Wolbachia and Cytoplasmic Incompatibility in the California *Culex pipiens* Mosquito Species Complex: Parameter Estimates and Infection Dynamics in Natural Populations

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ABSTRACT

Before maternally inherited bacterial symbionts like Wolbachia, which cause cytoplasmic incompatibility (CI; reduced hatch rate) when infected males mate with uninfected females, can be used in a program to control vector-borne diseases it is essential to understand their dynamics of infection in natural arthropod vector populations. Our study had four goals: (1) quantify the number of Wolbachia strains circulating in the California *Culex pipiens* species complex, (2) investigate Wolbachia infection frequencies and distribution in natural California populations, (3) estimate the parameters that govern Wolbachia spread among *Cx. pipiens* under laboratory and field conditions, and (4) use these values to estimate equilibrium levels and compare predicted infection prevalence levels to those observed in nature. Strain-specific PCR, *wsp* gene sequencing, and crossing experiments indicated that a single Wolbachia strain infects Californian *Cx. pipiens*. Infection frequency was near or at fixation in all populations sampled for 2 years along a >1000-km north-south transect. The combined statewide infection frequency was 99.4%. Incompatible crosses were 100% sterile under laboratory and field conditions. Sterility decreased negligibly with male age in the laboratory. Infection had no significant effect on female fecundity under laboratory or field conditions. Vertical transmission was >99% in the laboratory and ~98.6% in the field. Using field data, models predicted that Wolbachia will spread to fixation if infection exceeds an unstable equilibrium point above 1.4%. Our estimates accurately predicted infection frequencies in natural populations. If certain technical hurdles can be overcome, our data indicate that Wolbachia can invade vector populations as part of an applied transgenic strategy for vector-borne disease reduction.

In mosquitoes, the maternally inherited bacterial symbiont Wolbachia is associated with cytoplasmic incompatibility (CI), *i.e.*, reduced egg hatch when uninfected females mate with infected males. Matings between infected females and infected or uninfected males are fertile. Consequently, infected females have a reproductive advantage, allowing Wolbachia to spread rapidly through host populations (Turelli and Hoffmann 1999). The spread of Wolbachia has applied interest for the control of vector-borne diseases and pest insect populations (Pettigrew and O'Neill 1997). No vaccines for important vector-borne diseases such as malaria and dengue are available (Beaty 2000), and the evolution of insecticide resistance in important vector species is becoming an increasing problem (Hemingway and Ranson 2000). Due to these concerns, an effort is underway to create genetically modified vector arthropods that are unable to transmit pathogens (Pettigrew and O'Neill 1997). Effector molecules that block pathogen uptake and/or transmission by arthropod vectors have been identified, inserted into the insect germ line, and expressed in vector species (Ito *et al.* 2002). However, there is as yet no empirically demonstrated method to spread or "drive" these engineered genetic traits into vector populations to a high enough frequency to interrupt pathogen transmission cycles. Strategies exploiting Wolbachia spread to drive introduced transgenic traits into vector populations are currently under theoretical consideration (Turelli and Hoffmann 1999).

Before Wolbachia can be utilized in a vector-borne disease control strategy, it is essential to understand the dynamics of infection in natural vector populations to predict how introduced infections may behave. Models of Wolbachia dynamics in natural Drosophila populations predict that three kinds of information are critical for using Wolbachia in an applied manner to control disease: (1) the unstable equilibrium, *i.e.*, the introduction threshold of infected individuals that must be released for infection to become established in the population; (2) the stable equilibrium frequency that infection will ultimately reach; and (3) how long (in generations) invasion will take from a given introduction level (Turelli and Hoffmann 1999). Although there has

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been considerable theoretical and experimental work involving CI in mosquitoes (reviewed in Hoffmann and Turelli 1997), there have been no studies on infection dynamics in natural mosquito or other vector populations. The dynamics of Wolbachia spread have been adequately investigated only in *Drosophila simulans* (Turelli and Hoffman 1995) and *D. melanogaster* (Hoffmann *et al.* 1994, 1998). Model validation and estimates of equilibrium levels based on data from natural mosquito populations are necessary for designing transgenic vector control strategies and will provide a conceptual framework for asking comparative questions about Wolbachia-arthropod interactions.

The goals of this study were to: (1) quantify the number of Wolbachia strains circulating in populations of mosquitoes in the *Culex pipiens* species complex in California; (2) investigate Wolbachia infection frequency and distribution in natural *Cx. pipiens* populations; (3) under laboratory and field conditions, estimate the parameters that govern Wolbachia spread in *Cx. pipiens*; and (4) use field parameter values to estimate Wolbachia equilibrium levels in *Cx. pipiens* and compare theoretical results to observed levels in nature. The California *Cx. pipiens* species complex includes two subspecies: *Cx. p. pipiens* in northern California and *Cx. p. quinquefasciatus* in the south, with extensive gene flow and hybridization between these two extremes (Urbanelli *et al.* 1997; Cornel *et al.* 2003). We used molecular techniques and crossing experiments to determine Wolbachia strain composition in natural populations and used a PCR-based assay to investigate infection frequency and distribution along a north-south transect for 2 years. Under laboratory and field conditions, we estimated the parameters that govern Wolbachia spread (vertical transmission, CI, and fecundity) and examined the effect of male age on CI expression in the laboratory. Parameter estimates were used in models of Wolbachia spread to predict stable and unstable equilibrium levels and time to equilibrium. Predictions were compared to field observations. The implications of dynamics in Wolbachia spread were considered within the context of using transgenic mosquitoes to reduce disease.

