Fine-scale landscape genomics of *Aedes aegypti* reveals loss of *Wolbachia* transinfection, dispersal barrier and potential for occasional long distance movement

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Abstract

The endosymbiotic bacterium *Wolbachia* is a promising tool for controlling arboviral diseases transmitted by the mosquito *Aedes aegypti*, and can spread by itself through wild mosquito populations following local introductions. Recent *Wolbachia* introductions into *Ae. aegypti* populations in Cairns, Australia, have produced a slower than anticipated spread that could be due to: i) non-perfect transmission of *Wolbachia* from mother to offspring; ii) dispersal being leptokurtically biased towards long distances in *Ae. aegypti* adults; and iii) geographical barriers to *Ae. aegypti* dispersal. We investigated these three potential causes using double-digest restriction-site associated DNA sequencing and *Wolbachia* screening in 161 *Ae. aegypti* collected from Cairns in 2015. We detected differential *Wolbachia* status among full siblings in one out of 10 *Wolbachia*-infected matrilineages, providing the first evidence for transmission failure in this host/*Wolbachia* system. Full-siblings were also found in ovitraps up to 1312 m apart, indicating a potential for long-distance movement in *Ae. aegypti* females that are generally considered weak dispersers. We also detected a small but significant barrier effect of Cairns highways on *Ae. aegypti* dispersal using distance-based redundancy analysis (dbRDA) and a patch-based simulation analysis. These findings together may explain the slow spread of *Wolbachia* infection through the *Ae. aegypti* population in Cairns, and highlight the utility of genome-wide SNPs for fine-scale ecological investigations. Our approach could be extended to other host/*Wolbachia* systems that are increasingly considered for the biocontrol of disease vectors and pests.

Introduction

The mosquito *Aedes aegypti* (Diptera, Culicinae) is the primary vector of arboviral diseases like Dengue, Zika and Chikungunya fever that are posing an increasing burden to human health worldwide (Weaver and Lecuit, 2015; Bogoch *et al.,* 2016). The conventional approaches to combatting these diseases have involved the suppression of *Ae. aegypti* populations through source reduction or insecticide-based programs, but these have demonstrated a limited efficacy that cannot combat worldwide escalation of disease risk (Focks *et* al., 2000; Gubler, 2002). One of the new biocontrol strategies involves releases of the virus-inhibiting, endosymbiotic bacterium *Wolbachia* into *Ae. aegypti* populations (McGraw and O'Neill, 2013). Once widespread in the host populations, *Wolbachia* are expected to diminish the disease transmission rate enough to prevent outbreaks (Ferguson *et al.*, 2015). *Aedes aegypti* does not naturally carry the *Wolbachia,* but several *Wolbachia* strains have been successfully transferred into *Ae. aegypti* from other hosts like *Drosophila melanogaster* (Walker *et al.*, 2011) and *Ae. albopictus* (Xi *et al.* 2005). The strain *w*Mel, originating from *D. melanogaster*, has proven suitable for field deployments in *Ae. aegypti* given its substantial viral blockage (Ferguson *et al.*, 2015), relatively small fitness cost (Walker *et al.*, 2011), high transmission fidelity from mother to offspring (Hoffmann *et al.*, 2014) and complete cytoplasmic incompatibility (Blagrove *et al.*, 2013). Cytoplasmic incompatibility (CI) is a phenomenon where offspring of uninfected females mated with *Wolbachia*-infected males are almost always unviable, whereas offspring of *Wolbachia*-infected females are always viable regardless of the male infection status (Hoffmann and Turelli, 1997). Because CI greatly reduces the relative fitness of uninfected females when *Wolbachia*-infected males are common, it drives establishment of *Wolbachia* in isolated mosquito populations (Caspari and Watson, 1959). However, *Wolbachia* infection also imposes some fitness cost in the host, such as reduced larval competitive ability (Ross *et al.,* 2016a). The interaction between costs and benefits produces a critical frequency of *Wolbachia* infection ($\hat{p}$)

that needs to be exceeded for *Wolbachia* to invade the mosquito population (Caspari and Watson, 1959; Hoffmann *et al.,* 1990; Turelli, 2010).

Releases of *w*Mel-infected *Ae. aegypti* into two sites near Cairns, Australia, have confirmed that *Wolbachia* can establish stably in quasi-isolated habitat patches (Hoffmann *et al.*, 2011; 2014). A subsequent study with releases centred within continuous mosquito habitat demonstrated the successful spread of the invasion into surrounding habitat following releases of large enough size, so that the area covered by the infection grew ≈70-85% over the ensuing 18 months (Schmidt *et al.,* submitted). However, this rate of spread of approximately 100-200 m/year is slow compared with the much more rapid spread observed in some natural infections of other insects of 100 km/year or more (Kriesner *et al.* 2013; Turelli and Hoffmann, 1991). Slow *Wolbachia* spread in Cairns may be a product of various biological and environmental factors (Barton and Turelli, 2011; Turelli and Barton, 2016), three of which are investigated in this study: i) the occasional loss of *Wolbachia* from mother to offspring; ii) a leptokurtic dispersal kernel towards long-distance movement of *Ae. aegypti* adults; and iii) the presence of one or more barriers to mosquito dispersal. Understanding the factors that produced slow spread in Cairns is important for designing future *Wolbachia* release strategies that maximise the area invaded while minimising both the cost and the time taken to achieve invasion.

Maternal transmission of *w*Mel in *Ae. aegypti* lab populations is 100% ($\mu = 0$) (Walker *et al.,* 2011; Ross *et al.,* 2016a). However, lab populations subjected to fluctuating temperatures similar to those expected in the field have shown considerable decreases in *w*Mel infection density (Ross *et al.* 2016b), which could lead to reduced transmission fidelity of the infection to offspring (Clancy and Hoffmann, 1998; Ikeda *et al.,* 2003). We looked for evidence of maternal transmission failure ($\mu > 0$) in the field by comparing the infection status of all individuals belonging to a given matrilineage. In a matrilineage containing individuals of different infection status, those without the *w*Mel infection will have failed to inherit it.

