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Authors
Kriesner, Peter
Conner, William R
Weeks, Andrew R
et al.

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Persistence of a Wolbachia infection frequency cline in Drosophila melanogaster and the possible role of reproductive dormancy

Peter Kriesner, 1 William R. Conner, 2 Andrew R. Weeks, 1 Michael Turelli, 2 and Ary A. Hoffmann 1,3

1 School of BioSciences, Bio21 Institute, The University of Melbourne, Parkville, 3010, Australia
2 Department of Evolution and Ecology, University of California, Davis, 95616, California
3 E-mail: ary@unimelb.edu.au

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Field populations of arthropods are often polymorphic for Wolbachia but the factors maintaining intermediate Wolbachia frequencies are generally not understood. In Drosophila melanogaster, Wolbachia frequencies are highly variable across the globe. We document the persistence of a Wolbachia infection frequency cline in D. melanogaster populations from eastern Australia across at least 20 years, with frequencies generally high in the tropics but lower in cool temperate regions. The results are interpreted using a model of frequency dynamics incorporating cytoplasmic incompatibility (CI), imperfect maternal transmission and Wolbachia effects on fitness. Clinal variation is less pronounced in eastern North America which may reflect annual recolonization at higher latitudes. Limited samples from Africa from latitudes matching our tropical and subtropical samples from Australia and North America show comparably high infection frequencies, but some equatorial samples show lower frequencies. Adult dormancy across cold periods may contribute to the Australian Wolbachia cline. Infected flies exposed to cold conditions for an extended period had reduced fecundity and viability, an effect not evident in unexposed controls. These fitness costs may contribute to the relatively low Wolbachia frequencies in Australian temperate areas; whereas different processes, including CI induced by young males, may contribute to higher frequencies in tropical locations.

KEY WORDS: Cytoplasmic incompatibility, deleterious effects, diapause, mutualism, transmission-selection equilibria.

Wolbachia bacteria are endosymbionts that infect a large fraction of insect species (Jeyaprakash and Hoy 2000; Werren and Windsor 2000; Hilgenboecker et al. 2008; Zug and Hammerstein 2012; Weinert et al. 2015) with the potential to spread rapidly within populations through cytoplasmic incompatibility (CI), male killing and other effects on insect reproduction (O’Neill et al. 1997). Well-documented examples include the very rapid spread of the wRi strain of Wolbachia through the Drosophila simulans population in California (Turelli and Hoffmann 1991, 1995) and more recently eastern Australia (Kriesner et al. 2013), and the spread of a male-killing strain in the tropical butterfly Hypolimnas bolina (Dyson et al. 2002). This invasion ability has become particularly important in mosquito populations where Wolbachia suppress arbovirus transmission by mosquitoes (Moreira et al. 2009; Bian et al. 2010; Walker et al. 2011) and where deliberate introductions and invasions are being undertaken (Hoffmann et al. 2011) with the potential to control vector-borne disease agents such as dengue virus.

Some Wolbachia infections are known to induce significant CI and reach high frequencies in natural populations associated with a balance between CI and imperfect maternal transmission (Turelli and Hoffmann 1995; Rasgon and Scott 2003; Jaenike 2009; Narita et al. 2009). However, there are also many polymorphic infections that produce little CI or other reproductive manipulation (Hoffmann et al. 1996; Zhang et al. 2013; Hamm et al. 2014). The reasons for these polymorphisms are not entirely
clear. Although imperfect maternal transmission can account for infection frequencies somewhat below 100% (as in the case of well-studied Drosophila infections; Turelli and Hoffmann 1995; Unkless and Jaenike 2012), there has been little progress toward explaining infection frequencies that are low to moderate (e.g., Jiggins et al. 2001; Tagami and Miura 2004) or geographic variation in infection rates within species (Shoemaker et al. 2003; Ahrens and Shoemaker 2005).

Unfortunately, identifying factors that influence Wolbachia infection frequency in field populations presents a number of challenges: it requires accurate host species identification, molecular diagnostic tools to distinguish strains and other endosymbionts potentially present in a given population, and capacity to rear hosts in controlled environments to assess effects on host reproduction and maternal transmission fidelity. Consequently, few host-Wolbachia systems apart from those in some Drosophila are well characterized in nature.

The existence in field populations of weak or non-CI-inducing strains as wAu in D. simulans (Hoffmann et al. 1996), wMel in D. melanogaster (Hoffmann 1988), wSuz in D. suzukii (Hamm et al. 2014), and other systems including wLug in Nilaparvata lugens (Zhang et al. 2010) and wOri in Tetramychus phaselaus (Zhao et al. 2013) leads to the expectation that some Wolbachia variants, at least under some circumstances, provide a net fitness benefit to hosts (Hoffmann and Turelli 1997). Initial attempts to identify positive Wolbachia fitness effects either failed (Harcombe and Hoffmann 2004; Montenegro et al. 2006) or produced mixed results (Fry et al. 2004). However, likely candidates for such host fitness benefits have emerged more recently in the form of virus protection (Hedges et al. 2008; Teixeira et al. 2008; Osborne et al. 2009), nutritional supplementation (Brownlie et al. 2009; Hosokawa et al. 2010; Moriyama et al. 2015), and iron metabolism (Gill et al. 2014). In field populations, the magnitude of these effects is not yet characterized and would require multiple samples of individuals across multiple time points. For instance, Kriesner et al. (2013) pointed out that the spread of Wolbachia strain wAu and subsequently wRi in eastern Australian D. simulans detected by repeated population sampling could best be explained by inferring substantial fitness benefits for both strains.

In contrast to the very rapid spread to a high frequency of Wolbachia wRi through D. simulans populations in eastern Australia and California, there is variability in Wolbachia infection frequencies in D. melanogaster populations from the Australian east coast (Hoffmann et al. 1994; Hoffmann et al. 1998), with high infection frequencies in tropical and warmer temperate regions (above ~26°S latitude) and lower frequencies for mid to cooler temperate regions. For laboratory stocks of D. melanogaster that carry Wolbachia, infection frequency is generally near 100%. Friberg et al. (2011) found that long-term persistence of Wolbachia at high frequency in D. melanogaster laboratory stocks could be explained by an absence of fitness costs, near perfect maternal transmission, and significant levels of CI. However, the Wolbachia infection in field populations of D. melanogaster shows imperfect maternal transmission and induces only weak CI (Hoffmann et al. 1998) unless crosses involve very young males (Reynolds and Hoffmann 2002).

If natural infection frequencies represent a balance between positive and negative fitness effects of Wolbachia (potentially context dependent) and imperfect maternal transmission, clinal variation in frequencies might be explained by spatially varying effects with greater net benefits at tropical and warmer temperate latitudes. Alternatively, clines may reflect latitudinal variation in maternal transmission rates. Drosophila melanogaster is afrotropical in origin and has undergone a relatively recent human-mediated worldwide expansion, having arrived in Europe about 10,000–15,000 years ago after the last glaciation (Lachaise et al. 1988; Stephan and Li 2007) but reaching North America only in the past 150 years (David and Capy 1988; Keller 2007), and invading Australia from the north around 100 years ago (Hoffmann and Weeks 2007). It has adapted relatively recently to temperate climatic conditions with extended cold winter periods.