MATERIALS AND METHODS

General methods

Experimental *Cx. pipiens* colonies: Laboratory mosquito colonies were reared in 30-cm^3 screen cages. Cages were kept in an environmental chamber at 27°–29°, 80–90% relative humidity on a 16:8-hr light:dark cycle. Larvae were fed a 1:2:2 mix of ground fish food, rabbit pellets, and bovine liver powder. Adult mosquitoes were allowed access to a cotton wick soaked in 10% sucrose solution as a carbohydrate source. Eggs to maintain the colonies and for the experiments outlined below were obtained by feeding mosquitoes twice each week on a 2- to 18-day-old chicken. A Wolbachia-infected *Cx. p. pipiens* colony was established from a larval collection from Lincoln, California (Placer County) during August 1999 (strain LIN). Mosquitoes in that colony had male genitalia morphology (DV/D ratio; *i.e.,* the distance between the dorsal and ventral arms of the male phallosome divided by the distance between the two dorsal arms) within the accepted range for *Cx. p. pipiens* (Sundaraman 1949). They were identified as *Cx. p. pipiens* by amplification of a subspecies-specific PCR fragment (Crabtree *et al.* 1997) and by a subspecies-specific restriction digest of the acetylcholinesterase gene (Bourguet *et al.* 1998). The Wolbachia-infected colony was maintained for 15 generations after which it was split into two colonies that were maintained in separate rooms at a standing population size of approximately 2000 adults per colony. Larvae from one colony were cleared of Wolbachia infection by a modification of an existing protocol (Portarro and Barr 1975). Newly hatched larvae were reared in a solution of tetracycline (50 ppm for two generations, 100 ppm for four generations, 200 ppm for two generations) to clear them of Wolbachia infection (strain LINT). The addition of food at the outset of rearing avoided larval mortality as previously reported (Portarro and Barr 1975). Wolbachia infection was undetectable by PCR amplification of the 16S rDNA gene (see *Wolbachia-specific PCR assay* below) after the third generation of treatment. To control for maternal effects, mosquitoes were not reared in tetracycline for a minimum of four generations before being used for any of the experiments outlined below.

DNA extraction: DNA was extracted from individual mosquitoes by salt extraction/ethanol precipitation (Black and DuTeau 1997) and reconstituted in deionized water. DNA samples were stored at −20° until used for PCR amplification.

Wolbachia strain composition in natural *Cx. pipiens* populations

Strain-specific PCR and sequencing: To examine Wolbachia strain composition, *Cx. pipiens* spp. were collected from natural development sites as larvae or adults. Five specimens were randomly chosen from each of six equidistant sites spanning the collection range (Anderson, Elk Grove, Madera, Hanford, Lancaster, and Palm Desert; Figure 2). Infection type was first grouped into "A" or "B" supergroups using *fts*Z A- and B-specific primers (Werren *et al.* 1995) and then classified by *wsp*-specific primers into a subgroup (Zhou *et al.* 1998). A 558-bp fragment from the Wolbachia *wsp* gene was also amplified by general *wsp* primers 81F and 691R (Zhou *et al.* 1998) from the same specimens subjected to strain-specific PCR. Fragments were separated by agarose gel electrophoresis, purified with Qiaquick PCR purification spin columns (QIAGEN, Valencia, CA), and sequenced in both directions using an ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). Sequence data were aligned using Sequencher DNA Sequence Analysis Software v. 4.0.5 (Gene Codes Corporation, Ann Arbor, MI). All PCR was carried out in a GeneAmp PCR system 9700 thermocycler (Perkin-Elmer Applied Biosystems), using Ready-To-Go (RTG) PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the conditions and primers outlined in the respective protocols.

Bidirectional cytoplasmic incompatibility assay: *Cx. pipiens* spp. colonies were established from larvae collected from Anderson (strain CPS) and Palm Desert (strain CVQ), which were at the extreme northern and southern ends of the collection range (Figure 1). Mosquitoes were reared for several generations before the experiment to maximize the likelihood that mosquitoes would mate and blood-feed in the laboratory. Because mating was poor in individual pair matings we performed mass matings, which had a relatively high success rate. Reciprocal mass crosses were performed with 3-day-old males and females (75 males to 50 females). *Cx. pipiens* females are for the most part monogamous (Kitzmiller and Laven 1958);

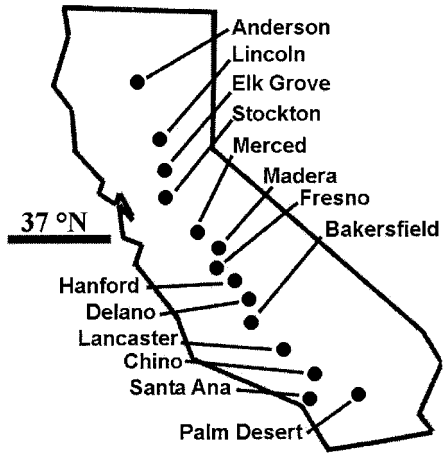

FIGURE 1.—Approximate *Cx. pipiens* spp. collection locations. Strain-specific PCR and sequencing were performed on individuals from Anderson, Elk Grove, Madera, Hanford, Lancaster, and Palm Desert.