If host dispersal distances follow a leptokurtic distribution, *Wolbachia* spread is expected to be slower than if the distribution is Gaussian (Turelli and Barton, 2016). Under leptokurtic dispersal, long-distance migrants infected with *Wolbachia* will be too infrequent to be able to initiate *Wolbachia* spread in a new location. Dispersal in *Ae. aegypti* is generally thought to represent a combination of short-range active flight of adults and long-range passive movement of eggs (Brown *et al.,* 2011; Rašić *et al.,* 2015a). *Ae. aegypti* females lay batches of eggs through multiple acts of oviposition (Reiter, 2007) and over multiple gonotrophic cycles (Christophers, 1960). This means that full-siblings can be found at different sites. The distance of separation between sampled full-siblings is indicative of female flight ranges (or passive movement such as following entry into vehicles) within a single gonotrophic cycle or over two gonotrophic cycles. We can compare this distance to those obtained from the traditional approaches such mark-release-recapture (MRR) studies with fluorescent dust marking. MMR studies have produced a wide range of estimates for female flight distance, ranging from 50-100 m (McDonald, 1977; Muir and Kay, 1998; Harrington *et al.,* 2005; Russell *et al.,* 2005; Maciel-de-Freitas *et al.,* 2007; 2010) to over 800 m within a single gonotrophic cycle (Shannon and Davis, 1930; Reiter *et al.,* 1995; Honório *et al.,* 2003).

Highways have been proposed as likely barriers to *Ae. aegypti*'s dispersal. For example, patches separated by a 120 m-wide highway in Trinidad were found to have different frequencies of mitochondrial haplotypes (Hemme *et al.*, 2010). A MRR study in Cairns with releases centred next to a ≈20 m-wide road recorded lower recapture rates at sites across it (Russell *et al.,* 2005). Other studies have indirectly inferred an inhibitive effect of highways on *Ae. aegypti* dispersal based on the observed dynamics of *Wolbachia* invasions in their vicinity. In Gordonvale, Queensland, *Wolbachia* failed to invade the region across a highway even after several years (Turelli and Barton, 2016), and similar dynamics was recently observed in urban Cairns (Schmidt *et al.,* submitted). Here we explicitly tested the hypothesis that *Ae aegypti's* genetic structure in Cairns is affected by the highways acting

as barriers to dispersal against the alternative hypotheses of isolation-by-distance and the patchy and asynchronous releases of *Wolbachia*-infected mosquitoes in the region.

We screened *Aedes aegypti* collected from Cairns in 2015 for the *w*Mel transinfection and genotyped at genome-wide single nucleotide polymorphisms (SNPs) using double-digest restriction-site associated DNA sequencing (ddRADseq; Peterson *et al.,* 2012). SNP datasets produced with ddRADseq have been used to elucidate genetic structure in *Ae. aegypti* at broad geographic scales (Rašić *et al.,* 2014a; 2014b; 2015a; 2015b) as well as within cities (Rašić *et al.,* 2015a; 2015b), and have demonstrated power superior to microsatellites when inferring relationships between *Ae. aegypti* individuals and populations (Rašić *et al.,* 2014a).

Methods

Study site and sample collection

We deployed 110 ovitraps within the properties of consenting householders in Cairns, Australia between 13 and 16 April, 2015. The traps covered a 3300 x 1900 m region of central Cairns, which we partitioned into 6 "plots" for reference: Cairns North West (CNW), Cairns North East (CNE), Parramatta Park North (PPN), Parramatta Park South (PPS), Westcourt (WC) and Bungalow (BN) (Figure 1). Partitions were established based on geographic location, location of highways, and *Wolbachia* release history. Each of these had three possible assignments: the locational groupings of Cairns North, Parramatta Park and Westcourt/Bungalow; the highway groupings of southeast of Bruce Highway, west of both highways, or northeast of Captain Cook Highway; and the *Wolbachia* release groupings of releases in 2013, releases in 2014, or no history of releases.

Within each plot, ovitraps were deployed in a quasi-random pattern where we avoided setting traps in contiguous dwellings. Mosquito eggs were collected from a combination of bungalows, multi-storey apartment complexes and local businesses, particularly along each highway. Our sampling period of mid-April coincided with the end of the region's monsoonal wet season when there was a high abundance of *Ae. aegypti*. Each ovitrap consisted of a black plastic bucket filled halfway with an infusion of water and alfalfa (lucerne) pellets to attract gravid female *Ae. aegypti* (Ritchie, 2001), which oviposit on strips of red felt clipped to the bucket and extending into the liquid. With a generation time of >14 days in *Ae. aegypti* (Christophers, 1960), we allowed only a brief window of time for oviposition to ensure that our samples were not spread over multiple mosquito generations. Traps were left for 5-7 days, then the felt strips were removed and dried. Dried strips of mosquito eggs were transferred into the laboratory and hatched by immersing all strips from each trap into vessels filled with RO (reverse osmosis) water, with 2–3 grains of yeast and one quarter of a crushed tablet of TetraMin tropical fish food (Tetra, Melle, Germany). After three

days the water, food and yeast were replaced, with additional food given to vessels containing many larvae. Emerging virgin adults were transferred to freezing ethanol and stored at -20°C until DNA extraction.

Genomic DNA was extracted using Roche DNA Isolation Kit for Cells and Tissues (Roche, Pleasanton, CA, USA), with an additional step of RNAse treatment. Of the 110 ovitraps deployed, 74 produced adult *Ae. aegypti*, from which we selected 161 individuals for sequencing. As we expected ovitraps to contain many full-siblings from the same oviposition (Apostol *et al.,*1993; Hoffmann *et al.,* 2014; Rašić *et al.,* 2014a), we limited the number of samples per ovitrap to three individuals to avoid potential biasing effects during analyses of population structure (Goldberg and Waits, 2010).

Wolbachia *infection screening*

All individuals from Cairns were screened for *Wolbachia* using the protocol of Lee *et al.* (2012). The infection is diagnosed with polymerase chain reactions (PCR) run on the Roche LightCycler® 480 system (384-well format). For each mosquito, PCR was performed using three primer sets, *Aedes* universal primers (*mRpS6*_F/*mRpS6*_R), *Ae. aegypti*-specific primers (*aRpS6*_F/*aRpS6*_R) and *Wolbachia*-specific primers (*w1*_F/*w1*_R). A sample was scored as *Wolbachia* positive when there was robust amplification of all three primer sets *mRpS6*, *aRpS6* and *w1,* while an *Ae. aegypti* sample that was *Wolbachia* negative amplified only *mRpS6*, and *aRpS6.* Each PCR was run using three positive *Wolbachia* controls and three negative *Wolbachia* controls. *Wolbachia* titre, defined as the ratio of *Wolbachia* gene copies to host gene copies, was estimated using $2^{[\mathrm{cp(A)}-\mathrm{cp(W)}]}$, where cp(A) is the crossing point of the *aRpS6* marker and cp(W) is the crossing point of the *w1* marker (Lee *et al.*, 2012). For each individual we averaged the results of three PCR replicates.