Although a few species employ polyphenism or undertake long-distance migration, most insects undergo some form of reproductive latency as an adaptation to seasonally adverse conditions encountered during colder months of the year in temperate regions, or at the onset of dry seasons (Taubert et al. 1986). For many species, this entails a distinct physiological condition, usually instigated in embryonic, larval, or pupal stages known as true diapause, with distinct initiation and termination phases. Drosophila melanogaster individuals however overwinter at low temperatures as adults (Izquierdo 1991; Mitrovski and Hoffmann 2001). Here, we follow the terminology of Kostal (2006) and refer to this response as dormancy rather than diapause. Reproductive dormancy in females is characterized by a weak developmental arrest of oocyte maturation, generally at previtellogenic stages, induced by ambient temperatures of 12°C or below and a short photoperiod (Saunders et al. 1989).

Schmidt et al. (2005) found polymorphism for incidence of what they termed diapause (defined as oocyte development arrest at or prior to stage 7) in D. melanogaster, with frequencies of arrested individuals increasing clinally with latitude along the U.S. east coast under laboratory conditions of 12°C with 10:14 h light:dark photoperiod. However, Lee et al. (2011) did not find a latitudinal cline for this phenotype in flies from eastern Australia, although there was clinal variation in the ability of females to retain eggs (Mitrovski and Hoffmann 2001). Although cold stress does not lead to the removal of Wolbachia infections from hosts (Li et al. 2014), it is possible that Wolbachia might influence
dormancy responses and thereby the fitness of overwintering flies.

In this article, we revisit clinal patterns in D. melanogaster and focus on two main issues. First, we examine the temporal stability of clinal frequency variation in eastern Australia by combining new datasets with published data from the last 20 years when there has been warming in this region (www.bom.gov.au). We also provide information on Wolbachia infection frequencies from North America, Africa, and Eurasia for comparison. Second, we investigate fitness effects of natural Wolbachia infections during reproductive dormancy using D. melanogaster samples from cool temperate locations on three separate continents. We present a theoretical analysis that indicates the magnitude of fitness effects, maternal transmission, and CI needed to explain observed infection frequencies.

**Materials and Methods**

**FIELD COLLECTIONS FOR ESTIMATING WOLBACHIA INFECTION FREQUENCY**

We assayed D. melanogaster field samples collected from various localities along the east coast of mainland Australia and Tasmania during 2004, 2005, 2011, and 2014 for Wolbachia infection status. Field flies were collected either from rotting fruit placed out in the field to attract adults, or from piles of rotting fruit located at field sites where thousands of flies were found. Most flies assayed were field males but in some cases isofemale lines were established and field females were assayed by polymerase chain reaction (PCR) after they produced offspring. Species identity was established—directly, or from male F1 offspring in the case of females—by examining male genital arch morphology. Flies were preserved in 100% ethanol and stored at −20°C until use in PCR.

Genomic DNA was extracted from adult flies using a Chelex-based method. We detected Wolbachia using either a standard PCR method (for the 2004 and 2005 collections), or a real-time PCR/high-resolution melt (RT/HTM) method. We also screened some samples from North America with the standard method to supplement published data from this continent. The standard screening involved the wsp primers wsp81F and wsp691R and a concurrent PCR assay targeting arthropod-specific 28S rDNA (Turelli and Hoffmann 1995). Positive controls using single-copy nuclear genes are essential because failure to detect a Wolbachia PCR product could be a failed PCR or failure to extract DNA. PCR products were visualized on 1% agarose gels alongside a standard. We considered an individual infected when both the Wolbachia-specific primers and nuclear controls produced fragments of appropriate sizes.

For the RT/HTM method, we amplified a ~340 bp fragment of the wsp gene (Lee et al. 2012). This assay included D. melanogaster specific primers for the host gene RpS6 as an internal control to test for the presence of DNA and correct PCR conditions.

**BIOINFORMATIC ASSAYS OF WOLBACHIA INFECTION FREQUENCIES IN AFRICA**

Lack et al. (2015) reported whole genome data from 246 African lines of D. melanogaster. We obtained the raw sequencing reads for 122 lines from the National Centre for Biotechnology Information short read archive (http://www.ncbi.nlm.nih.gov/sra). Information on locations and dates of the African samples are provided in Table S2. We determined infection status of these lines as described in Methods S1.

**REPRODUCTIVE DORMANCY INDUCTION**

To test the hypothesis that Wolbachia infection has a fitness effect on flies that undergo reproductive dormancy, we established isofemale laboratory lines from D. melanogaster females collected in 2013 from cool temperate locations on three separate continents—Hawthorn (Australia, latitude: 37°49’S), Edinburgh (UK, latitude: 55°56’N), and Portland, ME (US, latitude: 43°40’N). We determined Wolbachia infection status for each line using original mothers and F1 offspring with the previously described RT/HTM method. Of these, 12/32 lines from Hawthorn (HAW), 2/13 from Edinburgh (EDIN), and 20/23 from Maine (MNE) were infected. Lines of known status from the respective locations were subsequently used to create infected (w+) and uninfected (w−) mass-bred lines designated HAW_w+, HAW_w−, EDIN_w+, EDIN_w−, MNE_w+, and MNE_w−.

For each of the HAW_w+, HAW_w−, MNE_w+, and EDIN_w− mass-bred lines, we combined F1 offspring of seven mated females from each isofemale line with the appropriate infection status to initiate the mass-bred line. To initiate the MNE_w− mass-bred line, we collected 30 virgin females from each of the three uninfected Maine isofemale lines and crossed these en masse with five males from each of the remaining 20 infected Maine lines. We then combined these now mated females with a further seven previously mated females from each of the three uninfected Maine lines. To initiate the EDIN_w+ mass-bred line, we collected 40 virgin females from each of the two infected Edinburgh isofemale lines, and crossed these en masse with eight males from each of the remaining 11 uninfected Edinburgh lines. We then combined these mated females with a further 12 previously mated females from each of the two infected Edinburgh lines. These steps were taken to ensure that the mass-bred lines being compared had a high level of genetic diversity. All mass-bred lines were maintained in bottles with a census population size >200 at either 25 or 19°C. We then created a further six mass-bred lines by introgressing Wolbachia infections from each of the three locations into the genetic background of the other locations by mating 50+ virgin infected females with an equivalent number...
Table 1. Mass-bred lines used in dormancy experiments.