it is unlikely that multiple fertilization events distorted our results. The 3:2 ratio of males to females was chosen to minimize the probability that individual males would mate with multiple females, because multiple male matings have been shown to reduce CI expression (HOFFMANN and TURELLI 1997). Experimental crosses were performed in 3.8-liter bucket cages with CPS F_3 and CVQ F_7. Due to low insemination and oviposition rates in the CPS male × CVQ female cross, that cross was repeated using CPS F_5 and CVQ F_9 and the data were pooled (squared-ranks test, $z = 0.73$, $P = 0.23$). Females were allowed to mate for 5 days and then allowed to feed once on a 2-week-old chicken. After blood-feeding, engorged females were individually placed into 10-cm cylindrical cages that contained a 50-ml cup filled with strained water from larval pans as an oviposition stimulus and a cotton pad soaked in 10% sucrose as a carbohydrate source. After each female oviposited, it was checked for insemination by dissection and microscopic examination (×100) for the presence of sperm in the spermethecae. Egg rafts produced from uninseminated females were not included in hatch rate analysis. Eggs per female were counted under a dissecting scope, placed in a 50-ml cup containing deionized water, and allowed to hatch. Hatch rates were calculated by counting the number of unhatched eggs after 2 days and treatments were statistically compared by Mann-Whitney *U*-test (SOKAL and ROHLF 1997). Confidence intervals were estimated by the BCa bootstrapping procedure with 5000 replicates (EFRON and TIBSHIRANI 1993).

Wolbachia spatial/temporal distribution and infection frequencies

Experimental field collections: *Culex pipiens* spp. were collected as larvae and/or as adults from 14 locations along a north-south transect in California during the summer of 1999 and from 12 locations along the same transect during the summer of 2000 (collections were not performed in Fresno or Bakersfield during 2000; Figure 1). Specimens were returned to the University of California-Davis (UCD), and immatures were reared to adulthood and stored at −80° until further processing. Collected adults were killed at −80° and stored at that temperature until further processing (see below). Differences in infection frequency among sites, collection years, and sexes were statistically compared by *G*-test (SOKAL and ROHLF 1997). Confidence intervals were estimated from the binomial distribution.

Wolbachia-specific PCR assay: PCR was conducted using a primer multiplex system, which consisted of two primer sets that amplified two regions simultaneously. Primers 99F and 994R (O'NEILL *et al.* 1992) amplify a 0.9-kb fragment from Wolbachia 16S rDNA and are designed to be specific to Wolbachia of all strains. Primers 12SA1 and 12SB1 (SIMON *et al.* 1991) are universal for insect mitochondrial DNA and amplify an ~0.4-kb fragment. Successful amplification of the latter fragment was used as a control to verify the quality of extracted DNA. RTG PCR beads were used for PCR reactions to ensure standardized amplification. Fragments were amplified as described by O'NEILL *et al.* (1992) except that the annealing temperature was raised to 54°. PCR products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. As a positive control, known Wolbachia-infected *D. simulans* or *Cx. pipiens* specimens were included in every reaction. Tetracycline-cured *Cx. pipiens* specimens were included in every reaction as a negative control.

Infection parameter estimates—laboratory and field

Vertical transmission: The DNA stain 4′,6-diamidino-2-phenylindole-2-HCl (DAPI; HOFFMANN *et al.* 1994) was used to visualize the presence of Wolbachia in infected embryos. Females were induced to oviposit using an artificial crepuscular lighting period and strained water from larval pans as an oviposition stimulus. Embryos were fixed within 3 hr of oviposition. Because the fixation protocol outlined by HOFFMANN *et al.* (1994) proved destructive to Culex embryos, an alternative fixation procedure was developed. Entire egg rafts were individually placed (without dechorionating) into 20-ml scintillation vials containing 5 ml of fixation buffer (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris, 3.7% formaldehyde, pH 7.2) and 5 ml *n*-heptane. Vials were incubated at room temperature with gentle agitation for 15 min. The aqueous layer was removed and replaced with 10 ml 100% methanol, and the vials were incubated at room temperature with gentle agitation for 10 min. The organic heptane layer was removed and 10 ml 100% methanol was added. Vials were gently shaken for 15 sec and the fixed embryos were allowed to sink to the bottom. Embryos were collected from the side of the vial, removed with a large-bore pipette tip, washed twice with 100% methanol, and stored in 100% methanol at −20° until stained for microscopy. For microscopy, fixed embryos were washed twice in PBST (1× PBS, 0.1% Triton X-100), twice in PBS, and stained in a 1-μg/ml solution of DAPI in PBS for 15 min at room temperature with gentle agitation. Stained embryos were washed once in PBS, mounted in 80% PBS, 20% glycerol and viewed at ×100 on a Nikon Microphot-SA compound microscope with epifluorescent optics. In infected embryos, Wolbachia are visible as specific fluorescence at both the anterior and posterior poles. In uninfected embryos, only background fluorescence is detectable (Figure 2). Transmission rate per female was calculated as the number of embryos with visible Wolbachia over the total number examined.

Laboratory: Egg rafts from laboratory LIN and LINT females were processed in the above manner. LINT specimens served as a negative control.

Field: Gravid females were captured between May 2000 and July 2002 from a Hanford, California, dairy using a gravid female trap (DU and MILLAR 1999) or by aspiration from walk-in resting boxes (SERVICE 1993). Specimens were transported alive to UCD and induced to oviposit, and egg rafts were processed as described above.