To provide a comparison between lab-raised and field-raised mosquitoes, we also assayed 23 field-caught adult mosquitoes from Gordonvale. This small town 23 km south of Cairns has been subjected to intensive *Wolbachia* releases in 2011 (c.f. Hoffmann *et al.,* 2011). The sample ("Gv13"), was taken in January 2013, after the *Wolbachia* invasion had successfully established and remained at near-fixation (Hoffmann *et al*., 2014). Adult mosquitoes were collected using BG Sentinel traps (Biogents AG, Weissenburgstr. 22, 93055, Regensburg, Germany), and were processed and assayed in a manner identical to those from Cairns.

SNP discovery

Double-digest RADseq library preparation

We applied the method of Rašić *et al.,* (2014a) for our double-digest RAD-seq (ddRAD-seq) library preparation, but modified the protocol to select for a smaller size range of genomic fragments to accommodate a larger number of mosquitoes per library. An initial digestion of 100 ng of genomic DNA from each individual was performed in a 40 µL reaction, using 10 units each of *NlaIII* and *MluCI* restriction enzymes (New England Biolabs, Beverly MA, USA), NEB CutSmart® buffer, and water. Digestions were run for 3 hours at 37°C with no heat kill step, and the products were cleaned with 60 µL Ampure XP™ paramagnetic beads (Beckman Coulter, Brea, CA). These were ligated to modified Illumina P1 and P2 adapters overnight at 16°C with 1000 units of T4 ligase (New England Biolabs, Beverly, MA, USA), before undergoing heat-deactivation at 65°C for 10 minutes.

Size selection of fragments between 350–450 bp was performed using a Pippin-Prep 2% gel cassette (Sage Sciences, Beverly, MA). Final libraries were created by pooling eight 10 µL PCR reactions per library, each consisting of 1 µL size-selected DNA, 5µL of Phusion High Fidelity 2× Master mix (New England Biolabs, Beverly MA, USA) and 2 µL of 10 µM standard Illumina P1 and P2

primers, run for 12 PCR cycles. These were cleaned and concentrated using an 0.8× concentration of Ampure XP™ paramagnetic beads (Beckman Coulter, Brea, CA) to make the final libraries. Three libraries containing a total of 161 *Ae. aegypti* were sequenced in three Illumina HiSeq2500 lanes using 100 bp paired-end chemistry.

Processing

Raw fastq sequences were processed within the customized pipeline (Rašić *et al.,* 2014a), and reads were filtered based on a minimum phred score of 13 and trimmed to the same length of 90 bp. High-quality reads were aligned sequentially to the *Ae. aegypti* reference nuclear genome AaegL1 (Nene *et al*., 2007), and the *Ae. aegypti* reference mitochondrial genome (Behura *et al*. 2011), using the program Bowtie (Langmead *et al.,* 2009). We allowed for up to 3 mismatches in the alignment seed, and uniquely aligned reads were analysed with the Stacks pipeline (Catchen *et al.,* 2013), which we used to call genotypes at RAD stacks of a minimum depth of 5 reads. Among the individuals retained after quality filtering, we observed an average read depth of 15.6 reads.

The Stacks program Populations was used to export VCF files that were then further manipulated with the program VCFtools (Danecek *et al.,* 2011). We first removed individuals with >20% missing data, leaving 134 individuals. We then extracted the 42183 SNPs that were present in at least 75% of individuals. We applied further filtering to retain only those loci that were at Hardy-Weinberg Equilibrium and with minor allele frequencies of ≥0.05. To avoid using markers in high linkage disequilibrium, data were thinned so that no single SNP was within 250 kbp of another. As *Aedes* genome is thought to contain approximately 2.1 megabases per cM (Brown *et al.*, 2001), 250 kbp roughly corresponds to eight SNPs per map unit, a sampling density that has been shown to largely eradicate the effects of linkage in SNPs (Cho and Dupuis, 2009). Our final dataset had 3784 unlinked and informative SNPs for analyses of relatedness and genetic structure.

Relatedness Analysis

We used the program SPAGeDi (Hardy and Veckmans, 2002) to calculate Loiselle's *k* (Loiselle *et al.,* 1995) among individuals. First degree kin relations (full-sib or parent/offspring) can be ascertained with only hundreds of SNPs (Tokarska *et al.,* 2009; Cramer *et al.,* 2011; Sellars *et al.,* 2014; Weinman *et al.,* 2015), and with the thousands of SNPs available through ddRADseq this confidence can be extended to include second-degree relations (half-sib or grandparent/grandchild) (Rašić *et al.,* 2014; Bateson *et al.,* 2016; Munshi-South *et al.,* 2016; Shultz *et al.,* 2016). Considering that our cross-sectional study design provided a period for sampling (7 days) shorter than that required for mosquito development (>14 days; Christophers, 1960), we assumed that related individuals were of the same generation, and thus those showing first degree levels of relatedness were considered to be full-siblings and those with second degree relatedness were considered half-siblings. The estimated pairwise kinship $k > 0.1875$ represented full-sibs, and $0.1875 > k > 0.09375$ represented half-sibs, with each pair being assigned the most likely kinship category (Iacchei *et al.,* 2013). We considered half-siblings to be paternal because polyandry is much rarer than polygyny in wild *Ae. aegypti* (Richardson *et al.,* 2015). Therefore, we assumed full-siblings to come from the same matrilineage, and half-sibs from different matrilineages.

Wolbachia *loss*

Using the results from *Wolbachia* screening and relatedness analysis, we identified infected matrilineages as being any group of full-sibs containing at least one individual infected with *Wolbachia*. Any uninfected individual within an infected matrilineage was considered to have either failed to inherit the infection from its mother or to have lost it during development. By comparing

the number of these individuals with the total number of full-sibs from infected matrilineages, we could estimate the rate of infection loss per generation (μ).

