Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila

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Estimates of *Wolbachia* density in the eggs, testes and whole flies of drosophilid hosts have been unable to predict the lack of cytoplasmic incompatibility (CI) expression in so-called *mod*[−] variants. Consequently, the working hypothesis has been that CI expression, although related to *Wolbachia* density, is also governed by unknown factors that are influenced by both host and bacterial genomes. Here, we compare the behaviour of the *mod*[−] over-replicating *Wolbachia popcorn* strain in its native *Drosophila melanogaster* host to the same strain transinfected into a novel host, namely *Drosophila simulans*. We report that (i) the *popcorn* strain is a close relative of other *D. melanogaster* infections, (ii) the *mod*[−] status of *popcorn* in *D. melanogaster* appears to result from its inability to colonize sperm bundles, (iii) *popcorn* is present in the bundles in *D. simulans* and induces strong CI expression, which demonstrates that the bacterial strain does not lack the genetic machinery for inducing CI and that there is host-species-specific control over *Wolbachia* tissue tropism, and (iv) infection of sperm bundles by the *mod*[−] *D. simulans wCof* strain indicates that there are several independent routes by which a strain can be a CI non-expressor.

Keywords: *Wolbachia*; *popcorn*; cytoplasmic incompatibility; sperm modification

1. INTRODUCTION

Wolbachia are ubiquitous bacteria that infect 16–76% of all insect species surveyed (Werren *et al.* 1995; Jeyaprasaksh & Hoy 2000). As obligate intracellular inhabitants of host germ-line tissue, they are capable of inducing a range of reproductive abnormalities (O'Neill *et al.* 1997). *Wolbachia* infections in *Drosophila* are best known for mediating a failure of karyogamy (Callaini *et al.* 1997) that is known as cytoplasmic incompatibility (CI). *Wolbachia* introduce a 'modification' in immature sperm by an unknown mechanism that can only be 'rescued' following fertilization of an egg with similar infection status. The result is that a high proportion of the embryos produced by matings between infected males and either uninfected females or females carrying a heterologous *Wolbachia* strain are non-viable. The bacterium is predominantly transmitted transovarially, therefore the infection tends to spread rapidly into populations, as infected females have greater fitness relative to their sympatric uninfected counterparts (Turelli & Hoffmann 1991).

The strain *popcorn* is a *Wolbachia* infection described in *Drosophila melanogaster* that differs from most strains in two major ways. First, males infected with this strain are unable to induce CI (Min & Benzer 1997), thereby making it a member of a class of so-called '*mod*[−]' variants or CI non-expressors (Holden *et al.* 1993; Giordano *et al.* 1995; Hoffmann *et al.* 1996). Second, the strain is known to heavily infect somatic tissues, including the brain, nervous tissue and flight muscle (Min & Benzer 1997). Densities of *popcorn* increase in these locations as flies age in a temperature-dependent manner. *D. melanogaster* carrying *popcorn* infections suffer from reduced lifespans, presumably due to excessive bacterial replication, in particular in their nervous tissues (Min & Benzer 1997).

Numerous studies have attempted to relate the *Wolbachia* density in eggs, testes, ovaries and entire flies to the strength of the CI (the proportion of eggs not hatched when uninfected females are mated with infected males) and modification status. Although clearly involved in determining the level of CI expression, density measurements are not entirely predictive (Boyle *et al.* 1993; Hoffmann *et al.* 1994; Giordano *et al.* 1995; Bourtzis *et al.* 1996, 1998). This may simply reflect the evaluation of densities in inappropriate tissues, as CI intensity appears to be well correlated with the number of infected sperm cysts as opposed to the *Wolbachia* densities of entire testes tissue (Solignac *et al.* 1994; Poinot *et al.* 1998).