<table>
<thead>
<tr>
<th>Expected nuclear genetic background</th>
<th>Infection status</th>
<th>Wolbachia source</th>
<th>Line designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hawthorn: 100%</td>
<td>+</td>
<td>Hawthorn</td>
<td>HAW_w+</td>
</tr>
<tr>
<td>Hawthorn: 100%</td>
<td>−</td>
<td></td>
<td>HAW_w−</td>
</tr>
<tr>
<td>Edinburgh: 100%</td>
<td>+</td>
<td>Edinburgh</td>
<td>EDIN_w+</td>
</tr>
<tr>
<td>Edinburgh: 100%</td>
<td>−</td>
<td></td>
<td>EDIN_w−</td>
</tr>
<tr>
<td>Maine: 100%</td>
<td>+</td>
<td>Maine</td>
<td>MNE_w+</td>
</tr>
<tr>
<td>Maine: 100%</td>
<td>−</td>
<td></td>
<td>MNE_w−</td>
</tr>
<tr>
<td>Hawthorn: 96.9%; Maine: 3.1%</td>
<td>+</td>
<td>Maine</td>
<td>HAWwMN</td>
</tr>
<tr>
<td>Hawthorn 96.9%; Edinburgh 3.1%</td>
<td>+</td>
<td>Edinburgh</td>
<td>HAWwED</td>
</tr>
<tr>
<td>Edinburgh 96.9%; Hawthorn 3.1%</td>
<td>+</td>
<td>Hawthorn</td>
<td>EDINwHW</td>
</tr>
<tr>
<td>Edinburgh 96.9%; Maine 3.1%</td>
<td>+</td>
<td>Maine</td>
<td>EDINwMN</td>
</tr>
<tr>
<td>Maine 96.9%; Hawthorn 3.1%</td>
<td>+</td>
<td>Hawthorn</td>
<td>MNEwHW</td>
</tr>
<tr>
<td>Maine 96.9%; Edinburgh 3.1%</td>
<td>+</td>
<td>Edinburgh</td>
<td>MNEwED</td>
</tr>
</tbody>
</table>

of infected males. We then undertook repeated backcrossing for five generations. A summary of mass-bred lines is given in Table 1.

Wolbachia infections of D. melanogaster field populations and laboratory stocks are not all identical and can be broadly classified into two monophyletic groups of variants: wMel-like or wMelCS-like (Chrostek et al. 2013), with the former being hypothesized to have largely replaced the latter in field populations globally within the past 100 years (Riegler et al. 2005). However, Richardson et al. (2012) in analyzing genomic data from the DGRP and DPGP lines found this replacement commenced several thousand years ago and is not yet complete. There have also been at least some reports of wMelCS-like variants found in field populations more recently (Nunes et al. 2008; Richardson et al. 2012; Ilinsky 2013; Versace et al. 2014). As Chrostek et al. (2013) found significant differences in fitness effects between these two categories of variants in a common genetic background, we assayed each infected mass-bred line for Wolbachia variant type using primers designed for the WD1310 locus (Riegler et al. 2005). This locus is diagnostic for discriminating wMel and wMelCS variants (Riegler et al. 2005; Woolfit et al. 2013) as the latter contain an IS5 element insertion absent in wMel-like variants. Sanger sequence data from each amplicon obtained were invariant from that of the published wMel genome (Wu et al. 2004).

OVARIOLE DEVELOPMENTAL ARREST
Virgin females collected within 4 h of eclosion were placed in chilled phosphate buffered saline Tween 20 (PBST) buffer. Ovaries were dissected and examined under 40–100× magnification. The most advanced oocyte from each set of ovaries was determined following King (1970). Treatment differences in arrest stages were assessed using logistic regression models (glm) in R version 3.2 (2015).

FECUNDITY AND VIABILITY
Batches of one-day-old mated females from each experimental line were subjected to 9½:14½ h light:dark with temperature range of 9.0–9.6°C. For each line, 15–17 females were transferred individually to glass vials with plastic spoons holding ~2 ml of treacle medium, brushed with 5% w/v live baker’s yeast solution and allowed to air dry for 1–2 h. These vials were held at 25°C under 12:12 h light:dark. Females were transferred to new vials with fresh spoons daily for seven days. Eggs were counted, and then left to develop in vials with culture medium at 25°C for a further 12 days to assess egg to adult viability. Most emerging progeny were preserved in 100% ethanol at −20°C.

Fecundity and viability data were not normally distributed. Wilcoxon rank sum tests (wilcox.test in R) were therefore used to test for differences between infected and uninfected lines, whereas Kruskal–Wallis rank sum tests (kruskal.test in R) were used to detect differences in lines grouped by genetic background or Wolbachia origin. Pairwise comparisons between all lines were performed with Wilcoxon rank sum tests following Holm correction (pairwise.wilcox.test in R).

WING SIZE
Wings from F1 progeny of females subjected to dormancy-inducing conditions and control flies (one wing per fly) were mounted on microscope slides and examined under 100× magnification. Two distances between landmarks representing wing length and wing width (see Fig. S1) were scored using Nikon NIS
WOLBACHIA DENSITY

To obtain ratios of relative Wolbachia density compared to a single-copy host nuclear gene, we used a quantitative PCR (qPCR) method with the Roche LightCycler 480 system in a 384-well format and primers designed to amplify a 139 bp fragment of the wsp gene region conserved across a variety of Supergroup A strains (wspFQALL 5′-GCATTGTTTAAYAAGGACGA-3′ and wspRQALL 5′-GGAGTGATGGCATATCTTCAAT-3′) for Wolbachia (Osborne et al. 2009) and a second set of primers to amplify a 135 bp region of the rpl2125 gene region which encodes the large RNA polymerase II subunit (qprpl2125F 5′-AGCCGTTGAGTGTGGTGC-3′ and qrpII215R 5′-TGAGGTTGATGGTGATTG-3′) for the host (Correa and Ballard 2012). Amplification efficiency of each gene was determined by constructing a standard curve using serial dilutions of gDNA from infected flies. Reactions were performed in triplicate and relative copy number of Wolbachia was calculated by the Pfaffl Method (Pfaffl 2001) using Drosophila rpl2125 as a reference gene for wsp. Genomic DNA was extracted from adult whole bodies using a Chelex-based method. The qPCR protocol consisted of an initial denaturation step of 10 min at 95°C, with 42 cycles of 10 sec at 95°C, 15 sec at 58°C, and 30 sec at 72°C. Melting curves were analyzed after each run to confirm specificity of amplified products.

Differences in relative Wolbachia density between experimental lines were performed with a linear model fit (lm in R), and the different lines were compared pairwise with a Tukey’s test.

Results

WOLBACHIA INFECTION FREQUENCY ESTIMATES

Our data for east Australian D. melanogaster populations (Fig. 1B–E) exhibit a pattern: Wolbachia frequency is generally high in the tropics, but declines with more southerly latitude in temperate regions. This is broadly consistent with earlier results (Hoffmann et al. 1994; Hoffmann et al. 1998; Fig. 1A). Detailed results are shown in Table S1.

