19	36.3 ± 15.8	0.852 ± 0.265
R♀ × W♂	38	39.9 ± 13.3	0.905 ± 0.239
R × R	108	37.5 ± 13.6	0.888 ± 0.189

Egg number and fraction hatched values are means ± SD.
[a] Number of crosses contributing to mean egg numbers; for egg hatch, there are two fewer W♀ × R♂ and three fewer R × R.

cates a statistically significant difference (Kruskal-Wallis H = 9.43, d.f. = 2, $P < 0.01$). This suggests that infected females may have slightly higher hatch rates, in contrast to previous results for egg-to-adult viability in infected and uninfected lab strains (HOFFMANN *et al.* 1990). There is a small but statistically significant difference between the hatch rates produced by R females mated to R *vs.* W males (0.888 *vs.* 0.905, Mann-Whitney U = 1330, $P < 0.01$), with a higher hatch rate observed with W males, as might be expected from imperfect maternal transmission. The pooled hatch rate from the compatible crosses, 0.888, is much larger than the hatch rate from the incompatible cross, 0.482. This implies a relative hatch rate of $\hat{H} = 0.543$ for the incompatible crosses. From 1000 bootstrap replicates, a 95% bootstrap confidence interval for H is (0.42, 0.66).

Effect of male age on incompatibility: Three of the field collections used to estimate maternal transmission and incompatibility in nature were also used to examine the effects of male age.

March 1993, males from Riverside and Winters: The means and standard deviations of the hatch rates produced by mating males of known age to W_W females are displayed in Figure 5. We included in our analyses only hatch rates estimated from ≥10 eggs. Because of unexpectedly high temperatures (in excess of 30°), many of the Winters replicates were lost before the males were 1 week old. Hence, we were forced to pool results over successive day-classes (*e.g.*, results from 1- and 2-day-old males were pooled and assigned a weighted average age). Moreover, because the frequency of type R was so high in Riverside (~94%), too few W males were obtained to present the W data from the two populations separately. Consistent with previous analyses of infected males from laboratory stocks (HOFFMANN *et al.* 1986, 1990), Figure 5 shows that the level of incompatibility decreases with male age. Comparing the hatch rates produced by R *vs.* W males, we estimate the relative hatch rates from incompatible crosses involving males of ages 1–2 days, 7–8 days and 13–14 days to be: 0.06, 0.16 and 0.50, respectively, for the Riverside sample, and 0.11, 0.19 and 0.24, respectively, for the

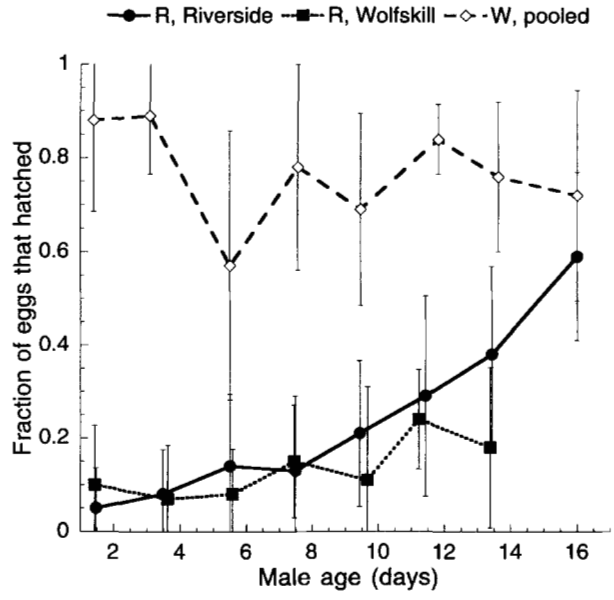

FIGURE 5.—Relationship between mean hatch rate of offspring from uninfected females and male age for infected males from Riverside and Winters and for uninfected males from both populations. The error bars indicate one standard deviation (not standard error of the mean). The means are based on hatch rates produced by: 28, 23, 30, 27, 21, 21, 14 and eight males for the Riverside R sample; 26, 24, 14, nine, six, nine and eight males for the Winters R sample; and 15, 11, four, nine, 13, 11, 19 and five males for the W sample, pooled from Riverside and Winters.

Winters sample. In general, the levels of incompatibility estimated from these males of known age are systematically higher than the estimates obtained from wild-caught flies, except for the oldest Riverside males. This suggests that even older males should be examined to obtain incompatibility levels comparable to those in the field. This was done in the next experiment, which also included additional controls.