Statistical analysis: Wolbachia transmission rates between laboratory and field females were statistically compared by Mann-

FIGURE 2.—*Cx. pipiens* embryos stained with DAPI and visualized by epifluorescence microscopy. Wolbachia-infected LIN embryos (right) show high bacterial concentration at posterior and anterior poles. Distribution of Wolbachia in naturally infected, field-collected embryos is similar to LIN, but there is a greater variation in titer (not shown). Uninfected LINT embryos (left) exhibit some background fluorescence but have no visible Wolbachia symbionts at the poles or in the cytoplasm. Bar, 0.25 mm.

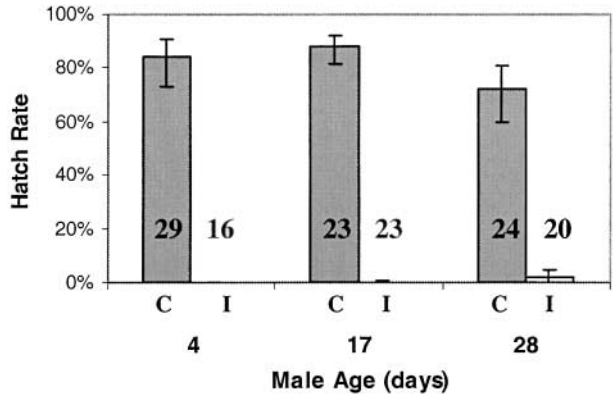

FIGURE 3.—CI changes with male *Cx. pipiens* age under laboratory conditions. Three-day-old LINT females were mated to 4-day-old, 17-day-old, or 28-day-old LINT (C, compatible cross) or LIN (I, incompatible cross) males. Error bars represent 95% bootstrap confidence intervals (5000 replicates). Numbers represent sample size. Compatible crosses are significantly different from incompatible crosses ($P < 0.0001$).

Whitney *U*-test (SOKAL and ROHLF 1997). Confidence intervals were estimated by bootstrapping (5000 replicates).

Cytoplasmic incompatibility: As described in *Bidirectional cytoplasmic incompatibility assay*, we conducted mass crosses due to poor mating success in individual pair-mating attempts.

Laboratory: All four possible LIN × LINT crosses were conducted using 3- to 5-day-old mosquitoes. Mosquitoes were held as a group in 3.8-liter bucket cages (75 males, 50 females) and were allowed to mate for 5 days (four to five replicate cages per treatment). Blood-feeding, female isolation, oviposition, insemination check, and hatch rate determination were carried out as described in *Bidirectional cytoplasmic incompatibility assay*. Treatment hatch rates were statistically compared by Kruskal-Wallis test followed by pairwise comparisons using the Dwass simultaneous test procedure with a Bonferroni correction for multiple tests (SOKAL and ROHLF 1997). Confidence intervals were calculated by bootstrapping (5000 replicates).

Field: Wild males were collected by net from mating swarms and/or by aspiration from resting boxes and transported live to UCD during summer-autumn 2000. To determine if field CI levels fluctuated over time, the experiment was replicated four times. Three collections were made from Hanford, California (August 10, September 16, and October 7) and one from Elk Grove, California (October 6). Depending on the number of wild males collected, there were one to three replicate cages per treatment per collection. In each cage, 20–40 wild males were mass mated to 20 3-day-old LIN or LINT females and the experiment was carried out as described for laboratory CI estimates. Hatch rates between treatments were statistically compared by Mann-Whitney *U*-test. Confidence intervals were calculated by bootstrapping (5000 replicates).

CI changes with male age in laboratory: Hatch rates from matings of three infected (LIN) or uninfected (LINT) male age cohorts were examined (4, 17, and 28 days; Figure 3). Three-day-old LINT females were mass mated to males in each treatment/age cohort (75 males to 50 females). Females were allowed to mate for 5 days and then offered blood meal. Female isolation, oviposition, insemination check, and hatch rate determination were carried out as described in *Bidirectional cytoplasmic incompatibility assay*. Hatch rates for various crosses were statistically compared by a Kruskal-Wallis test followed by pairwise comparisons and Bonferroni correction as previously described. Confidence intervals were calculated by bootstrapping (5000 replicates).

Fecundity: *Laboratory:* In 3.8-liter bucket cages, LIN and LINT females were mated to males of similar infection status (50–75 males to 30–50 females), blood fed, and allowed to oviposit (six to eight replicate cages per treatment). Eggs per raft were counted under a dissecting microscope.

Field: LIN or LINT first instar colony larvae were placed in 30-cm^3 submerged field cages at a density of ~0.1 larvae/cm^3 in rice ponds exposed to natural environmental conditions on the Kearny Agricultural Field Station (Parlier, CA). Infected and uninfected cages (two replicate cages) were reared in the same pond with two replicate ponds. Larvae were maintained under field conditions to pupation. Pupae were transported to UCD, adults were allowed to emerge, and fecundity differences were assayed as described above.

Statistical analysis: We used a two-factor ANOVA to determine the effect of infection status (Wolbachia infected or uninfected) and rearing environment (laboratory or field) on the number of eggs produced per female.

Model dynamics: Mathematical models (TURELLI AND HOFFMANN 1999) were parameterized using data estimated from our field experiments to predict: (1) Wolbachia stable equilibrium levels, (2) Wolbachia introduction thresholds, and (3) time (in generations) to stable equilibrium. Predicted stable levels were compared to our field infection prevalence data.