Long-distance host movement

We considered the distance of separation between sampled full-sibs indicative of female movement range. Full-sibs found within a single ovitrap would likely be from the same gonotrophic cycle, while those further apart would likely be from one or possibly two gonotrophic cycles. The distance of separation between half-sibs was more difficult to characterise, but would reflect the total movement of the male and two females involved. Although advective dispersal of mosquitoes by humans is usually thought to target eggs rather than adults, we could not rule out human interference in the observed "natural" flight ranges. However, there is no difference between natural and advective adult dispersal in the effect produced on a *Wolbachia* invasion. A measure of flight range used in this study is therefore a composite of mosquito active flight and possible advective human movement, and is more informative for understanding *Wolbachia* invasion success than a measure that considers mosquito flight in isolation.

Barriers to dispersal

Landscape Resistance Modelling

Analyses of population structure are generally thought to be at risk of bias when full-sibs are included in analyses (Goldberg and Waits, 2010; Porras-Hurtado *et al.,* 2013), though specific testing of this effect has produced ambiguous results (Peterman *et al.,* 2016). For the proceeding distance-based redundancy analyses (dbRDA, Legendre and Anderson, 1999), we erred on the side of caution

and sampled one individual from each matrilineage with the smallest percentage of missing data, leaving 100 individuals.

We tested the hypotheses that highways had a significant effect on *Ae. aegypti* genetic structure in Cairns (H_1), and that genetic structure was significantly affected by recent releases of *Wolbachia* (H_2). For H_1, we considered each individual as occupying a position southeast of Bruce Highway, west of both highways, or northeast of Captain Cook Highway (Fig 1), and from this assigned them a score of 0, 1 or 2 respectively, so that the distance between two individuals' scores represented the number of highways between them, calling this variable "highways". We approached H_2 similarly, but with the distances representing the time since *Wolbachia* releases were commenced in the area (Fig 1). Thus each individual also received a score of 0, 1 or 2, dependent on whether the plot it occupied underwent releases in 2013, 2014 or had never underwent releases, with this variable denoted "releases". Plots with 2013 releases were considered more 'distant' from those never experiencing releases than those with 2014 releases, as potential structuring effects such as selection or reductions in diversity would presumably display a greater effect over time. Treating the geographical or temporal separation of individuals as additive variables analogous to landscape resistance surfaces avoids issues associated with detecting discrete barriers (Landguth *et al.,* 2010).

We performed partial dbRDA to test H_1 and H_2 as explicit hypotheses, while controlling for the potentially confounding effects of isolation by distance and latitudinal and longitudinal clining. We placed potentially confounding variables (Easting and Northing UTM coordinates and a binary variable representing *Wolbachia* infection status for each individual) inside a conditional matrix. The dependent variable was a distance matrix of Rousset's *a* scores (Rousset, 2000) calculated for each pair of individuals using the program SPAGeDi (Hardy and Vekemans, 2002).

All remaining model procedures were performed in the package VEGAN. The dbRDA models were built using the function *capscale*. For all models, we applied the effects of the conditional matrix described above, after which we tested for predictive power of the variables for highways and releases. We built three models, one implementing both predictor variables and the other two implementing each variable in isolation. We assessed the statistical significance of predictor variables with the function *anova.cca,* using 99999 permutations to test for the marginal significance of each term after accounting for the effects of the others. The entire procedure was then repeated with the binary variable representing *Wolbachia* infection status removed from the conditional matrix and used as a predictor variable alongside highways and releases. While constructing models, we calculated variance-inflation factors (VIF) to check for multicollinearity between predictor variables, using the function *vif.cca*. All VIFs were < 1.1, so none were rejected.

Type I Error Testing

Although ordination methods such as dbRDA are thought to detect genetic structure with greater power than traditional Mantel tests (Legendre and Fourtin, 2010; Cushman *et al.,* 2013), they also suffer from elevated risk of Type I error (Kierepka and Latch, 2015a). Specifically, Type I errors can be caused if hypotheses of geographical influence are proposed against only null hypotheses of panmixia, rather than against alternative geographical hypotheses (Cushman *et al.,* 2006; Cushman and Landguth, 2010). We adapted the method of Kierepka and Latch (2015b) to test for Type I error in our models of population structure.

We used CDPOP to simulate the field site without any highways, *Wolbachia* infections, or mosquito release histories, thus creating an artificial environment in which only isolation by distance could explain the pattern of structuring. We designated 1000 locations as the positions of individual mosquitoes, which included the locations of the 100 individuals used in dbRDA and 893 new

locations placed at random throughout our field site. We used parameters coding for high recruitment and high mortality, and negative exponential dispersal approximating a leptokurtic dispersal kernel with no maximum limit to dispersal, but where dispersal over 300 m was relatively rare. Dispersal was unbiased by sex, as while males generally disperse more readily early in their lives, females tend to travel greater distances over their lifetimes (Sheppard *et al.,* 1969; McDonald, 1977). The CDPOP input file listing all relevant parameters is supplied in Supplementary Information A.

We allowed CDPOP to generate genotypes for each individual, so that the population would initially be at maximum genetic diversity. We reduced the number of SNPs from our data set so that the real and simulated sets would have similar numbers of effective alleles. Effective allele counts have been found to correspond well with analytical power across different types of genetic data (Wang, 2006; Hauser *et al.,* 2011). We used the method of Kimura and Crow (1964) to calculate the number of effective alleles in our 3784 loci set, which gave 5618 effective alleles, corresponding to 2809 biallelic loci at maximum diversity.

We constructed 100 simulations and ran each for 80 discrete generations, after which we sampled the genotypes of the mosquitoes now present at the original 100 locations. These samples were tested for isolation by distance using Mantel tests, and showed similar (slightly higher) levels of isolation by distance (r, $\bar{x} = 0.082$, $\sigma = 0.022$, all $P < 0.05$) as that present in the empirical data ($r = 0.047$, $P < 0.05$). All Mantel tests were run using the function *mantel* in the R package VEGAN (Oksanen *et al.*, 2007). For each sample we constructed new dbRDA models with the same parameters as the observed data, and tested for the marginal significance of highways and timing of *Wolbachia* releases using ANOVAs. We allowed for a maximum of 5 of the simulated samples to show significance for either of the predictor variables, as any more than that would indicate the risk of Type I error from the residual isolation by distance in the data.

Simulation of Wolbachia *Spread and Barrier Strength*

We created a spreadsheet-based simulator of mosquito activity and *Wolbachia* spread following a release of *Wolbachia*-infected mosquitoes. After the successful releases at northern Parramatta Park (PP) in Cairns in 2013, the infection was seen to spread south rapidly to the Bruce Highway, but did not successfully invade the region south of the highway (Schmidt *et al.,* submitted). Using this simulator, we modelled the spread in an artificial PP devoid of any highway barriers to dispersal, and compared these results against the observed spread at PP. We then used the findings from this comparison to estimate highway barrier strength.