To assess the consistency of the relationship between wMel infection frequency and latitude for the eastern Australian data, we performed logistic regression. There was a highly significant negative association between wMel infection frequency and southerly latitude overall (b = −0.173 ± 0.006, P < 2 × 10^{-16}), and for each of the six sampling periods considered separately (P < 2.47 × 10^{-8}; see Fig. 2A). There was no significant association when data only for tropical locations (i.e., north of latitude 23.5°S) were considered (P = 0.657). For the data from subtropical and temperate locations only (i.e., below latitude 23.5°S), the negative association between infection frequency and latitude was greater (b = −0.198 ± 0.008, P < 2 × 10^{-16}). When “year” was included as a factor, the logistic regression model for the 1994–1996 sampling period differed significantly from 1993 (P = 0.019), 2004 (P = 7.26 × 10^{-7}), 2011 (P = 0.00025), and 2014 (P = 3.83 × 10^{-6}). The average gradient (latitude: year interaction) for the 1994–1996 model also differed significantly from that of models for each of the other sampling periods. However, when the 1994–1996 data are excluded, differences between the remaining models are not significant (see Fig. 2B). In a number of cases, the 1994–1996 data (Hoffmann et al. 1998) comprised samples obtained from the same location at several different time points. Infection frequencies at two subtropical locations (Gold Coast and Coffs Harbour) varied over time (19.6–95% and 11.1–80% infected, respectively) during this period. Reasons for this temporal variation are unclear, but there is no obvious seasonal effect.

To investigate whether a clinal pattern might be found elsewhere, we assayed field D. melanogaster adults from various localities along the east coast of North America during 2011 and 2013. These results, combined with previously published data (Verspoor and Haddrill 2011; Early and Clark 2013; Huang et al. 2014; Webster et al. 2015), are summarized by latitude of collection locality in Figure 1F, with a complete list in Table S1. We did not find a significant linear association between northerly latitude and wMel frequency for the North American data as a whole (P = 0.148). However, when data from the seven locations above 38°N were excluded, there was a significant negative association (b = −0.36 ± 0.025, P = 1.52 × 10^{-5}) which, along with high infection frequencies observed in Panama City (latitude ~9°N; 28/30 = 93.3% infected) and at three separate tropical locations in Brazil (latitude ~8-23°S; 284/300 = 94.7% infected; Ventura et al. 2012) is consistent with the Australian pattern. For locations above 38°N, average daily minimum temperatures (e.g., worldweatheronline.com 2015) are expected to consistently fall well below freezing during winter months, whereas locations further south (down to ~33°N) behave less consistently with rare freezing conditions.

We also examined Wolbachia infection frequencies from Africa and Eurasia, combining estimates from our bioinformatics analyses with previously published data (Ilinsky and Zakharov 2007; Verspoor and Haddrill 2011; Richardson et al. 2012; Early and Clark 2013; Webster et al. 2015). These data are summarized in Figure 3A and B and a full list provided in Table S2.
The latitudes sampled in southern Africa below about 16.5°S correspond to the tropical and subtropical regions sampled in eastern Australia and North America and show similarly high infection frequencies. There are likely to be few if any locations in southern Africa where conditions are sufficiently cold to induce extended periods of reproductive dormancy among fly populations. African equatorial samples show highly variable infection levels, for reasons we do not understand—from over 96% in samples from Rwanda to less than 10% (and statistically heterogeneous) in Accra, Ghana, where large samples were available. Two separate samples obtained from one North African location (Marrakesh) also had variable infection frequencies (83.3 and 34.1%).

Ilinsky and Zakharov (2007) analyzed *D. melanogaster* isofemale lines from almost 100 locations throughout Eastern Europe and Central Asia over a considerable timespan. There was no latitudinal or other geographic pattern apparent in their data. However, in nearly all cases, the original populations sampled were from locations that experience prolonged periods below freezing during winter. Western Europe generally experiences very different climatic conditions to those in central Asia at equivalent latitudes. Limited sampling from Western Europe suggests infection frequencies that roughly correspond to those found in temperate regions in eastern Australia, but again there were variable infection frequencies for separate samples from each of the two most northern locations from Western Europe (Table S2: Sussex and Edinburgh, UK; 10.8–68.4%, and 24.2–98% infected, respectively). Taken together, our data suggest that Wolbachia infection frequencies are mostly high to very high for *D. melanogaster* populations from tropical and subtropical climates, but with some notable exceptions in Africa. For populations in cool temperate climates, there is evidence for lower infection frequencies.

**Figure 2.** Logistic regression models with 95% confidence intervals for each of the five sampling periods from eastern Australia for locations below (south of) latitude 24°S.
possibly related to conditions experienced during winter months, but higher frequencies at very high latitudes where permanent populations are unlikely outdoors.

**REPRODUCTIVE DORMANCY INDUCTION**

We investigated dormancy responses of infected and uninfected flies from each of our mass-bred lines (see Methods) by subjecting newly eclosed females to 10:14 h light:dark at 10.0–10.6°C; or 9½:14½ h light:dark at 9.0–9.6°C. In many cases, the most advanced oocyte dissected was either previtellogenic or on the cusp of vitelline formation and between stages 7 and 8 in size.

Significantly, fewer females had ovaries containing at least one oocyte advanced beyond stage 7/8 under the second dormancy condition (Fisher’s exact test [fisher.test in R], \( P = 0.0007 \); Fig. 4). Under the first condition, a few more infected females produced oocytes that had developed past stage 7/8 than uninfected females did, but this was not significant (Fisher’s exact test, \( P = 0.118 \)). In this case, the smaller sample size (\( N = 180 \)) provides reduced power (pwr.chisq.test from pwr package in R [Champely 2015], power = 0.7446 at the \( \alpha = 0.05 \) level) to detect an effect size (0.195) at least as large as that observed between the two dormancy conditions.
Most advanced egg stage at 10° to 10.6° C

0 5 10 15 20 25 30
s13 s12 s10 s9 s8 s7

HAW w+ HAW uninf EDIN w+ EDIN uninf MAINE w+ MAINE uninf

Most advanced egg stage at 9° to 9.6° C

0 5 10 15 20
s13 s12 s10 s9 s8 s7

HAW w+ HAW uninf EDIN w+ EDIN uninf MAINE w+ MAINE uninf

Figure 4. Frequency distribution of the most advanced egg development stage among females sampled from D. melanogaster infected and uninfected mass-bred lines. The flies underwent one of two reproductive dormancy-inducing conditions: (1) temperature range 10.0–10.6°C with 10:14 h light:dark (left) and (2) 9.0–9.6°C with 9½:14¾ h light:dark (right).