RESULTS

Wolbachia strain composition

Subgroup-specific PCR: PCR amplification of Wolbachia *ftsZ* sequences using A supergroup-specific primers was not successful (data not shown). Amplification using B supergroup-specific primers succeeded. Further strain characterization with specific Wolbachia B subgroups-specific primer sets was conducted. B subgroups tested

TABLE 1

***Wolbachia* infection frequency estimates for mosquitoes in the *Cx. pipiens* complex collected in California during 1999 and 2000**

	1999			2000		
Location	*N*	Frequency	95% C.I.	*N*	Frequency	95% C.I.
Anderson	40	1.00	(0.93)	45	1.00	(0.94)
Lincoln	77	1.00	(0.96)	16	1.00	(0.83)
Elk Grove	50	0.98	(0.89, 0.9995)	12	1.00	(0.78)
Stockton	50	1.00	(0.94)	30	1.00	(0.91)
Merced	50	1.00	(0.93)	30	0.93	(0.78, 0.992)
Madera	50	1.00	(0.94)	30	1.00	(0.91)
Fresno	72	0.99	(0.93, 0.9997)	—	—	—
Bakersfield	50	1.00	(0.94)	—	—	—
Hanford	50	1.00	(0.94)	25	1.00	(0.89)
Delano	48	1.00	(0.94)	30	0.97	(0.83, 0.999)
Lancaster	29	1.00	(0.90)	30	1.00	(0.91)
Chino	50	1.00	(0.94)	16	1.00	(0.83)
Orange Co.	50	0.98	(0.89, 0.9995)	30	0.97	(0.83, 0.999)
Coachella	100	1.00	(0.971)	30	1.00	(0.91)
Total	766	0.996	(0.99, 0.9992)	324	0.988	(0.97, 0.997)
Total both years	1090	0.994	(0.99, 0.997)			

Confidence intervals (C.I.) were calculated from the binomial distribution.

included *w*Ori, *w*Dei, *w*Con, and *w*Pip. All specimens were found to be infected with the Wolbachia strain *w*Pip.

Wolbachia *wsp* sequence analysis: Analysis of the Wolbachia *wsp* gene nucleotide sequence was undertaken to control for the possibility that strain-specific PCR might not detect noncharacterized Wolbachia strains and/or variation outside the PCR priming regions. The *wsp* gene sequence was chosen for analysis because it is the most highly variable region known in the Wolbachia genome (Zhou *et al.* 1998; van Meer *et al.* 1999). All sequences obtained ($N = 30$) were identical within and between populations, with no detected ambiguous bases to indicate the presence of multiple strains. The *wsp* sequence in the California *Cx. pipiens* complex is identical to that obtained from Asian *pipiens* complex members (*e.g.*, AF216859, AF216860). *Wsp* sequences from Anderson (Shasta County, *Cx. p. pipiens*), Hanford (Kings County, *Cx. p. pipiens/Cx. p. quinquefasciatus* hybrid), and Palm Desert (Riverside County, *Cx. p. quinquefasciatus*) were submitted to GenBank (accession nos. AF301010, AF301011, and AF301012).

Bidirectional cytoplasmic incompatibility: There was no statistically significant difference in hatch rates from reciprocal crosses made between mosquitoes from the extreme northern [Shasta (CPS)] and southern [Coachella (CVQ)] ends of California [CPS female × CVQ male, 0.969 (95% bootstrap CI, 0.956, 0.978), $N = 27$; CVQ female × CPS male, 0.968 (0.952, 0.978), $N = 23$, Mann-Whitney U, $P = 0.8153$]. This result, coupled with analysis of statewide *wsp* sequences, indicates that California *Cx. pipiens* complex mosquitoes are infected with a single strain of Wolbachia with respect to CI attributes.

Wolbachia spatial/temporal distribution and infection frequencies in California

During 1999, the total statewide infection frequency (Table 1) was 99.6% ($n = 766$; female, 100%, $n = 386$; male, 99.2%, $n = 380$), with no statistically significant differences among sites ($G = 4.55$, d.f. = 13, $P = 0.98$). During 2000, the total statewide infection frequency was 98.8% ($n = 324$; female, 98.4%, $n = 247$; male, 100%, $n = 77$; Table 1). Again, there were no statistically significant differences among sites ($G = 5.44$, d.f. = 11, $P = 0.91$). Total infection frequency was not significantly different between years ($G = 1.14$, d.f. = 1, $P = 0.29$) or sexes (female, 99.4%, $n = 633$; male, 99.3%, $n = 457$; $G = 0.001$, d.f. = 1, $P = 0.97$). Our data indicate that Wolbachia is at or close to fixation throughout the California *Cx. pipiens* complex.

Wolbachia infection parameter estimates

Vertical transmission: In infected embryos a mass of Wolbachia symbionts is clearly visible in the anterior pole just under the micropile, consistent with previous transmission electron microscopy observations (Yen and Barr 1974). We also observed high numbers of symbionts in the posterior pole where the pole (or germ) cells form, similar to what has been observed in Drosophila (Kose and Karr 1995; Figure 2). Symbionts were distributed throughout the cytoplasm, but in much lower numbers compared to the poles. Uninfected embryos showed some background fluorescence, but were easily distinguishable from infected embryos. It should be noted

TABLE 2

Mean hatch rate in all four possible crosses between LIN (infected) and LINT (uninfected) *Cx. pipiens*

Cross	*N*	Mean	95% C.I.
LIN × LIN	16	0.657	0.492–0.819
LIN × LINT	35	0.801	0.657–0.883
LINT × LIN[a]	33	0	—
LINT × LINT	64	0.852	0.779–0.896

Crosses were female × male.