November 1993, males from Ivanhoe: We compared the fraction of eggs that hatched when males of known age from four different, replicated treatments were mated to W_W females. We used: infected Ivanhoe males, collected as they eclosed from rotting fruit and aged in outdoor cages; R_R males aged on rotting fruit in outdoor cages; R_R males aged in the laboratory; and W_W males aged in the laboratory. The infection status of the Ivanhoe males was determined by PCR after they had mated, and we present data from only the infected males. Too few uninfected Ivanhoe males were found to estimate mean hatch rates (only three were found of all ages). The data means are displayed in Figure 6; and the means, standard deviations and sample sizes are presented in Tables 8 and 9 in APPENDIX. The W_W controls in these experiments show rather low egg hatch, whereas the R_R lines aged in nature show hatch rates at each age higher than those of our infected Ivanhoe males. Our *a priori* expectation was that the R_R males aged in the field cages would produce signifi-

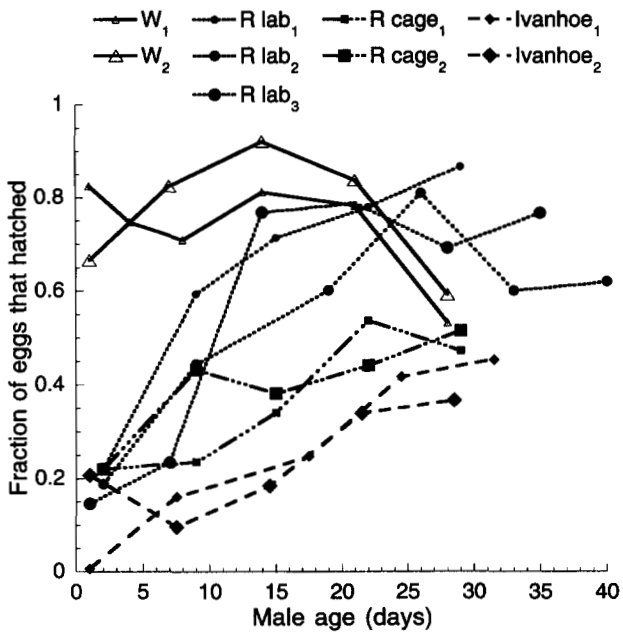

FIGURE 6.—Relationship between mean hatch rate of offspring from uninfected females and male age for replicates of four different treatments.

cantly lower hatch rates than the Ivanhoe males. The data obviously do not support this.

The replicates for each treatment are fairly consistent. At essentially every age, infected Ivanhoe males were least compatible with W_W females, the R_R males held in the field cages were intermediate, and the R_R males held in the lab were most compatible. Taking averages of the means over replicates, we estimate the relative hatch rates from incompatible crosses involving males of ages 1–2 days, 7–9 days, 14–18 days, 21–25 days and 28–33 days to be: 0.15, 0.17, 0.25, 0.47 and 0.73, respectively, for Ivanhoe; 0.29, 0.44, 0.41, 0.60 and 0.89 for R_R held in field cages; and 0.25, 0.55, 0.79, 0.98 and 1.25 for R_R held in the lab. The high relative hatch rates for the oldest age class reflects the fact that there was a decrease in the hatch rates of 28–33-day-old W_W males mated to W_W females. A similar result appears in Figure 3 of HOFFMANN *et al.* (1990). The declining hatch rate for the oldest W_W males was associated with an increasing fraction of crosses in which all eggs were unhatched. This was observed to a lesser degree with old males from the R_R stock and may be an artifact of inbreeding in these 9-yr-old laboratory stocks. Thus, the incompatibility levels of the infected males may have been systematically underestimated, accounting for the apparent "super-compatibility" of the 28–33-day-old R_R males held in the laboratory. However, inbreeding does not account for the unexpected differences between the infected males from the three different treatments. Differences between the R_R lab and field cage males probably reflect environmental conditions. The cages were held in an unheated lathe house from November 9 until December 29, 1993, and ambient temperatures were systematically lower here than in the laboratory (it froze on at least two nights). Differences between the Ivanhoe and R_R flies held in cages are difficult to explain.

DISCUSSION

Work on the population and transmission biology of CI has been hampered by the necessity of performing replicated progeny tests on isofemale lines to determine infection status. Two techniques now allow us to assay individual flies: DAPI (O'NEILL and KARR 1990) and PCR (O'NEILL *et al.* 1992). We have cross-validated these alternative techniques and performed several experiments that depend critically on knowing whether individual wild-caught flies are infected. We will discuss the new techniques and our estimates of parameter values in nature, and then use them to interpret our data concerning the frequency of mtDNA variants and incompatibility types in natural populations.

Assays for infection status and incompatibility type: We have independently tested several hundred isofemale lines for Wolbachia infection using PCR, DAPI and progeny testing. With very few possible exceptions, both PCR and DAPI were reliable indicators of Wolbachia infection. Some discrepancies are expected because of imperfect maternal transmission. In most populations surveyed, infection implies unidirectional incompatibility with uninfected lines. All such infected lines from North, Central and South America, Africa, Hawaii and New Caledonia were either incompatibility type R, first identified by HOFFMANN *et al.* (1986) in flies from Riverside in southern California, or incompatibility type S, first identified in Hawaiian flies by O'NEILL and KARR (1990), then shown to pervade several Pacific and Indian Ocean island populations by MONTCHAMP-MOREAU *et al.* (1991). We have now found a new class of infected flies that are PCR-positive for Wolbachia but act as uninfected flies (incompatibility type W) in progeny tests with type R and W lines. We denote these incompatibility type A, because they were first reported from Australia (A. A. HOFFMANN, unpublished data). This type is prevalent in southern Florida, where it has coexisted with the uninfected W type for $\geq$2 yr, and it co-occurs with both R and W in a sample from Ecuador. The new incompatibility type will be discussed further below. Overall, our experience indicates that infection frequencies in natural populations can be reliably and rapidly determined by PCR. However, it is essential that the incompatibility types of several infected lines be determined by progeny tests when surveying new geographical locations. Moreover, we recommend that four males be assayed from putative uninfected lines to account for the possibility of imperfect maternal transmission, unless PCR is carried out directly on females from the field.