FECONDITY AND VIABILITY ASSAYS

We introgressed the Wolbachia infection from each mass-bred line into each of the other two genetic backgrounds to create a further six infected mass-bred lines denoted HAW_{ED}, HAW_{MN}, EDIN_{HW}, EDIN_{MN}, MNE_{HW}, and MNE_{ED} (see Methods). We then subjected females from these lines to conditions inducing dormancy and scored for daily fecundity over seven days and viability of F1 offspring. Overall, very few eggs were produced within the first 24 h for any of the lines ($n_{max} = 14$, mean = 0.357).

As expected for this type of assay (e.g., Weeks et al. 2007), overall fecundity was variable, but increased significantly ($P = 0.020$, adjusted $r^2 = 0.631$) over the postdormancy period, peaking around day 5. There was little change in the number of viable offspring per female until after day 5 when it tended to decline (Fig. S2A and B).

Uninfected females from the original mass-bred lines produced significantly more eggs (on average 1.365× more) than infected females from these lines (Wilcoxon rank sum test, $P = 0.017$; Fig. 5A) and this pattern is consistent across the postdormancy period (Fig. S2A). In contrast, the difference between uninfected and infected females from these same lines without the induction period was small (0.996×) and not significant (Wilcoxon rank sum test, $P = 0.814$; Fig. 5C).

When all infected females (from the three original and six introgressed lines) that underwent dormancy induction are considered together, uninfected females produced 1.48× the number of eggs compared to infected females (Wilcoxon rank sum test, $P = 0.0005$). The difference in average fecundity between infected females from the original and introgressed lines was not significant (Wilcoxon rank sum test, $P = 0.265$), and there was also no significant effect among exposed females for either genetic background or Wolbachia origin—overall, or when original or introgressed lines were considered separately (Kruskal–Wallis rank sum tests, $P > 0.05$).

The number of viable offspring produced by uninfected females was higher (on average 1.58× more) than the number produced by infected females from the original lines when exposed to dormancy induction (Wilcoxon rank sum test, $P = 0.046$; Fig. 5B), whereas the difference between uninfected and infected unexposed control females from these same lines was small (1.013×) and not significant (Wilcoxon rank sum test, $P = 0.882$; Fig. 5D). When the infected females from all lines (original and introgressed) were considered, the uninfected females produced 2.17× the number of viable F1 offspring compared to infected females following dormancy induction (Wilcoxon rank sum test, $P = 0.0002$; Fig. 5B) and there was a difference between the original and introgressed infected females (Wilcoxon rank sum test, $P = 0.029$). There was no significant effect for either genetic background or Wolbachia origin on this trait (data not shown). The difference between uninfected and infected females from these same lines without induction was small (0.996×) and not significant (Wilcoxon rank sum test, $P = 0.814$).

EFFECT OF WOLBACHIA ON BODY (WING) SIZE OF F1 OFFSPRING

We used wing size (length and width) as a proxy for overall body size (Karan et al. 1998) from 84 individuals for each of the lines ($N = 504$ in total) using wing landmarks. These measures were strongly correlated ($r^2 = 0.89$). Each batch of 84 comprised F1
Figure 5. (A) Total fecundity of females over seven days following release of dormancy induction, and (B) number of viable F1 offspring that resulted from the eggs produced, contrasted with (C) fecundity and (D) viability for unexposed control lines over the same period.
offspring arising from eggs produced on day 2 (N = 36) or day 6 (N = 32) postdormancy and unexposed controls (N = 16).

Females subjected to dormancy produced significantly smaller ♀ offspring than controls did for both wing length and width (P < 0.0001) but ♀ F1s arising from day 2 eggs were similar in size to those arising from day 6 eggs (Tukey’s test of pairwise comparisons; Fig. 6A). Similar results were obtained for ♂ F1s (Fig. S3A). Infected females were significantly larger than uninfected females for wing length (P = 0.001). This difference was evident both in unexposed controls (Fig. 6B) and in ♀ F1s produced by dormancy-exposed mothers (Fig. 6C). Size differences between infected and uninfected males were not significant (Fig. S3B and C).

**EFFECT OF WOLBACHIA DENSITY ON FECUNDITY AND EGG VIABILITY**

For the infected mothers from the original (N = 42) and introgressed (N = 72) lines that underwent induced dormancy, along with some female F1 offspring (N = 14) and other females from the original mass-bred lines (N = 18), we assayed Wolbachia density using qPCR. For the mothers that experienced the dormancy-induction period, there was a difference in average Wolbachia density between the original and introgressed lines (Wilcoxon rank sum test, P = 4.1 × 10^{-9}), and these were therefore treated separately.

All females from the original lines produced at least some eggs; and there was a significant negative correlation (adjusted r^2 = 0.1073; Spearman’s rank correlation, P = 0.027; Fig. 7A) between number of eggs produced over seven days and Wolbachia density. Of the 42 females, 29 produced viable offspring, and the relative Wolbachia density of females that did not produce offspring was on average 1.40 × the density of females which did (marginally nonsignificant, Wilcoxon rank sum test, P = 0.060). However, there was no correlation between the number of viable F1 offspring with Wolbachia density (Spearman’s rank correlation, P = 0.3) and no effect of genetic background (Kruskal–Wallis rank sum test, P = 0.169).

For females from the introgressed lines, 68 of 72 individuals produced at least some eggs. For females producing eggs, there was a significant negative correlation (adjusted r^2 = 0.067; Spearman’s rank correlation, P = 0.009) between number of eggs over six days and Wolbachia density (Fig. 7B). For the introgressed-line females overall, 40 of 72 produced some viable offspring. The mean relative Wolbachia density of introgressed-line females that did not produce viable offspring was significantly higher (1.72 ×) than that of females from the same lines which did (Wilcoxon rank sum test, P = 0.002). There was no significant effect of either genetic background (Kruskal–Wallis rank sum test, P = 0.142) or Wolbachia origin (Kruskal–Wallis rank sum test, P = 0.358). For females from the introgressed lines which produced at least some viable offspring, those with higher relative Wolbachia density tended to produce fewer viable offspring (adjusted r^2 = 0.065) but this was not significant (Spearman’s rank correlation, P = 0.093).

**MATHEMATICAL ANALYSES**

To explore the forces that may be responsible for the ubiquitous wMel polymorphisms and the persistent cline in eastern Australia, we consider an idealized discrete-generation model for Wolbachia infection-frequency dynamics introduced in Hoffmann et al. (1990). The model incorporates imperfect maternal transmission, CI, and effects of Wolbachia infection on host fitness, modeled as differences in fecundity (Hoffmann and Turelli 1997). We assume that on average a fraction μ of the ova produced by an infected female are uninfected, and that uninfected ova from infected females are as susceptible to CI as are ova from uninfected females (see Carrington et al. 2011 for empirical support in D. simulans). Embryos produced from fertilizations of uninfected ova by sperm from infected males hatch with frequency H = 1 – s_h relative to the fraction of embryos that hatch from the three compatible fertilizations, all of which are assumed to produce equal hatch frequencies. Finally, we assume that the relative fecundity of infected females is F.