The simulator was adapted from Nemo2 (http://nemo2.sourceforge.net/, Guillaume and Rougemont, 2006), in which gene flow and the spread of heritable phenotypes is recorded through a series of adjoining habitat patches covering the area of interest. We did not consider gene flow in our analyses; our adapted simulator considered the spread of *Wolbachia* through these patches, but initial frequencies of *Wolbachia* (p_{init}) could be assigned patch by patch, an option not available in Nemo2. Thus we assigned high p_{init} to patches within the release area, and p_{init} = 0 for all other patches. Parameters used in simulations are listed in Table S1 and described in detail in Supplementary Information B.

We divided the PP study site from Schmidt *et al.* (submitted) into 74 hexagonal patches, each of area 4.6 ha, so that their centrums were spaced 250 m from each other (Figure S1). The simulator models a *Wolbachia* release as a series of 22 discrete generations, representing the 2 years of spread recorded at PP (assuming 5 generations per dry season and 6 per wet season, ref). The sequential steps of a generation were as follows: (i) dispersal, (ii) mating, (iii) infection loss and (iv) population regulation. Each of these life cycle events was modelled with probability functions described in supplementary information A. As studies have reported varying estimates for dispersal in *Ae. aegypti*,

we used three different probability functions to model dispersal: inverse square/Gaussian dispersal (Inv), leptokurtic dispersal up to 450 m (L450) and leptokurtic dispersal up to 800 m (L800).

Given there was no highway barrier effect included in the simulations, we hypothesised that the final infection frequencies (p_{final}) of patches separated by highway would be overestimated, relative to those of other patches outside the release zone. However, *Wolbachia* spread can also be impeded by sharp increases in host density (Barton and Turelli, 2011). To control for this effect, we incorporated a patch carrying capacity calculated from one of three habitat models: Null Habitat Model (NHM); Linear Habitat Model (LHM); and Exponential Habitat Model (EHM). We used data collected for three characteristics of human dwellings in Cairns that may act as measures of habitat quality: construction material(s); architectural style; and window screening. These are described in further detail in Supplementary Information B.

Table 1 summarises the 15 treatments used for simulations. Dispersal models are Inverse Square Dispersal (Inv), Short-range, Leptokurtic Dispersal (L450), and Long-range, Leptokurtic Dispersal (L800). For each LHM and EHM treatment, we added a second treatment incorporating dispersal cues (_cues). In models using dispersal cues, a multiplier was added to each dispersal probability, that would increase or decrease dispersal into a patch in direct proportion to the patches quality of habitat relative to the average.

For each treatment we ran 100 simulations, and from these calculated an average p_{final} for each patch. We calculated adjusted R-squared between the observed p_{final} and the simulated p_{final}, and used linear regression to test for significance of relationship, with each analysis calculated for: all patches (A, n=49), patches entirely or partially within the release zone (Z, n=16), patches offsite but ahead of the highway (O, n=23) and patches offsite and across the highway (H, n=10).

To distinguish the effects of highways in stopping the *Wolbachia* invasion, we calculated the average overestimation of p_{final} in O and H patches for each treatment. We hypothesised that if

overestimation of $p(H)_{final}$ > overestimation of $p(O)_{final}$ across different treatments, this would indicate that highways had acted as barriers to the spread of *Wolbachia* at PP. However, the presence of a steep habitat density gradient at the same location as the Bruce Highway suggests an alternative explanation for inhibited spread: that the invasion is being slowed not by dispersal restrictions of highways, but by a sharp increase in density from the area surrounding Bruce Highway to southern PP. If this is the case, we expect the overestimation of $p(H)_{final}$ > overestimation of $p(O)_{final}$ when habitat is homogeneous (NHM) but not when habitat quality is modelled as either a linear (LHM) or exponential (EHM) variable.

Finally, we ran new simulations following the same procedure, but with a variable strength of highway barrier effect incorporated into each dispersal function. We modelled highway barriers in two ways and analysed each in turn. For the first, we reduced the likelihood of a mosquito dispersing between patches separated by highways from 5% to 25%, increasing in 5% increments. For the second, we treated patches separated by highways as having a greater distance of separation, from 25 m to 125 m, increasing in 25 m increments.

Results

Wolbachia *Infection Screening*

Of the 161 Cairns mosquitoes, 60 were infected with *Wolbachia*. The six samples from PPN were all infected, while all of PPS and CNE were uninfected; the remaining plots had a mixture of infection rates (Figure 1). All individuals caught in the post-release Gordonvale sample (Gv13) were infected. *Wolbachia* titre scores at Gv13 ($\bar{x}$ = 18.4, σ = 10.8) were almost three times higher than those at Cairns ($\bar{x}$ = 6.6, σ = 3.8; t-test with unequal variances, $P < 0.001$), but were also three times as dispersed (Figure 2). One infected mosquito at Gv13 had a titre of just one gene copy for every three *Aedes* gene copies, while the lowest titre in Cairns was more than three times this amount. The highest titre in Gv13 was 56 *Wolbachia* gene copies for each *Aedes* gene copy, more than double the highest recorded in Cairns.

Wolbachia *loss*

We identified 31 full-sib groups ($0.203 \leq k \leq 0.487$) in Cairns, 10 from the *Wolbachia*-infected matrilineages (containing 21 individuals) and 21 from uninfected matrilineages. Among 21 individuals from the *Wolbachia*-infected matrilineages, we recorded a single case of infection loss in CNW. The sample from this matrilineage consisted of a pair of full-sibs (k = 0.376), one of which carried the infection (titre = 6.15) and one of which did not. If we treat this observed rate of loss as representative of the *Ae. aegypti/w*Mel system in Cairns, then it can be used to calculate the likelihood of a female mosquito failing to transmit *Wolbachia* to at least one of her offspring. As there were 10 infected matrilineages, this likelihood is one in ten (0.10). Alternatively, we can consider the probability of infection loss among offspring within infected matrilineages, which gives a

likelihood of loss of one in 21 (μ = 0.048), although the 95% binomial confidence intervals are substantial around this estimate (0.001, 0.238).