Maternal transmission: Three of our four experi-

ments estimate v, the fraction of uninfected progeny produced by infected females; and only one directly estimates μ, the fraction of uninfected ova produced by infected females. As argued above, $v \leq \mu$ (see Equation 2); so we will discuss all of the results together as conservative estimates of μ. Two of our three new assays of wild-caught females produce estimates near 0.045, with 95% confidence intervals spanning roughly 0.03–0.09. One of these was made from Ivanhoe in April 1993, the other was from Riverside, ~300 km away. Our third estimate, $\hat{v} = 0$, was based on a November 1993 sample of wild-caught females, captured from the same Ivanhoe orchard that yielded $\hat{\mu} \geq 0.047$ in April 1993. This indicates temporal variation of an unknown cause in the reliability of maternal transmission in nature. HOFFMANN *et al.* (1990) previously documented imperfect maternal transmission with wild-caught females from a third California population at Piru, implying that $\hat{\mu} \geq 0.016$. Our data in Figure 3, showing between 0 and 45% uninfected sublines from different females, reinforces the tentative conclusion of HOFFMANN *et al.* (1990) that infected females from the same population can differ appreciably in the fidelity of Wolbachia transmission.

The weighted average of our estimates from wild-caught females yields $\hat{\mu} \geq 0.032$. This is close to the value of $\mu = 0.04$, inferred by assuming that the long-term frequency of R observed in Riverside (0.94) represents an equilibrium between imperfect maternal transmission and cytoplasmic incompatibility with $H = 0.55$ and $F = 0.95$ (TURELLI *et al.* 1992). Here H denotes the relative hatch rate from incompatible crosses and F denotes the relative fecundity of infected females. If we apply Equation (2) to correct the possible bias in the estimates of μ, the weighted average of our four estimates is 0.042, assuming that $H = 0.55$ and that the frequency of R was 0.44 at Piru, 0.80 at Ivanhoe for our first estimate, and 0.94 at Riverside.

HOFFMANN *et al.* (1990) found no examples of imperfect maternal transmission among R_R lines that had been held in the laboratory for several years, even after subjecting them to temperature extremes or using 3-week-old females to set up the sublines. They examined 396 sublines in total, yielding a binomial 95% upper bound on v of 0.008. To determine whether this low value is a specific property of the R_R line or a more general feature of infected lines maintained for many generations in the laboratory, we replicated our assay of Ivanhoe females from the collection that yielded $\hat{v} = 0.047$, using isofemale lines maintained in the laboratory for 6 months (12 generations). The lab-held females gave $\hat{v} = 0.010$ (95% confidence interval 0.001–0.037), indicating that the fidelity of maternal transmission increases with laboratory culture. This may be attributable directly to laboratory conditions increasing infection levels or may reflect evolutionary changes in the host or parasite genomes (TURELLI 1994).

Maternal transmission *vs.* incompatibility: Our analysis of females collected as virgins from rotting fruit shows that as the fidelity of maternal Wolbachia transmission increases, infected females tend to become increasingly compatible with young R_R males (Figure 4). These data suggest that uninfected or weakly infected ova produced by infected females are incompatible with sperm from infected males. The data in Figure 4 are consistent with the conjecture of HOFFMANN *et al.* (1990) that infected females in nature vary in their levels of intracellular Wolbachia density. They are also consistent with BREEUWER and WERREN's (1993) demonstration that bacterial density affects cytoplasmic incompatibility in *Nasonia vitripennis*. They found that females from lines characterized by low infection levels are incompatible with males from lines with higher infection levels. Covariation of Wolbachia density with level of incompatibility has also been reported in comparisons between *D. simulans* and *D. melanogaster* (BOYLE *et al.* 1993) and among strains of *D. melanogaster* (SOLIGNAC *et al.* 1994). These results and ours suggest that it may be insufficient to characterize California *simulans* lines as R *vs.* W, because the infected class, "type R," may be heterogeneous in its compatibility properties. We examined this directly with wild-caught flies (see below).

Incompatibility and fecundity in the field: We report six new experiments that estimate H, the relative hatch rate from incompatible *vs.* compatible crosses. Four experiments are repeats of the "female assay" developed by HOFFMANN *et al.* (1990), one uses a new assay based on mating wild-caught males to uninfected females from laboratory stocks, and one involves mating wild-caught males to virgin females collected from rotting fruit. The average of the four new female-assay estimates, weighted by the number of females in each experiment, is $\hat{H} = 0.58$, very similar to the average, $\hat{H} = 0.57$, obtained from three 1988–1989 samples of the Piru population (HOFFMANN *et al.* 1990). Our male assay yielded $\hat{H} = 0.43$ (95% confidence interval 0.35–0.56), and our combined male and female assay yielded $\hat{H} = 0.54$ (0.42–0.66). The weighted average of all six estimates from this paper and the three Piru estimates from HOFFMANN *et al.* (1990) is $\hat{H} = 0.55$. We have found appreciable unidirectional incompatibility in California orchard populations in the early spring, midsummer and late fall. Our data do not indicate systematic seasonal variation in H; however only our most recent assays have sufficient power to detect it, and these have not been replicated across seasons.