[a] Mean is significantly different ($P < 0.0001$) from all other means after correcting for multiple comparisons. Confidence intervals were calculated by bootstrapping (5000 replicates).

that the DAPI technique estimates Wolbachia transmission rate to embryos. We assume that this rate is linked to infection in the adult (*i.e.*, infected embryos develop into infected adults), but did not explicitly test this.

Laboratory: We individually examined 3153 DAPI-stained embryos produced by 30 LIN females. Transmission estimates per female ranged from 0.872 to 1.0. Twenty-six embryos were observed without visible Wolbachia symbionts. These embryos were indistinguishable from control LINT embryos with no observable Wolbachia symbionts (Figure 2); we therefore infer that these embryos were devoid of Wolbachia. The mean transmission rate in laboratory females was estimated to be 0.9902 (95% bootstrap C.I.: 0.974, 0.996).

Field: Thirty-five wild females were successfully induced to oviposit in the laboratory, which produced a total of 4304 embryos. Transmission estimates per female ranged from 0.893 to 1.0. We observed, in total, 70 DAPI-negative embryos. Putatively negative embryos were indistinguishable from LINT controls. The mean transmission rate for all collections was estimated to be 0.986 (0.975, 0.992).

There was no significant difference in transmission rate between laboratory- and field-reared females (Mann-Whitney *U*, $P = 0.275$). However, in many cases the Wolbachia titer in positive field embryos was qualitatively less than that observed in laboratory-reared LIN embryos. Field embryo titers within single rafts also varied considerably, although no formal quantification was attempted. Uninfected embryos were more or less randomly distributed across females (*i.e.*, uninfected embryos were not produced from a few females) under both lab and field conditions.

Cytoplasmic incompatibility: *Laboratory:* Analyses indicated that hatch rates from at least one cross differed significantly (Kruskal-Wallis, d.f. = 3, $P < 0.0001$). Pairwise comparisons indicated that hatch rates for all three compatible crosses (LIN male × LIN female, LINT male × LIN female, LINT male × LINT female) were not significantly different from one another ($P > 0.15$), but were all significantly different from the incompatible cross (LIN male × LINT female; $P < 0.0001$). No larvae were produced from any incompatible cross, indicating a laboratory CI level of 100% (Table 2).

Field: All estimates for mosquitoes collected at different times and two locations indicated that CI is 100% in the field (Table 3). Out of 18,314 eggs produced by 83 females involved in incompatible crosses, one larva was produced (Hanford, August 10). We were not able to determine the sex or infection status of the larva due to its death prior to pupation. Confidence intervals for the Elk Grove compatible cross should be considered cautiously due to small sample size ($N = 3$) and little variation in hatch rate between samples.

CI changes with male age in laboratory: Analyses indicated that hatch rates from at least one age/compatibility treatment differed significantly (Kruskal-Wallis, d.f. = 5, $P < 0.0001$). Pairwise comparisons indicated that hatch rates within compatibility crosses were not significantly different across the male ages we examined (compatible, $P > 0.08$; incompatible, $P > 0.6$), but were significantly different between compatibility treatments ($P < 0.0001$). If CI expression in field males is similar, we expect that male age is not a significant modulator of CI expression under field conditions. We did not explicitly test the age of males in the field, because there is no method available to reliably age-grade wild male *Cx. pipiens* complex mosquitoes.

Fecundity: There was no significant effect of infection status on female fecundity under laboratory or field conditions. The mean number of eggs produced by infected and uninfected females was almost identical (laboratory infected, 233.06 ± 60.05 SD, $n = 69$; laboratory uninfected, 233.99 ± 59.84 SD, $n = 83$; field infected, 187.7 ± 59.16 SD, $n = 102$; field uninfected, 189.34 ± 51.09 SD, $n = 92$; $P = 0.84$). With our sample sizes under both laboratory and field conditions, we had 95% power to detect a 15% difference at $\alpha = 0.05$. Increasing the sample size would increase our ability to detect smaller differences in the future. There was a highly significant effect of rearing environment (laboratory *vs.* field; $P < 0.0001$), which was independent of infection status ($P = 0.95$). Mosquitoes of either infection status reared under field conditions laid significantly fewer eggs.

Predicted dynamics: Unlike some *Cx. pipiens* populations (Laven 1967; O'Neill and Patterson 1992; Guillemaud *et al.* 1997), our data indicate that a single Wolbachia strain/crossing type is present in California and as such, a model describing the dynamics of a single strain is appropriate. Using previously defined terminology (Turelli and Hoffmann 1999), Wolbachia infection parameters are the following: μ is the percentage of uninfected offspring from an infected female ($\mu = 0$ if transmission is 100%), H is the relative hatch rate of an incompatible *vs.* compatible cross ($H = 0$ if CI is 100%), and F is the relative fecundity of an infected *vs.* uninfected female ($F = 1$ if there is no effect on fecundity). Assuming random mating and discrete gen-

TABLE 3

Hatch rates of eggs obtained from LIN and LINT *Cx. pipiens* females mated to wild males collected during 2000

		Wild males × LIN females			Wild males × LINT females			
Collection	Date	*N*	Mean	95% C.I.	*N*	Mean	95% C.I.	*P*
Hanford	August	15	0.692	0.437–0.876	18	1.95×10^{-4}	0–5.54×10^{-4}	<0.0001
Hanford	September	14	0.857	0.589–0.937	19	0	0	<0.0001
Hanford	October	18	0.811	0.614–0.917	27	0	0	<0.0001
Elk Grove	October	3	0.935	0.904–0.953	19	0	0	<0.0001
Total		50	0.796	0.684–0.873	83	4.00×10^{-5}	0–1.2×10^{-4}	<0.0001