Long-distance movement

The 31 full-sib groups contained 43 full-sib pairs, most of which were found within single ovitraps. The two pairs were found in different plots (Figure 3): one pair was split between CNW and CNE (239 m separation, *k*=0.203); the other between CNW and PPS (1312 m, *k*=0.235). Eight of the 27 half-sib pairs ($0.0989 \leq k \leq 0.187$) were found across multiple traps (47-560 m separation), though none of these were located in different plots. There was a gap in *k* scores (Δk = 8.6%) between the most closely related half-sibs (*k*=0.187) and the most distantly related full-sibs (*k*=0.203), indicating that the cut-off point in relatedness between full-sibs and half-sibs (*k*=0.1875) is valid. This 8.6% difference between the two categories is much greater than the difference between the top two half-sib pairs (Δk = 0.9%) and the bottom two full-sib pairs (Δk = 2.9%).

Barriers to dispersal

Landscape Resistance Modelling

ANOVAs performed on partial dbRDA models showed that, of the variables representing "highways", "releases", and "*Wolbachia* infection", only "highways" was predictive of genetic structure (Table 2; $1.55 \leq$ F-value ≤ 1.64; $P < 0.05$). "Highways" was a significant effect in all models, and based on the partial Eta squared (η_p^2, Tabachnick and Fidell, 1996), this variable explained 1.7% of the variance in each model. Supplementary Information C describes additional analyses of genetic structure among groups, with results that are in accordance with these findings.

Type I Error Assessment

Only two out of the 100 simulations with only isolation by distance displayed genetic structure correlating with "highways" at the $P < 0.05$ level. This was observed regardless of the "releases" variable being included in the models. The result suggests that the structuring effect of "highways" observed in the real data is unlikely a Type I error caused by sampling bias or residual autocorrelation.

Simulation of Wolbachia *Spread and Barrier Strength*

Using the adjusted R-squared between the observed p_{final} and the simulated p_{final} for all patches as a measure of performance, the treatments that performed best were those in which habitat was assumed to be homogeneous (NHM) and those in which habitat quality was modelled as either a linear (LHM) or exponential (EHM) variable but with dispersal cues. For every treatment, H patches (offsite patches across the highway) had lower adjusted R-squared scores than O patches (offsite patches before the highway), and linear regression found no significant relationship between observed $p(H)_{final}$ and simulated $p(H)_{final}$.

We observed a greater overestimation of $p(H)_{final}$ relative to $p(O)_{final}$ for all 15 treatments. Relative overestimation of $p(H)_{final}$ ranged from 0.014 (L450_NHM) to 0.174 (L800_EHM), with an average of 0.086 across all treatments (paired t-test, $P < 0.001$). Relative overestimation was higher for L800 and EHM treatments and lower for L450 treatments. When we removed treatments that had performed poorly (adjusted R-squared for all patches < 0.8), relative overestimation was reduced to 0.065 but was still significant (paired t-test, $P < 0.01$). These results support the findings from our

molecular analyses that highways in Cairns act as barriers to mosquito dispersal, and by extension, to the spread of *Wolbachia*.

We introduced highway barrier effects into the treatments that were considered to have performed well (adjusted R-squared > 0.8), and by increasing barrier strength incrementally we determined the level at which overestimation of $p(H)_{final}$ = overestimation of $p(O)_{final}$, representing the "true" strength of the highway barrier effect. By modelling the effect as a reduction in dispersal likelihoods, we determined that a 15% reduction was required to remove overestimation (Figure 4a). Modelling it as an increase in distance between patches showed that barrier strength corresponded to an extra 30-35 m of separation (Figure 4b). In both cases, increasing the strength of the barrier effect not only reduced $p(H)_{final}$, but also increased $p(O)_{final}$, as more infected mosquitoes remained within the highway borders and fewer uninfected individuals dispersed from H patches into O patches. This relationship was approximately linear; for every 10% reduction in dispersal or 25 m of additional distance between O and H patches, $p(H)_{final}$ decreased by 0.03 and $p(O)_{final}$ increased by 0.01.

Discussion

Our study produced three main findings: i) the *w*Mel infection in *Ae. aegypti* in Cairns fails to transmit maternally at some non-zero frequency ($\mu > 0$); ii) eggs laid by the same female were collected in ovitraps 1312 m apart; and iii) highways exert a significant influence on *Ae. aegypti* genetic structure. These phenomena describe infection loss, long-distance host movement and barriers to dispersal respectively, and each may have a slowing effect on *w*Mel invasion as observed in the spread of *Wolbachia* through Cairns (Schmidt *et al*., submitted; Turelli and Barton, 2016).

Wolbachia loss

We provide the first evidence of maternal transmission failure (μ) in the *Ae. aegypti*/*w*Mel system, and from it an estimate for the rate of successful transmission in Cairns of around 95%. While this estimate is tentative due to a small sample size in our study, it is approximately equal to the frequencies of infection observed at Gordonvale and Yorkey's Knob, two quasi-isolated release sites near Cairns that were successfully invaded with *w*Mel in 2011 (Hoffmann *et al.,* 2011) but which have maintained infection rates close to 95% for years without reaching fixation (Hoffmann *et al.,* 2014). This suggests that the inability of the *Wolbachia* infection to reach complete fixation (100%) at these sites can be explained partly or entirely in terms of μ. Although transmission of *w*Mel in *Ae. aegypti* has previously been recorded as perfect or quasi-perfect in the laboratory (Walker *et al.,* 2011) or in the field (Hoffmann *et al.,* 2014), a transmission rate of 95% is also comparable to the 96.7% rate of the *w*AlbA *Wolbachia* infection observed among field-collected *Aedes albopictus* (Kittayapong *et al.,* 2002). An alternative explanation would also include dispersal of uninfected mosquitoes into each site, either by natural dispersal from adjoining uninfected habitats, or by advective dispersal from other human population centres. Under assumptions of $\mu = 0$, uninfected

migration rates of 3-6% into each site would be required to maintain the observed frequencies of infection (Hoffmann *et al.,* 2014).