Each of our assays involves assumptions. The female assay assumes random mating between R and W. HOFFMANN *et al.* (1990) found no evidence for nonrandom mating in the laboratory, and the quantitative agreement of our male-based and female-based estimates offers further support for this assumption. The male-based assay assumes that the age and condition of the

uninfected females do not affect the relative hatch rate of incompatible *vs.* compatible crosses. Support for this assumption is provided by our male-assay experiment in which we compared relative hatch rates in crosses with 2- *vs.* 12-day-old females and found no significant difference. Given the greater statistical power of the male assay over the female assay, in which the proportion of incompatible crosses must be estimated, the former seems to be the method of choice for future evaluations of incompatibility in nature. An additional advantage of male assays is that they can be applied even after the Wolbachia infection is at a high frequency in a population.

The most powerful incompatibility assay involves mating wild-caught males to virgin females collected from rotting fruit at a site known to be highly polymorphic for alternative compatibility types. When individual males can be assayed for infection status, this provides data from each possible cross and allows a direct test of the assumption that matings between infected individuals produce the same hatch rate as matings between uninfected individuals. Over the hundreds of progeny tests we have performed with newly established isofemale lines, ~5–20% have to be repeated because the initial tests produce ambiguous results. For instance, some lines initially produce intermediate levels of egg hatch when mated to both R_R males and W_W females. In subsequent tests, these lines eventually become type R or type W. The initial ambiguity is presumably related to highly imperfect maternal transmission, as we have found for some wild-caught females. Given that "type R" may represent a continuum of infection levels, which manifests in both maternal transmission efficiency and compatibility with young R_R males (see Figure 4), we might expect that R × R matings in nature produce a lower egg hatch than W × W matings, because the former may include some partially incompatible crosses. In light of this, it is perhaps surprising that our combined male-and-female assay (Table 7) provides no indication of partial incompatibility between type R flies. Further support for the hypothesis of little or no incompatibility between type R flies in nature is provided by the very high egg hatch rates observed in R females from Piru (HOFFMANN *et al.* 1990). Perhaps few if any infected males in nature are as incompatible with uninfected females as are young R_R males.

HOFFMANN *et al.* (1990) and NIGRO and PROUT (1990) found that infected females from laboratory stocks have fecundities 10–20% lower than uninfected females derived from the same stocks. In contrast, the three Piru samples of HOFFMANN *et al.* (1990) and our five new assays of wild-caught females show no significant differences in the fecundities of R *vs.* W females. The largest of our experiments, each with >150 females, produced 95% confidence intervals of F, the relative fecundity of R females, of 0.94–1.24 and 0.93–1.18. The weighted average of the relative fecundities estimated from all eight experiments is $\hat{F} = 1.01$, suggesting that infected females from nature do not have lower fecundity than uninfected females when tested in the lab.

Effect of male age on incompatibility: Our first experiment, using males from two different populations, found age-specific incompatibilities comparable to those found by HOFFMANN *et al.* (1990) for an infected laboratory stock. HOFFMANN *et al.* (1990) found a relative hatch rate of 0.42 from incompatible crosses using 12-day-old males. With males that were 13–14 days old, we found relative hatch rates of 0.50 for our Riverside sample and 0.24 for our Winters sample. Other flies collected contemporaneously from these populations were used to estimate the level of incompatibility in nature and the fidelity of maternal Wolbachia transmission. A March 1993 sample from Winters yielded $\hat{H} = 0.71$ (0.48–0.89) using a female assay (Table 6), and another yielded $\hat{H} = 0.54$ (0.42–0.66) from a combined male-and-female assay. To achieve these incompatibility levels, matings in this population must involve males more than 2 weeks old, implying generation times of at least 3.5 weeks. Our April 1993 Riverside sample yielded $\hat{\mu} = 0.047$ (0.026–0.080), which is close to our indirect estimate of $\mu = 0.04$, based on observed levels of incompatibility in the field and the apparently stable frequency of type R in Riverside. The consistency of the incompatibility and transmission estimates from these samples with the averages over several experiments suggests that the males in the age experiments may be fairly typical of wild-caught California *simulans*.

Our second experiment found unexpectedly low levels of incompatibility for the reference R_R stock in comparison with a November 1993 sample from Ivanhoe. Other flies from the Ivanhoe collection were used to estimate H and μ. Our male assay gave $\hat{H} = 0.43$ (0.35–0.56), indicating a relatively high level of incompatibility. Our maternal transmission assay produced a 95% upper bound on μ of only 0.007, considerably below our indirect estimate and our other three direct estimates from wild-caught females. Both the incompatibility and transmission data suggest that infection levels within individuals may have been unusually high in Ivanhoe at this time. This may account for the fact that the infected Ivanhoe males were apparently less compatible with uninfected females than our reference R_R males. We do not know why the levels of incompatibility observed for R_R in this experiment, especially for young males, were so much lower than in previous and later experiments. Nevertheless, the age-specific levels of incompatibility estimated from the Ivanhoe males reach the value $\hat{H} = 0.43$, estimated from nature, only after the males are more than 2 weeks old.