From Hoffmann et al. (1998) and Reynolds and Hoffmann (2002), we know that wMel is imperfectly maternally transmitted in natural Australian populations and that it causes little CI unless males are extremely young. Hence, it may seem plausible that the frequency of wMel can be understood by ignoring the low level of CI and considering an equilibrium between imperfect transmission and positive fitness effects, F > 1 (Hoffmann and Turelli 1997). If F(1 – μ) > 1, the stable transmission-selection equilibrium frequency is

\[ \hat{p} = 1 - \frac{\mu F}{F - 1}, \]

which increases from 0 toward 1 – μ as F increases from 1/(1 – μ). However, as shown below, even very weak CI can appreciably increase equilibria predicted from equation (1) with s_h = 0.

Turelli and Hoffmann (1995) focused on the properties of equilibria produced by imperfect transmission and CI when infected females suffer reduced fecundity, that is, \(F < 1\). Here, we revisit the equilibria assuming that Wolbachia enhances fecundity. Hoffmann and Turelli (1997) conjectured that advantageous Wolbachia-induced fitness effects must be common for infections that do not induce CI. Now, based on the observed spatial and temporal dynamics of wAu and wRi in Australian D. simulans (Kriesner et al. 2013), it seems plausible that even CI-causing natural Wolbachia infections routinely enhance host fitness. According to the Hoffmann et al. (1990) model, adult infection...
Figure 6. Wing length for unexposed control females and F1 females arising from eggs produced by dormancy-exposed mothers. (A) Controls compared to F1s arising from eggs produced on day 2 or day 6 following dormancy release, (B) uninfected compared to infected controls, and (C) uninfected compared to infected F1s.

frequencies, denoted \(p_t\), change between generations as

\[ p_{t+1} = \frac{p_t F(1 - \mu)}{1 + p_t(F - 1 - s_h) + p^2 s_h(1 - \mu F) + 1 - F(1 - \mu)} \approx p_t F(1 - \mu) \]

for \(p_t \approx 0\).

Whether it causes CI, a Wolbachia infection will tend to increase when rare only if

\(F(1 - \mu) > 1\). The fecundity parameter \(F\) approximates more general fitness effects. Given that our interest is in understanding polymorphic variation in Wolbachia infections, we need to understand the stable (and unstable) polymorphic equilibria (i.e., \(0 < \hat{p} < 1\)) produced by (2). Nonzero equilibria of (2) satisfy

\[ \hat{p}^3 s_h (1 - F\mu) + p (F - 1 - s_h) + 1 - F(1 - \mu) = 0. \]  

First note that if \(F < 1\), the coefficients of the powers of \(p\) in equation (3) are positive, negative, and positive, respectively. Thus, by Descartes’ rule of signs, equation (3) has either two positive roots or none. With small \(\mu\) and moderate CI (see conditions (4) in Turelli and Hoffmann 1995), there are two positive
Figure 7. Relative amount of Wolbachia genomic DNA (calculated using host rpl215 as a reference gene) for mothers that underwent induced dormancy compared to number of eggs produced for: (A) original mass-bred lines and (B) introgressed mass-bred lines. Each point represents an average of three PCR replicates. Shaded areas indicate 95% confidence intervals for the gradient of fitted linear models.

solutions between 0 and 1, corresponding to unstable and stable polymorphic equilibria satisfying $0 < \hat{p}_u < \hat{p}_s < 1$. In contrast, with positive fitness effects such that $F(1 - \mu) > 1$, CI (i.e., $H < 1$), and $F\mu < 1$, the coefficients of equation (3) change sign only once. This produces only one equilibrium between 0 and 1, it is stable and given by

$$\hat{p} = \frac{s_h + 1 - F + \sqrt{(s_h + 1 - F)^2 + 4s_h[F(1 - \mu) - 1][1 - F\mu]}}{2s_h(1 - F\mu)}.$$  \hspace{1cm} (4)

As expected, $\hat{p} = 1$ if $\mu = 0$. With $F(1 - \mu) > 1$ and $\mu > 0$, the stable equilibrium can be anywhere in $(0, 1)$; whereas for $F < 1$, the stable equilibrium generally exceeds $\frac{1}{2}$. To understand the implications of (4), we need plausible parameter values for wMel.

The intensity of CI ($s_h$) and maternal loss ($\mu$) have been estimated in natural Australian populations. Hoffmann et al. (1998) used field-collected males and females from four different locations ranging from near the middle of the cline (Coffs Harbour, latitude 30°19′S) to the tropical end in far north Queensland (Cairns, latitude 16°54′S). None of the assays found statistically significant evidence for CI, but the resulting confidence intervals are generally broad, with four of six estimates being consistent with low levels of CI, on the order of $s_h = 0.05$. In contrast, Reynolds
Minimum stable polymorphic equilibrium frequencies of $Wolbachia$ infections among adults assuming imperfect maternal transmission (quantified by $\mu$, the loss parameter), fitness effects (quantified by $F$, the relative fecundity of infected females), and cytoplasmic incompatibility (quantified by $s_h = 1 - H$, where $H$ is the relative hatch rate of embryos produced by incompatible fertilizations). The polymorphic equilibria are solutions of equation (4). For $F(1 - \mu) > 1$, blue lines show the unique stable equilibrium. For $F(1 - \mu) < 1$, black and red lines show the stable and unstable polymorphic equilibria, respectively, and zero is the alternative stable equilibrium.

and Hoffmann (2002) used one-day-old infected males derived from wild-collected larvae and pupae (from Coffs Harbour) and found average egg hatch frequencies of only 0.39, with 95% confidence interval (0.15, 0.64). Given that CI declines rapidly with male age in $D. melanogaster$, these data collectively suggest that for tropical and subtropical populations, $s_h$ values from 0 to 0.05 are plausible, but $s_h > 0.1$ is unlikely. Hoffmann et al. (1998) used wild-collected females from a mid-cline population (Gold Coast, latitude 30° 19′ S) to estimate the fidelity of maternal transmission. Their point estimate was $\mu = 0.026$, with 95% confidence interval (0.008, 0.059).