We observed considerable disparity in *Wolbachia* titre between Cairns and Gordonvale samples, with the Gordonvale sample having titres that were both higher on average and more variable. This likely reflects differences in sampling and processing between the two datasets. The Cairns ovitrap samples were raised under optimal laboratory conditions and stored as adults within a day of eclosion. In contrast, Gordonvale BG-Sentinel trap collections consisted of field-raised adults of variable age and physiological state (Williams *et al.,* 2006). The inclusion of blood-fed females in the Gordonvale sample may partially explain the occurrence of very high titres. *w*Mel titres within *Ae. aegypti* are known to double in blood-fed individuals (Frentiu *et al.,* 2014), which approximates the difference between the maximum titres from Gordonvale and Cairns. The very low titres recorded for some Gordonvale individuals, on the other hand, might result from high temperature fluctuations during development in the field (Ross *et al.,* 2016b), or from a reduction in titre over time as seen in the *w*AlbA infection in male *Ae. albopictus* (Tortosa *et al.,* 2010).

Long-distance movement

Analyses of local patterns of kinship have provided several useful inferences regarding *Ae. aegypti* ecology. The discovery of a full-sib pair at 1312 m separation indicates a single female traversing that distance, representing the extreme end of flight range in *Ae. aegypti*. However, contrary to a previous MRR study that identified long range dispersal of at least 840 m (Reiter *et al.,* 1995), we cannot be sure that this movement was within a single gonotrophic cycle. Seven days passed between deployment and sampling of the two ovitraps, which is long enough for an additional unobserved egg deposition somewhere between them (Christophers, 1960). If we assume that the flight distance of 1312 m reflects natural dispersal, an additional gonotrophic cycle is likely,

as the length of a single such cycle is rarely more than a few days, particularly when temperatures exceed 30°C (Christophers, 1960) as they did during our sampling. To assume a single gonotrophic cycle thus assumes the flight to have occurred over a shorter period of time, which entails an average daily speed of greater than the already high minimum estimate of 187 m/day over seven days. Previous estimates of average female flight speed in Cairns are almost an order of magnitude below this (Muir and Kay, 1998). With relatively heavy traffic along Mulgrave Road and Sheridan Street and an increased frequency of re-entering vehicles in commercial areas, the two highways may act as conduits for advective female dispersal. After allowing for mosquito "hitchhiking" along highways, the total distance between the ovitraps (measured from each trap to the nearest highway segment) is reduced to 277 m.

Using a continuous coefficient of relationship such as *k* to assign pairs of individuals to discrete kinship categories can be problematic when estimates are close to the critical cut-off values. There was some evidence of this in our estimates of half-sibship/unrelatedness, where some pairs with $k \approx 0.09375$ would be difficult to assign with confidence to either category. However, the categories of full-sibs and half-sibs were clearly separated from each other relative to the variability in *k* scores within each category. This reflects the power of genome-wide SNPs for inferring relationships (Blouin, 2003; Tokarska *et al.,* 2009; Cramer *et al.,* 2011; Sellars *et al.,* 2014; Weinman *et al.,* 2015). Inferring dispersal from relatedness also avoids potential biases resulting from lab-raised *Ae. aegypti* used in MRR studies failing to develop experience in local conditions that will inform their future oviposition choices (Kaur *et al.*, 2003; Ruktanonchai *et al*., 2015). Our findings are broadly consistent with the results of several MRR studies in *Ae. aegypti* adults (Shannon and Davis, 1930; Reiter *et al.,* 1995; Honório *et al.,* 2003; Reiter, 2007), and suggest that this method provides an alternative to MRR for studying dispersal.

Barriers to dispersal

While previous population genetic studies suggested that major roads could act as barriers to *Ae. aegypti* dispersal (e.g Hemme *et al.,* 2010), we provide evidence for such an effect through explicit hypothesis testing within a landscape genetics framework. We detected a minor but statistically significant barrier effect of highways, corresponding to 1.7% of dbRDA variance in genetic distance between individuals. Our simulations of the *Wolbachia* invasion progress south of Bruce Highway showed that barrier strength corresponded to an added 30-35 m of separation. Given the slow *w*Mel invasion dynamics observed at PP (Schmidt *et al.,* submitted) with the infection frequencies at the wave front being only slightly above the critical threshold ($\hat{p} \approx 0.35$ for *w*Mel in *Ae. aegypti* (Barton and Turelli, 2011)), an added "cost" to cross the highway could prove to be influential in preventing invasion. If we assume the restrictive effect of highways on dispersal to increase by highway width and highway traffic, then many urban highways in cities earmarked for future *w*Mel releases would likely function as effective barriers to spread.

On the other hand, the restrictive effect of highways helped strengthen invasion within the area enclosed by them. The habitat patches along these highway boundaries will have increased infection frequencies relative to patches in unsubdivided regions, and will serve to locally protect the *Wolbachia* frequency from the counteracting effect of uninfected immigrants. Following the 2013 *Wolbachia* releases in Cairns, the largest and most successfully invaded release site at Edge Hill/Whitfield recorded a large influx of uninfected *Ae. aegypti* at the start of the 2014/2015 wet season. This was likely in part due to the greater connectivity of Edge Hill/Whitfield with surrounding uninfected regions (Schmidt *et al.,* submitted). Prospective release sites in areas with such characteristics should be positioned adjacent to dispersal barriers such as highways, as this may help reduce the threat of reinvasion of uninfected mosquitoes. Additionally, the slow rate of spread (100-200 m/year, Schmidt *et al.,* submitted) observed at Cairns emphasizes the need to focus on area-

wide releases rather than rapid regional invasions, and denser release points even in unsubdivided regions (Turelli and Barton, 2016).

Conclusions

Our study demonstrates the utility of genome-wide markers for investigating ecological processes such as fidelity of *Wolbachia* transmission and mosquito dispersal at very fine spatial scales. Non-perfect maternal transmission of *w*Mel in *Ae. aegypti* may not occur in other *Wolbachia* strains such as *w*AlbB, whose density shows greater constancy under fluctuating high temperatures (Ross *et al.*, 2016b). On the other hand, leptokurtic dispersal and presence of barriers like highways are potential problems for any *Wolbachia* invasion strategy requiring spatial spread (Turelli and Barton, 2016). Our approach has provided empirical evidence for the processes predicted to slow down the spread of *w*Mel in *Ae. aegypti*, and could be extended to other host/*Wolbachia* systems that are increasingly considered for the biocontrol of disease vectors and pests.

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Data Archiving

Demultiplexed fastq files will be deposited at NCBI SRA by time of publication.

Author Contributions

G. Rašić, A.A. Hoffmann, and T. L. Schmidt conceived of and designed the study.

T. L. Schmidt, G. Rašić, and I. Filipović collected and dried the samples.

T. L. Schmidt performed the laboratory work and conducted the analyses, with assistance from G. Rašić, and computational support from I. Filipović.