Both of our age experiments are consistent in suggesting that males of known age from nature show levels of incompatibility comparable to those observed for laboratory stocks, and that males mating in nature may

often be 2 weeks old. Both points will be discussed further below.

mtDNA analyses: HALE and HOFFMANN (1990) and TURELLI *et al.* (1992) analyzed the joint frequency distribution of mtDNA variants among infected and uninfected flies in various California populations. They found that infected flies all carried mtDNA with the restriction-enzyme-defined variant B; whereas uninfected flies carried variants A and B, and the frequency of variant B rose among uninfected flies as the frequency of infected flies in the population increased. TURELLI *et al.* (1992) showed that this pattern was consistent with the recent northward spread of type R (and mtDNA variant B) from southern California and imperfect maternal transmission of type R. Pooling our October 1992 samples from Davis, Orland and Manton, the estimated frequency of type R is 0.53 in these populations; and the estimated frequency (and 95% confidence interval) of variant A among uninfected flies is 0.49 (0.38–0.61). These new data fit the empirical and theoretical relationships displayed in Figure 1 of TURELLI *et al.* (1992). Assuming that these populations began monomorphic for variant A before the infection invaded, the predicted frequency of variant A among uninfected flies is ~0.45 when the frequency of infected flies reaches 0.53. This prediction assumes $H = 0.55$, $F = 0.95$ and $\mu = 0.04$ (see TURELLI *et al.* 1992). Two southern California populations (Lake Cachuma and Piru) >500 km from the other populations showed very similar frequencies of variant A in 1988 when their infection frequencies were near 0.5. This suggests that the southern populations, like those analyzed in northern California, were initially uninfected and monomorphic for variant A, and that variant B was introduced from the maternal lineages carrying the incompatibility-causing infection.

Previously, mtDNA was examined from 92 infected lines, and all carried variant B. As argued by TURELLI *et al.* (1992), this indicates that the rate of paternal transmission in nature, τ, is <1%. In contrast, one of the 30 infected lines from 1992 carried variant A. There are at least four plausible explanations: paternal transmission, horizontal transmission, immigration, or line contamination. From the analysis of TURELLI *et al.* (1992), we expect that if paternal transmission occurs with a frequency of 0.01, variant B should have a frequency no greater than 0.9 among infected flies. Ignoring the 1988 data from Lake Cachuma and Piru, because we did not observe an initial increase of the infection there, 64 of the 65 R lines from populations known to have undergone a recent increase in infection frequency carry mtDNA variant B. Thus, we can reject $\tau = 0.01$ with $P < 0.01$. If we include the Lake Cachuma and Piru data, 87 of 88 R lines carry variant B. Following the analysis of TURELLI *et al.* (1992), this implies that we can reject $\tau = 0.005$ with $P < 0.05$. In contrast, both HOFFMANN and TURELLI (1988) and NIGRO and PROUT (1990) estimated $\tau = 0.01$–0.02 in laboratory populations. Both paternal transmission and horizontal transmission (which has similar effects) must be rare in natural populations.

Immigration is another possible explanation for the appearance of variant A among these infected lines. We do not know the frequency of mtDNA variants A and B outside of California, but we know that incompatibility type R has been present outside of California since at least 1985 (HOFFMANN and TURELLI 1988) and is now common throughout the continental US. Immigration can explain our data only if alternative mtDNA lineages have become infected with Wolbachia that produce incompatibility type R. This hypothesis still requires either paternal or horizontal transmission, but over a longer time-scale than covered by the increase of R in California. A more extensive geographical survey of mtDNA genotypes and incompatibility types is needed. We expect that very rare paternal transmission is more common than horizontal transmission, because the molecular clock analysis of MORAN and BAUMANN (1994) suggests a geological rather than ecological time scale for horizontal transmission across taxa.

Geographical surveys for infection frequency and type: We first use our parameter estimates to interpret geographical and temporal data on the frequency of type R in California, and then discuss our less extensive data from other locations.

CI-transmission equilibrium: As noted by FINE (1978) and elaborated by HOFFMANN *et al.* (1990) and TURELLI (1994), the simplest way to account for the persistence of uninfected individuals in predominantly infected populations is to postulate a balance between CI and imperfect maternal transmission. Assuming that the only factors significantly affecting local incompatibility-type frequencies are unidirectional CI, whose intensity is measured by $H = 1 - s_h$, imperfect maternal transmission, as measured by μ, and a possible fecundity deficit for infected females, as measured by $F = 1 - s_f$, there exists a stable equilibrium frequency

$$\hat{p}_s = \frac{s_f + s_h + \sqrt{(s_f + s_h)^2 - 4(s_f + \mu F)s_h(1 - \mu F)}}{2s_h(1 - \mu F)} > \frac{1}{2}, \quad (3)$$

whenever

$$1 > s_h > \frac{s_f}{1 - 2\mu F} \quad \text{and}$$

$$0 < \mu < \frac{1}{2}\left(1 - \sqrt{\frac{H(s_h - s_f^2)}{Fs_h}}\right). \quad (4)$$

This model assumes that uninfected ova from infected females are equivalent to ova from uninfected females (see TURELLI *et al.* 1992). It also ignores variation in μ

across females. When conditions (4) are satisfied, there is also an unstable equilibrium, p_u, with $0 < p_u < p_s$, whose value is obtained by reversing the sign of the square root in (3). Based on the experiments summarized above, our best estimates for these three parameters are $H = 0.55$, $\mu \geq 0.032$ and $F = 1$. Setting $\mu = 0.032$, the stable and unstable equilibria are $p_s = 0.96$ and $p_u = 0.08$. If we reduce F from 1 to 0.95, the equilibria become $p_s = 0.95$ and $p_u = 0.19$. This illustrates that for values of μ and s_h consistent with our *D. simulans* data, the position of the unstable equilibrium is much more sensitive to small fecundity deficits than the stable equilibrium.