Figure 8 illustrates the polymorphic equilibria produced by equation (3), focusing on values of $\mu$ (i.e., 0.01–0.05) and $s_h$ (i.e., 0–0.1) consistent with these data. For comparison, we present equilibria produced with $s_h = 0.5$, typical for $Wolbachia$ wRi in field populations of $D. simulans$ (Turelli and Hoffmann 1995; Carrington et al. 2011). A range of $F$ values is considered to illustrate combinations of parameters that might produce observed frequencies as stable polymorphisms. As noted above, without CI ($s_h = 0$), $\hat{p}$ increases from 0 toward 1 as $F$ increases. The rise is very steep if $\mu$ is very small, for example, $\mu = 0.01$, but becomes increasingly gradual for larger $\mu$, for example, $\mu = 0.05$. When $F(1 - \mu) > 1$, 0 is no longer a stable equilibrium (cf. eq. (2)). As $F$ increases, the new stable equilibrium can rise continuously from 0, as illustrated with $\mu = 0.05$ and $s_h = 0.05$; or increase discontinuously, as shown with $\mu = 0.05$ and $s_h = 0.1$. To understand this discontinuity, note that when $F(1 - \mu) = 1$, equation (3) produces a linear equation for the equilibrium whose positive solution is

$$\hat{p} = \frac{s_h - \frac{m}{1 - \mu}}{s_h (1 + \frac{\mu}{1 - \mu})}$$

whenever $s_h > \mu/(1 - \mu)$. Note that equation (5) provides the minimum stable polymorphic equilibrium for a given $s_h$ and $\mu$.

because $\hat{p}$ increases with $F$ beyond the threshold $F = 1/(1 - \mu)$. Using values of $s_h$ and $\mu$ that seem plausible for $D. melanogaster$, Figure 9 shows that the minimal stable $\hat{p}$ rises sharply with small increases in $s_h$.

In the Australiancline, observed frequencies range from about 0.3 up to about 0.9, but both lower and higher frequencies occur in equatorial Africa (see Fig. 3A). Several qualitative features of the predicted equilibria are notable. If maternal loss is uncommon, for example, $\mu = 0.01$, a stable equilibrium near 0.3 can be produced only if CI is essentially nonexistent, that is, $s_h << 0.05$. Indeed, even with $\mu = 0.026$, a stable $\hat{p} = 0.3$ cannot be produced if $s_h \geq 0.05$. Stable equilibria near 0.3 are much easier to explain with higher rates of maternal loss, for example, $\mu = 0.05$. Thus, if rates of maternal loss are low (e.g., $\mu \leq 0.026$), we expect that field populations which show consistently low $Wolbachia$ frequencies are unlikely to exhibit detectable CI—unless $Wolbachia$ effects other than viability, fecundity and imperfect transmission are significant (e.g., $Wolbachia$-mediated sperm displacement, cf.

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**Figure 8.** Equilibrium frequencies of $Wolbachia$ infections among adults assuming imperfect maternal transmission (quantified by $\mu$, the loss parameter), fitness effects (quantified by $F$, the relative fecundity of infected females), and cytoplasmic incompatibility (quantified by $s_h = 1 - H$, where $H$ is the relative hatch rate of embryos produced by incompatible fertilizations). The polymorphic equilibria are solutions of equation (4). For $F(1 - \mu) > 1$, blue lines show the unique stable equilibrium. For $F(1 - \mu) < 1$, black and red lines show the stable and unstable polymorphic equilibria, respectively, and zero is the alternative stable equilibrium.

**Figure 9.** Minimum stable polymorphic equilibrium frequencies, $\hat{p}$, of $Wolbachia$ infections among adults (from eq. 5), assuming $F(1 - \mu) > 1$. The minima are produced by assuming that $F = 1/(1 - \mu)$. The plots show $\hat{p}$ as a function of the magnitude of CI (i.e., values of $s_h$) for three values of $\mu$. 
Wolbachia. Wolbachia Chelymorpha alternans = D. simulans = females which survived an induced period of re-
fluence fitness particularly during egg diapause, < 1.03, suffices to pro-
D. melanogaster occurrence D. simulans 0.026 and little
Wolbachia Wol-
D. melanogaster FREQUENCY CLINE infections (Ahmed et al. 2015) beetles Wolbachia Wol-
D. melanogaster density and both fecundity and viability of offspring. D. melanogaster = /2264 fitness infection first detected
Wolbachia ¯ D. melanogaster Wolbachia = strains Aedes 0.05, as in California D. simulans. With no CI, a relatively
small fitness advantage, on the order of F = 1.03, suffices to pro-
duce p = 0.3 with μ = 0.026. In contrast, to produce equilibria near 0.9 with imperfect transmission near μ = 0.026 and little
or no CI (s_h ≤ 0.055), very strong selection, comparable to F = 1.3, favoring Wolbachia infection must be occurring. Equilibria
as large as p = 0.9 are more easily explained with significant CI (s_h > 0.05) or little maternal loss (μ < 0.026).

Discussion
The widespread variation in Wolbachia infection frequency previously observed among many D. melanogaster populations worldwide represents a conundrum. Simple models including CI predict invasion or loss depending on initial frequencies (Caspari and Watson 1959) or a balance between maternal leakage and CI leading to persistent uninfected individuals at a low frequency (Turelli and Hoffmann 1995). Yet intermediate frequencies appear typical of many Wolbachia infections (Ahmed et al. 2015; Weinert et al. 2015) and suggest that factors other than reproductive manipulation often dominate Wolbachia dynamics. Here we demonstrate that the Wolbachia infection first detected in D. melanogaster in eastern Australia (Hoffmann 1988; Hoffmann et al. 1994) shows a stable geographic pattern across 20 years. The stable cline is in sharp contrast to the rapid sweep in both California and eastern Australia of wRi in D. simulans.

Those sweeps were driven by strong CI (Turelli and Hoffmann 1991; Kriesner et al. 2013). Unlike the relative stability of the Wolbachia infection frequencies in D. melanogaster, the wAu infection in D. simulans increased in eastern Australia prior to being eliminated by the wRi invasion (Kriesner et al. 2013).

The clinal pattern in eastern Australia contrasts with data from North America. Where outdoor temperatures are likely to fall significantly below freezing for extended periods, fly populations may be re-established in the spring / summer from individuals that persist in artificially heated, human-constructed environments where a dormancy response is unlikely to be required. Alternatively where few or no flies survive winter outdoors, populations may be significantly supplemented via human-mediated fruit transport. Patterns of genetic variation in sigma viruses suggest persistent overwintering populations in cold locations (Wilfert and Jiggins 2014). This supports the hypothesis that populations in colder temperate regions are repopulated from local refugia rather than being replenished with summer migrants from warmer regions. In contrast, in south-eastern Australia, although eggs did not survive when placed outside over winter, adults persist and overwinter successfully to produce eggs (Mitrovski and Hoffmann 2001; Hoffmann et al. 2003). Therefore selection pressures at particularly high latitudes where no overwintering is possible may be different to those in areas where adults overwinter outside.

Our results indicate that Wolbachia-infected adult D. melanogaster females which survived an induced period of reproductive dormancy produced fewer eggs overall, fewer viable offspring and generally had a lower proportion of viable ova than uninfected females after the dormancy-inducing condition was released. This provides a clear advantage to uninfected individuals, particularly if there are abundant resources available directly after winter. Furthermore this cost was not simply related to the presence/absence of the infection but reflected a quantitative effect: among infected females there was a negative correlation between Wolbachia density and both fecundity and viability of offspring.