T. L. Schmidt wrote the manuscript with assistance from A.A. Hoffmann and G. Rašić.

Table 1: The 15 treatments used in simulations of spread at PP

	Inv_null	Inv_cues	L450_null	L450_cues	L800_null	L800_cues
NHM	I		II		III	
LHM	IV	V	VI	VII	VIII	IX
EHM	X	XI	XII	XIII	XIV	XV

Each treatment incorporated one habitat model and one dispersal model. The three habitat models used were: Null Habitat Model (NHM); Linear Habitat Model (LHM); and Exponential Habitat Model (EHM). The three dispersal models were Inverse Square Dispersal (Inv), Short-range, Leptokurtic Dispersal (L450), and Long-range, Leptokurtic Dispersal (L800). For each dispersal model, separate simulations were run in which dispersal cues were either omitted (*_null) or included (*_cues).

Table 2: Results of ANOVAs testing marginal significance of "highways" and "releases" variables in dbRDA

		sum of squares	F-value	P	η_p^2
analysed in isolation	HIGHWAYS	0.021	1.635	**0.021**	0.017
	RELEASES	0.016	1.241	0.163	0.013
analysed together	HIGHWAYS	0.020	1.555	**0.032**	0.017
	RELEASES	0.015	1.165	0.231	0.012

The two variables were each analysed in isolation in separate models, then together in a single model. In every case, "highways" was predictive of genetic structure while "releases" was not. Partial Eta squared (η_p^2) showed that "highways" accounted for 1.7% of the variation within each model.

Figure 1: Sampling locations of the 161 mosquitoes analysed with ddRADseq, set within the six sampling plots. Each sample was assigned a *Wolbachia* infection status, a score indicating its position relative to the two highways, and a score indicating when *Wolbachia* releases were carried out in the area. Plot abbreviations are: CNW (Cairns North West), CNE (Cairns North East), PPN (Parramatta Park North), PPS (Parramatta Park South), WC (Westcourt) and BN (Bungalow).

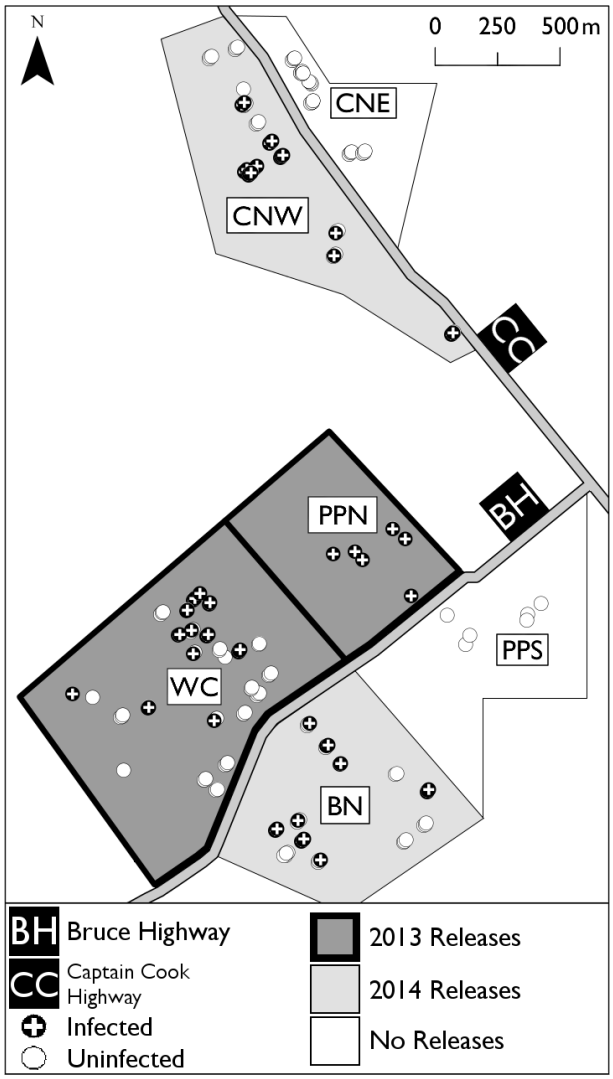

Figure 2: *Wolbachia* titres of infected individuals collected from Cairns and Gordonvale. Titres describe the the ratio of *Wolbachia* gene copies to *Aedes* gene copies. Titres were higher and more dispersed at Gordonvale than at Cairns.

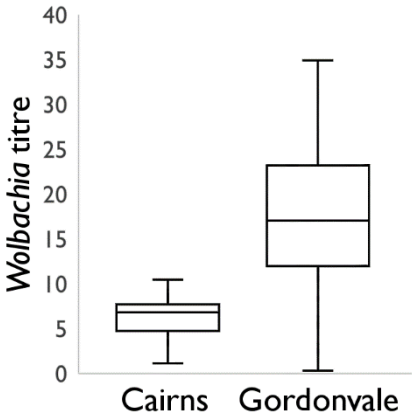

Figure 3: Loiselle's *k* estimates for sample pairs of relatedness $k > 0.046875$. Pairs of $0.09375 < k < 0.1875$ are most likely half-sibs, those of $k < 0.1875$ are most likely full-sibs. Most related pairs were found within the same trap, but separation distances of up to 1312m were observed.

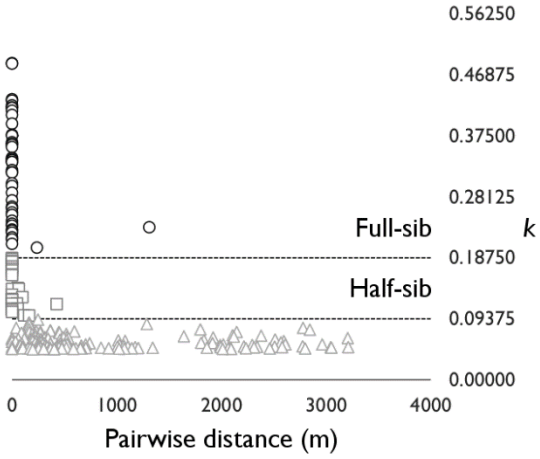

Figure 4: Highway barrier effects modelled as a reduction in dispersal likelihoods across the barrier (A) and an increase in separation distance between patches across the barrier (B). Increasing barrier strength lead to a decrease in p in H patches and a corresponding increase in O patches.