The prediction $p_s = 0.96$ is near 0.94, which is both the long-term average frequency observed in Riverside (see Figure 2) and the average of the frequencies of all California samples from Watsonville and more southern populations in 1992 (Table 1). Holding the other two parameter values fixed, the predicted stable equilibrium can be reduced from 0.96 to 0.94 by increasing H from 0.55 to 0.62, by increasing μ from 0.032 to 0.042, or by decreasing F from 1 to 0.85. Our data on the relative fecundity of wild-caught females indicate that F as low as 0.85 is unlikely, and the hypothesis that F is below 0.93 can be rejected in both of our largest experiments. In contrast, $H = 0.62$ falls outside the 95% confidence interval for only two of our nine estimates from orchard populations. Similarly, the value $\mu = 0.042$ is consistent with three of our four estimates from wild-caught females. Hence, the apparent equilibrium frequencies in predominantly infected California populations can be adequately understood as a balance between CI and imperfect maternal transmission, using parameter estimates very near those we have obtained. Additional support for this hypothesis and against the alternative hypothesis, that uninfected flies are maintained by immigration from uninfected source populations, comes from our analyses of mtDNA-variant frequencies among infected and uninfected flies. These indicate that as the infection spreads through a population, an increasing fraction of uninfected flies have infected maternal ancestors (TURELLI *et al.* 1992).

Despite the consistency of the CI-transmission balance hypothesis with our data, equilibria may also be influenced by direct curing of infected larvae by antibiotics occurring naturally on rotting fruit (STEVENS and WICKLOW 1992). With H near 0.5, larval curing is roughly twice as effective as imperfect maternal transmission in lowering p_s (TURELLI *et al.* 1992). Hence, we can also reduce p_s from 0.96 to 0.94 by supplementing $F = 1$, $H = 0.55$, and $\mu = 0.032$ with a larval curing frequency of ~0.5%. This suggests that imperfect maternal transmission is likely to be a more important determinant of the frequency of W in predominantly R populations than direct larval curing. However, variation in maternal transmission efficiency may be caused by antibiotics that can eliminate or reduce Wolbachia infections, helping to explain why fidelity is higher in the laboratory than nature. Females on laboratory media generally encounter only mold-inhibiting agents, such as propionic acid, that do not eliminate Wolbachia. The possible importance of naturally occurring antibiotics can be tested by transferring long-established R stocks, such as R_R, with μ near 0, to sets of replicated field cages stocked with either rotting fruit or laboratory medium, and comparing the estimates of μ from the two treatments after several generations.

We do not have enough longitudinal data from populations outside California to assess the robustness of CI-transmission equilibrium as a mechanism for maintaining uninfected flies. Ideally, we need estimates of maternal transmission efficiencies and incompatibility levels using wild-caught flies from a wide range of habitats. However, the frequencies observed in Mexico, southern Africa, Hawaii and New Caledonia (Table 3) suggest that stable near-fixation equilibria may occur in many locations and may involve both type R and type S Wolbachia. The presence of a low frequency of uninfected flies from populations near fixation for incompatibility type S has not been reported previously, but it suggests that parameter values may be similar to those estimated in California. We need to investigate the relative roles of the Wolbachia and Drosophila genomes in determining these parameter values.

Dynamics within populations: Longitudinal data from four California populations show fairly similar trajectories of increase for type R (Figure 2). Assuming that within-population dynamics are relatively insensitive to migration once the frequency of R exceeds 0.2, we can combine these observed rates of increase with our parameter estimates to infer generation times in nature. All four populations show increases of the frequency of R from ~0.3 to 0.8 in ~1 yr. Following HOFFMANN *et al.* (1990), we assume that p_t, the frequency of type R among adults in generation t, follows

$$p_{t+1} = \frac{p_t(1 - \mu)F}{1 - s_f p_t - s_h p_t(1 - p_t) - \mu s_h p_t^2 F}, \quad (5)$$

where $s_f = 1 - F$ and $s_h = 1 - H$. By iterating recursion (5) with $F = 1$, $H = 0.55$ and $\mu = 0.042$, we find that an increase from 0.3 to 0.8 should take about 14 generations (*vs.* 16 generations with $F = 1$, $H = 0.62$ and $\mu = 0.032$). This implies a generation time of ~1 month, assuming that reproduction occurs throughout the year. We have caught *D. simulans* in abundance from March through November. It may well reproduce year round in the relatively benign climates of the Central Valley and southern California. Although generation time is expected to change with temperature, these data suggest that the average generation time in these populations may be closer to a month that the standard 2-week regime maintained in the laboratory. Hence, mating males in nature may often be 2 or 3 weeks old. A more detailed assessment of generation length can be

made over shorter periods when the flies are known to be continually abundant. Our samples from northern California in August and October 1992 indicate that the frequency of R rose from ~0.37 to ~0.53. Recursion (5) implies that this requires five generations with $F = 1$, $H = 0.55$ and $\mu = 0.042$, which suggests a generation time closer to 2 weeks than 4 when the weather tends to be quite warm. This latter estimate of generation time suggests a rate of increase for R faster than observed in other locations and is difficult to reconcile with our field estimates of H and the change of H with male age. A two-week generation time would imply that females mate with males that are often <1 week old, and these matings would produce lower values of H than we observed.