Confidence intervals were calculated by bootstrapping (5000 replicates).

erations, the frequency of infected adults (p) at generation $t + 1$ has been shown to be

$$p_{t+1} = \frac{p_t(1 - \mu)F}{1 - p_t(1 - F) - (1 - H)(1 - p_t)p_t - \mu F p_t^2(1 - H)}. \quad (1)$$

In both laboratory and field experiments, we detected 100% CI and no evidence for Wolbachia-induced fecundity effects. Thus, our parameter estimates for CI and fecundity are $H = 0$ and $F = 1$. Equation 1 can be simplified to

$$p_{t+1} = \frac{p_t(1 - \mu)}{1 - p_t + p_t^2(1 - \mu)}. \quad (2)$$

Equation 2 predicts two equilibrium points for Wolbachia frequency in the population (ignoring the trivial equilibrium point $p = 0$). $p = 1$ is a stable equilibrium point, suggesting that Wolbachia will eventually reach fixation in the population after a successful introduction attempt. The equation predicts an unstable equilibrium point,

$$p' = \frac{\mu}{1 - \mu}, \quad (3)$$

which describes the introduction threshold of infected individuals that must be initially exceeded for Wolbachia to successfully invade the population. If infection frequency exceeds this unstable equilibrium point, Wolbachia will be expected to spread and reach the stable equilibria (*i.e.*, 1, or fixation).

On the basis of our field data, we obtained an estimate for μ ranging from $0.025 \geq \mu \geq 0.0077$. Using the mean value of $\mu = 0.014$, Wolbachia will be expected to spread if infection frequency exceeds a threshold level of 0.0142. Infection in California *Cx. pipiens* is predicted to reach fixation in ~30 generations with an initial introduction of $p = 0.05$ and in 12 generations with an initial introduction of $p = 0.15$ (Figure 4).

DISCUSSION

Successful vector-borne disease control strategies that aim to replace vector populations with transgenic insects will depend on a drive mechanism, such as CI-inducing Wolbachia (Turelli and Hoffmann 1999). Although there has been discussion of how Wolbachia might be expected to behave in natural mosquito populations during a transgenic insect introduction attempt, those conclusions were based on data estimated from Drosophila. Prior to our study, no data were available for Wolbachia infection dynamics in natural mosquito populations. Data from our study indicate that Wolbachia dynamics and equilibrium levels can be accurately estimated from field data in *Cx. pipiens*. It should be noted that the simplicity of the system is a potential drawback. Parameter values for transmission, CI, and fitness less ideal than those we detected would be potentially more informative for testing hypotheses regarding introduction thresholds and stable equilibrium levels. Nevertheless, the California *Cx. pipiens* complex represents an appropriate model system for studying Wolbachia dynamics in a vector insect, because the single infecting strain simplifies study of parameter interactions and Culex biology allows relatively easy parameter estimation under field and laboratory conditions.

Wolbachia DNA sequences from *Cx. pipiens* complex

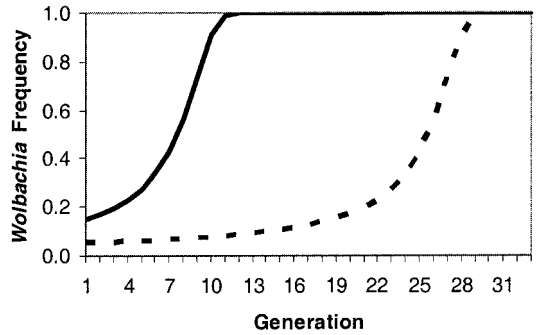

Figure 4.—Predicted dynamics of Wolbachia spread in *Cx. pipiens* using field-estimated parameter values ($F = 1$, $H = 0$, $\mu = 0.014$). Dotted line denotes initial infected introduction of 0.05. Solid line denotes infected introduction at 0.15. At a 5% introduction, infection is expected to reach fixation in 30 generations; with a 15% introduction, infection is expected to reach fixation in 12 generations.

members are known to display little molecular variation (Guillemaud *et al.* 1997). However, despite their lack of sequence variation, members of the *Cx. pipiens* complex worldwide exhibit considerable variation in hatch rates from crosses between different populations (Laven 1967; O'Neill and Patterson 1992; Guillemaud *et al.* 1997). In comparison to our results, Barr (1980, 1982) observed the presence of other rare compatibility types in California *Cx. pipiens*. Incompatibility in some of those strains was found to decrease over time under laboratory conditions and insemination status of females in experimental crosses was not examined. Rather, the distinction between incompatible egg rafts and uninseminated egg rafts was made on the basis of embryo morphology. In the course of our experiments, we found morphological characteristics to be inadequate for determining insemination status and, therefore, we checked female insemination directly by examining spermatheca. Variation in insemination patterns and resultant variation in hatch rates may explain differences between our observations and those of Barr (1980). Insemination has been confirmed in laboratory incompatible crosses between colonies derived from European Culex populations (Guillemaud *et al.* 1997), but it is possible that variation in insemination status may also contribute to the complexity of European crossing types (Laven 1967).