Thus, our results indicate a fitness deficit for Wolbachia-infected mothers over-wintering in climates with temperatures of 9–10°C or below for extended periods. If this time-limited cost is important in south-eastern Australian populations, it may drive annual cycles of Wolbachia infection frequency, with frequencies falling between late autumn and early spring. Our capacity to assess this prediction is limited. Sampling from the Melbourne region in early summer and again in late autumn did not indicate an infection frequency increase (actually a decrease, but not significantly so).

Previous research has shown that fecundity can be decreased by the wMel infection when flies are held in field cages in the tropics and temperate areas, although interactions between the infection and temperate/tropical genetic backgrounds may also occur (Olsen et al. 2001). Favorable fecundity effects of wMel have also been detected, although again with genetic background effects (Fry et al. 2004). Our dormancy results point to a wMel fitness cost, in specific environmental conditions, independent of genetic background. The quantitative relationships between Wolbachia and fecundity suggest that infection titer affects this cost, rather than just infection presence. The wide tissue distribution and high density of the wMelPop infection in D. melanogaster is thought to be associated with substantial fitness costs (Min and Benzer 1997) although a relationship between Wolbachia and fitness costs is not always clear cut (Hoffmann et al. 2015).

The influence of thermal dormancy on Wolbachia fitness effects has not been previously investigated. However in Aedes, Wolbachia influences fitness particularly during egg diapause, at least for the high-density wMelPop strain (McMeniman and O’Neill 2010; Yeap et al. 2011). Keller et al. (2004) found a stable clinal pattern of infection for one of two Wolbachia strains (wCah2) infecting Neotropical Chelymorpha alternans beetles based on sampling over four years in Panama and mtDNA haplo-
type analysis. They hypothesized that this pattern was associated with the incidence of dry season diapause in the Pacific side of the beetles’ range. In a meta-analysis of Wolbachia occurrence in moths and butterflies, Ahmed et al. (2015) found that intraspecific infection frequencies were systematically lower in colder
climates (and the fraction of species infected was also lower). A high proportion of species studied were found to harbor some level of Wolbachia infection suggesting these endosymbionts may be particularly influential for lepidopteran ecology. This raises the general issue of whether Wolbachia costs are more likely in areas where seasonal dormancy is protracted. Also, Morrow et al. (2015) detected Wolbachia infection at low to very low frequencies among Australian tephritid fruit fly populations in tropical locations, whereas the infection was absent in temperate populations. These authors hypothesized a relatively higher incidence of horizontal transfer in tropical regions as a possible explanation of this pattern, associated with higher biodiversity and transience of resulting infections. However, based on the current results, other factors such as overwintering dormancy could also be involved. The challenge is to collect phenotypic data from other insect groups on the impact of Wolbachia on hosts and particularly on their host’s dormancy. If species cannot be easily cultured or cured of Wolbachia, it might still be possible to test for changes in Wolbachia frequency before and after a dormant phase.

Although the effect of Wolbachia on viability appears to be related to density, the mechanism involved remains unclear. Total egg development time for Drosophila is expected to be 24 h or less at 25°C. Thus, flies released from dormancy-inducing conditions might be expected to rapidly purge any partly developed eggs that have accrued a high Wolbachia load or suffered deleterious effects, and replace these with fresh oocytes that did not experience any developmental delay. However, longer term effects might be expected if high Wolbachia densities influence the provisioning of eggs.

Although our data may help to explain the relatively lower frequency of the infection in southern areas, there remains the issue of accounting for the persistence of Wolbachia at low latitudes and the variability in Wolbachia frequencies in some equatorial locations. Field collections of Drosophila can utilize a variety of methods but these are not always specified. Some sampling methods may not provide a good representation of Wolbachia frequencies in a natural population, such as sampling multiple larvae from one breeding site which will often represent the offspring of one or a few females (Hoffmann and Nielsen 1985). It would be interesting to re-sample from equatorial locations with apparently low frequencies across time. On the other hand, some D. melanogaster populations may experience relatively higher viral prevalence so that having virus protection as a consequence of Wolbachia may be an advantage. The wMel strain provides virus protection in laboratory settings (Hedges et al. 2008; Teixeira et al. 2008), but there are no field data demonstrating protection (Webster et al. 2015). If the age distributions of mating males tend to be younger in tropical locations, our theoretical analysis indicates that even weak average CI produced by young wMel-infected males (Reynolds and Hoffmann 2002; Yamada et al. 2007) may appreciably raise wMel infection frequencies. As noted previously, if selection-transmission (eq. 1) produces a low equilibrium frequency, for example, \( \hat{p} = 0.32 \) with \( \mu = 0.026 \) and \( F = 1.04 \), adding even weak CI will appreciably raise the equilibrium infection frequency, for example, with \( s_b = 0.05, \mu = 0.026 \) and \( F = 1.04, \hat{p} = 0.63 \) (eq. 3). However, maintaining a high infection frequency, on the order of 90%, with even a moderate level of imperfect transmission (e.g., \( \mu = 0.015 \)) and weak CI (less than 10% increase in embryo death from incompatible fertilizations) requires significant net fitness advantage from Wolbachia infections. Such effects remain to be identified in nature.

In conclusion, we have demonstrated a stable cline of Wolbachia in D. melanogaster in eastern Australia, despite high rates of gene flow along this climatic gradient. We find a shallower cline in the eastern United States and no cline in Africa or Eurasia, but in these cases sampling has been more limited and other continents may not provide conditions that produce reproductive dormancy induced selection against wMel we expect in Australia. It will be interesting to track changes in the Australian cline across the next few years, particularly as there is evidence of genetic changes occurring in nuclear markers (Umina et al. 2005). As winter conditions become milder, an increase in Wolbachia frequency might be expected in southern parts of the gradient, unless other selective factors such as life-history changes act against them.

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DATA ARCHIVING

LITERATURE CITED
Wolbachia

Drosophila melanogaster’s Wolbachia

does not live by reproductive

Hypolimnas bolina

sequences found in 76% of sixty-three arthropod

Drosophila melanogaster

Drosophila

Drosophila simulans

Drosophila melanogaster

Drosophila melanogaster

Drosophila melanogaster

Drosophila melanogaster

Drosophila melanogaster

Wolbachia infection in strains for Wolbachia gonadal density in infection causing cytoplasmic incompatibility in...


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Wolbachia infection frequencies for D. melanogaster populations from eastern Australia and North America by location.

Table S2. Wolbachia infection frequencies for D. melanogaster populations from Africa and Eurasia by location.

Methods S1. Bioinformatic analysis.

Figure S1. Wing size measurements taken based on landmarks.

Figure S2. Trend in (A) average egg production per female per day after transfer from the dormancy inducing conditions to 25°C and 12:12 h light:dark, and (B) average number of viable F1 offspring per female that resulted from those eggs.

Figure S3. Wing length for unexposed control males and F1 males arising from eggs produced by dormancy-exposed mothers.