We have observed other frequency dynamics in natural populations that cannot be easily reconciled with our parameter estimates. The most extreme example is that the Piru population, in the Tehachapi Range, which separates the Central Valley from the Los Angeles basin, remained highly polymorphic in 1987 and 1989 (HOFFMANN *et al.* 1990), before finally reaching near-fixation for type R in 1992. As discussed in HOFFMANN *et al.* (1990), this may have been caused by a very low fidelity of maternal transmission. If true, this phenomenon was transient, because the infection frequency in October 1992 had a lower bound of 0.92. Variation in parameter values is also a plausible explanation for the temporary drop in the frequency of R observed in Ivanhoe in the spring of 1993 (Table 2). Our estimate $\hat{\mu} \geq 0.047$, suggests reduced fidelity of maternal transmission.

Columbia, South Carolina is the only population outside of California for which we have longitudinal data. The rise from $\hat{p} = 0.55$ in October 1993 to $\hat{p} = 0.97$ in July 1994 is consistent with the temporal dynamics displayed in Figure 2. However, this apparent steady rise is difficult to reconcile with the upper bound of 0.35 obtained from a sample of only seven individuals in early June 1994. One possible explanation is that this small sample contained relatives and hence did not accurately represent the local population.

Spatial spread: We have documented the northward spread of type R throughout California during the past 9 yr. As noted by TURELLI and HOFFMANN (1991), the initial wave of advance could be described by the standard Gaussian model for dispersal only by invoking dispersal distances more than an order of magnitude greater than direct estimates from Drosophila release-recapture experiments. In contrast to data from 1990, the 1992 data bear no resemblance to a Bartonian traveling wave (BARTON 1979; Figure 1). We found statistical homogeneity of infection frequencies over a 380-km transect from Davis to Yreka. In contrast, in 1990, the infection frequency in the center of the transition from high to low infection rates changed from 0.81 (in Ivanhoe) to 0.04 (in Davis) over ~350 km (Figure 1). The 1992 data imply a migration regime among these northern California populations that is more similar to Wright's "island model" than an "isolation by distance" model, such as a one- or two-dimensional stepping stone or a continuously distributed population (CLARK and BARTON 1990). These data are consistent with extinction-recolonization processes in which founder effects are important in taking infection frequencies in subpopulations above the unstable point, p_u, predicted by (5).

Data from Oregon and Washington suggest that the infection has spread to the Canadian border. Unfortunately, we do not have longitudinal data from these populations to confirm our expectation that type R has recently arrived. A joint analysis of mtDNA, characterized at a finer level than the restriction-enzyme variants A and B, along with infection frequencies and Wolbachia haplotypes, should allow us to determine whether the current pervasiveness of type R is recent, whether this incompatibility type is monophyletic, and whether it was introduced more than once into the continental US. Our strongest support outside of California for the recent-spread hypothesis comes from the southeastern US. Six lines obtained from North Carolina in 1985 were all uninfected, whereas six lines obtained in 1992 were all infected. This weak evidence is reinforced by our 1993–1994 South Carolina data, which document an intrapopulation spread of type R. Given that the infection was previously detected in an isofemale line derived in 1985 from Maryland, where type R is now near fixation, these data suggest a southward wave of advance of type R on the east coast of the US.

Type A: The presence of an infection (type A) in southern Florida and Ecuador that does not cause incompatibility in uninfected flies raises questions about the evolution of Wolbachia infections and their hosts. We know nothing about the cell biology of this new variant or its taxonomic affinity to the Wolbachia that cause incompatibility. The type A infection is not expected to spread in populations, because it does not cause incompatibility. If maternal transmission of the type A infection is not perfect, or if there are deleterious fitness effects associated with it, the infection will be rapidly lost from a population. The fact that type A flies appear to be widespread in America as well as Australia (A. A. HOFFMANN, unpublished data) suggests that parameter values for this infection are different from those associated with the R infection. In Australia, studies have shown that the A infection has perfect maternal transmission in the field and that it has no detectable deleterious effects on the fitness of its host (A. A. HOFFMANN, unpublished data). This infection may therefore behave like a neutral variant in natural populations. It is possible that these types of infections represent the end product of evolutionary changes in Wolbachia and host genomes. Following PROUT (1994), TURELLI (1994) has shown that a new Wolbachia strain may spread at

the expense of an ancestral strain even if it causes a lower level of incompatibility in crosses with uninfected females. This will happen if the new strain has a relatively higher rate of maternal transmission and/or smaller deleterious effect on its host, as long as the new variant remains compatible with the ancestral strain from which it is derived.