Most studies that attempt to quantify Wolbachia vertical transmission in the field rely on testing progeny of wild females for infection (Hoffmann *et al.* 1998; Kittayapong *et al* 2002a). However, if infection frequency in the population is high, and CI expression is strong, testing of female progeny may give a positively biased estimate of transmission. In this situation, field-collected females are likely to have mated with infected males and CI expression would potentially kill embryos that did not receive Wolbachia from their mother. Virgin females could be obtained from collection of pupae and mated to antibiotic-cured males in the laboratory to eliminate CI bias in hatch rates, but this would not be an accurate estimate of transmission under true field conditions due to laboratory rearing procedures. Unlike Drosophila (Hoffmann *et al.* 1998), *Cx. pipiens* cannot be reared under near-field conditions. Although it is difficult to evaluate whether progeny testing biased estimates of Wolbachia transmission in other insect systems, we felt that a conservative approach was prudent. DAPI staining addressed these concerns by allowing direct observation of symbionts in infected embryos. We suggest that DAPI staining of embryos should be considered when studying the dynamics of Wolbachia-insect interactions.

We initially hypothesized that CI expression might be less severe in the field than in the laboratory due to the presence of older males in field samples because CI was previously found to be significantly decreased by male age in *Cx. pipiens* (Singh *et al.* 1976). We observed 100% CI in all field crosses and considered the possibility that recent mass emergence events saturated mating swarms with young males. Because previous studies showed significant decreases in CI expression in matings from *Cx. pipiens* males older than 12 days (Singh *et al.* 1976), we examined CI in males up to 28 days old. Laboratory crosses with males of different ages indicated that male age was not an important factor in CI expression, a result that helps to explain our field data but that runs counter to previously published studies (Singh *et al.* 1976). Reduction in CI strength with male age is associated with the loss of the symbionts in developing sperm cysts with advancing male age (Clark *et al.* 2003). It is possible that (1) in the Culex strain we studied, there is no significant reduction in sperm cyst Wolbachia titer in older males or (2) differences in Wolbachia and/or mosquito genetic background may account for our inability to detect phenotypic differences compared to previous studies (McGraw *et al.* 2001; Clark *et al.* 2003). Similarly, no significant effect of male age on CI expression was observed in multiply infected (supergroups A and B) *Aedes albopictus*, even though CI was attenuated with male age in single-infected (supergroup A) males (Kittayapong *et al.* 2002b). It is interesting to note that the B infection in multiply infected *Ae. albopictus* (*w*AlbB) is closely related to the *w*Pip infection of *Cx. pipiens*. The lack of strong male age effects on CI expression may be a general property of *w*Pip-type Wolbachia strains.

In different arthropod hosts, Wolbachia has been shown to lower fecundity of infected females (Fleury *et al.* 2000), increase fecundity (Dobson *et al.* 2002), or have no effect (Zchori-Fein *et al.* 2000). Fluctuating asymmetry levels have been used to indirectly estimate Wolbachia-induced fitness effects in Drosophila field populations (Hoffmann *et al.* 1998), but due to the very low numbers of uninfected *Cx. pipiens* females detected in nature ($<1\%$) we were not able to obtain sufficient numbers of naturally uninfected females to perform those types of field experiments. As an alternative, we examined Wolbachia-induced fecundity effects using mosquitoes from our laboratory colony that were reared under field conditions and did not detect an effect of infection on fecundity. It is possible that Wolbachia may induce different fitness effects in different host genotypes. Conducting similar experiments with multiple mosquito strains collected from geographically diverse locations and habitats would help to clarify this issue.

Our survey data indicated that in the California *Cx. pipiens* complex Wolbachia infection is present in all populations sampled and is essentially at fixation, a result that agrees closely with model predictions. Populations where single negative individuals were observed in 1999 did not have negative individuals in 2000, indicating that the production of rare uninfected mosquitoes is likely a transitory local phenomenon. It is possible that local environmental factors such as temperature or naturally occurring antibiotics could rarely result in temporary clearing of infection (Clancy and Hoff-

MANN 1998). If clearing of infections does occur it is unlikely to be an important factor in affecting Wolbachia dynamics. It is also possible that negative results were due to Wolbachia titers below the threshold for detection by PCR. Lending credence to this alternative explanation is the fact that even at the extreme confidence levels calculated for our estimates of μ ($0.0077 \leq \mu \leq 0.025$) and H ($0 \leq H \leq 5.0 \times 10^{-5}$), for any value of $F > 0$, infection stable equilibrium levels are predicted to reach fixation.

Wolbachia with the characteristics of the California *Cx. pipiens* strain we studied would have potential for application in vector-borne disease control programs. Important characteristics for applied strategies include strong CI that does not attenuate appreciably with male age, no observable fecundity effects, and near-perfect vertical transmission. These values for infection parameters lead to the prediction that an economically viable low introduction threshold (<1.5%) will result in a stable equilibrium at fixation. As long as the transgenic trait is maternally transmitted with 100% fidelity, it will be expected to reach fixation in the population even if Wolbachia transmission is less than perfect (TURELLI and HOFFMANN 1999). It is important to note, however, that these characteristics may change according to species-specific host influences that will require empirical examination (MCGRAW *et al.* 2001; CLARK *et al.* 2003).

Our data are encouraging and support the idea that Wolbachia can invade vector populations as part of applied transgenic strategies for vector-borne disease control. However, major technical hurdles must be overcome before these types of strategies can be implemented. Obstacles include, but are not limited to, estimation of Wolbachia infection parameters under field conditions for multiple vector species, study of the fitness effects of transgenes in vector insects, transformation of Wolbachia, and studies to assess ecological processes relevant to releases of genetically modified vectors (TURELLI and HOFFMANN 1999; SCOTT *et al.* 2002). If these kinds of issues can be adequately addressed, Wolbachia have the potential to be an important part of novel efforts to control vector-borne diseases.

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