Future directions: Further monitoring of infection levels in populations will reveal whether the R infection continues to spread in North America and elsewhere as expected. A particularly interesting situation exists in Florida and Ecuador where the R infection should eventually displace the type A infection. As we have seen in California, monitoring infection frequencies can provide data on the biology of the host as well as the spread of the infection. We have already used this information to document migration patterns, generation times and the mating success of males of different ages in natural *D. simulans* populations.

Despite our success in understanding the dynamics of the R infection in natural populations, several questions remain unanswered. We know that maternal transmission rates and field incompatibility levels can vary within and among populations, but it is unclear if this variation is related to genetic variation among hosts and/or microbes or to antibiotics or other environmental conditions that influence infection levels. We have evidence that infected females can be partially incompatible with infected males, but it is not clear if the hatch rate of infected females in the field is ever significantly lower than the hatch rate produced by matings between uninfected individuals. The male age experiments raise questions about the infection levels of field *D. simulans* males compared with laboratory strains. Can levels in the field be higher than those in laboratory stocks? Are male and female infection levels always correlated? Quantifying infection levels in males (BRESSAC and ROUSSET 1993) and embryos (BOYLE *et al.* 1993) should provide answers.

Perhaps one of the most interesting areas for future research is evolutionary change in the host and Wolbachia genomes. We have predictions about the types of changes expected to occur (TURELLI 1994). They can be tested by comparing the effects of the R infection on *D. simulans* populations that have been recently infected with populations infected for many years. Microinjection experiments (BOYLE *et al.* 1993) can be used to transfer infections from their normal hosts to novel hosts. Such experiments will indicate whether the incompatibility and maternal transmission properties of an infection have evolved and whether such changes involve the host or Wolbachia genomes. Some recent data provide preliminary support for the prediction that incompatibility levels may decline through direct selection on the host (TURELLI 1994). *D. melanogaster* displays much lower levels of incompatibility than *D. simulans* (HOFFMANN 1988; HOFFMANN *et al.* 1994; SOLIGNAC *et al.* 1994). By analyzing mtDNA variation among infected lines, SOLIGNAC *et al.* (1994) have argued that the *D. melanogaster* infection may be much older than the R infection in *D. simulans*. Additional analysis of intraspecific and interspecific variation will reveal the time scale and direction of coevolutionary changes.

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APPENDIX: DATA FROM IVANHOE MALE-AGE EXPERIMENT

Tables 8 and 9 give the means, standard deviations and sample sizes of the age-specific egg-hatch data that are summarized in Figure 6.

TABLE 8

Age-specific hatch rates from crosses between Ivanhoe or W_W males and W_W females

Ivanhoe, rep 1			Ivanhoe, rep 2			W_W, rep 1			W_W, rep 2		
Age	Mean	*N*	Age	Mean	*N*	Age	Mean	*N*	Age	Mean	*N*
1	0.01 ± 0.01	10	1	0.21 ± 0.39	9	1	0.83 ± 0.24	17	1	0.66 ± 0.34	9
7.5	0.16 ± 0.21	15	7.5	0.10 ± 0.16	19	4	0.75 ± 0.38	10	8	0.82 ± 0.33	18
17.5	0.25 ± 0.13	14	14.5	0.18 ± 0.18	21	8	0.71 ± 0.26	18	14	0.92 ± 0.09	19
24.5	0.42 ± 0.22	11	21.5	0.33 ± 0.17	19	14	0.81 ± 0.26	20	21	0.84 ± 0.30	19
31.5	0.45 ± 0.13	5	28.5	0.37 ± 0.19	29	21	0.78 ± 0.32	16	28	0.59 ± 0.45	21
						28	0.53 ± 0.46	22			

Mean values are means ± SD.

TABLE 9

Age-specific hatch rates from crosses between R_R males held in the lab or in field cages and W_W females

Lab, rep 1			Lab, rep 2			Lab, rep 3			Cage, rep 1			Cage, rep 2		
Age	Mean	*N*	Age	Mean	*N*	Age	Mean	*N*	Age	Mean	*N*	Age	Mean	*N*
2[a]	0.22 ± 0.29	19	2	0.19 ± 0.17	10	1	0.15 ± 0.18	10	2[a]	0.22 ± 0.29	19	2[a]	0.22 ± 0.29	19
9	0.59 ± 0.21	18	9	0.44 ± 0.17	15	7	0.24 ± 0.19	19	9	0.24 ± 0.23	10	9	0.43 ± 0.34	5
15	0.71 ± 0.23	19	19	0.60 ± 0.41	14	14	0.77 ± 0.31	19	15	0.34 ± 0.23	9	15	0.38 ± 0.36	10
22	0.78 ± 0.31	17	26	0.81 ± 0.27	14	21	0.79 ± 0.29	19	22	0.54 ± 0.25	11	22	0.44 ± 0.35	10
29	0.87 ± 0.12	16	33	0.60 ± 0.43	13	28	0.69 ± 0.37	19	29	0.47 ± 0.29	2	29	0.51 ± 0.32	12
			40	0.62 ± 0.41	11	35	0.77 ± 0.37	20						

Mean values are means ± SD.

[a] All three of these groups, the first laboratory replicate and both field cages, were begun from a single collection of newly eclosed males. A single set of replicate crosses served to estimate the hatch rates from 2-day-old males for all three groups.