A related question that has been addressed by a range of transinfection studies has been determining the degree to which bacterial versus host genotypes contribute to the expression of the CI phenotype. By examining the behaviour of infections in novel bacterial strain–host species combinations it appears that host genomes often have a role in determining both the *Wolbachia* density and strength of CI (Boyle *et al.* 1993; Poinot *et al.* 1998). However, this is not always seen. Examples exist in which transfers into new host backgrounds do not alter *Wolbachia*-associated phenotypes (Giordano *et al.* 1995). In order to gain further insight into the factors responsible for density control and CI expression, we studied the behaviour of the *popcorn* strain, with its unique combination of phenotypes, in multiple-host backgrounds following embryonic microinjection of the *D. melanogaster popcorn* infection into an uninfected *Drosophila simulans* host. We then compared the modification status and *Wolbachia* density and distribution in the testes between the different *Drosophila* species. Our specific predictions were that the *popcorn* strain's inability to modify sperm in *D. melanogaster* is a characteristic associated with the bacterial genome and, therefore, that the *popcorn* strain would also be *mod*[−] in *D. simulans* and that we may be

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able to relate the patterns of modification status to the *Wolbachia* densities in testes.

2. MATERIAL AND METHODS

(a) Strain nomenclature

Wolbachia–host species combinations are named using a two-part designation, with the first part referring to the host species (Dmel and Dsim for *D. melanogaster* and *D. simulans*, respectively) and the second part indicating the bacterial strain (i.e. *w*MelPop and *w*Ri for the *popcorn* and *Riverside* infections, respectively). Any strain name followed by a 'T' (e.g. Dsim *w*RiT) indicates that the original host has been cured of its infection by standard methods of tetracycline treatment (Hoffmann *et al.* 1986).

(b) Embryonic microinjection

Egg cytoplasm from Dmel *w*MelPop (Min & Benzer 1997) was microinjected into Dsim *w*RiT (Hoffmann *et al.* 1986) using standard techniques (Ashburner 1989; Boyle *et al.* 1993; Sinkins *et al.* 1995). Embryos were incubated at 27 °C for 48 h. Hatched larvae were then transferred to vials containing a fruitfly diet (Sigma) and moved to 21 °C for all subsequent rearing.

(c) Rearing and screening for infection status

Surviving G₀ females were crossed with Dsim *w*RiT males and used for establishing isofemale lines. Following evidence of viable G₁ offspring, female parents were sacrificed and DNA was extracted using the STE/boil method (O'Neill *et al.* 1992). The presence of *Wolbachia* in the samples was detected by a polymerase chain reaction (PCR) using primers for the *wsp* gene (81F and 691R) (Braig *et al.* 1998). Amplification of DNA was carried out in 50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton-X 100, 2.5 mM MgCl₂, 0.25 mM dNTPs and 0.5 µM primers. The thermal cycling regime was as follows: 3 min denaturation at 94 °C, 35 cycles of denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C, followed by an extra 10 min extension step at 72 °C. The reactions were run in either a PTC-200 Thermal Cycler (MJ Research, San Francisco, CA, USA) or a Mastercycler (Eppendorf, Westbury, NY, USA). Approximately 350 embryos were injected. Nine out of nine G₀ males and seven out of 12 G₀ females were PCR positive for *Wolbachia*.

In order to ensure the stability of the infection in early generations, PCR screening was used for selecting only offspring from females that tested positive for *Wolbachia*, which were then used for seeding successive generations. After 11 generations of mating that included several out-crossings to Dsim *w*RiT males, we removed the selection regime and began rearing populations of flies in bottles. Several lines were lost during selection, such that descendants of a single original female comprised the entire line.

(d) Tests for sperm modification

Single mating pairs of 1–2 day old virgin flies (*n* = 75) were placed in separate empty food bottles with molasses plate lids dotted with a yeast suspension. The plates were collected every 24 h. The first set of plates containing 15 or more eggs was counted and saved for measuring egg hatchability. The number of unhatched eggs was recorded 72 h post-collection and the percentage of hatched eggs was calculated. Dsim *w*MelPop was assayed at generation 20 post-transinfection. The hatch rates in the cross of Dsim *w*RiT females with Dsim *w*MelPop males were unexpectedly low and so we examined 10 representative females for the presence of sperm in the spermathecae in order to

confirm that the flies were in fact mating. In crosses using older males (*n* = 50), the flies were first allowed to pre-mate at 12 days of age for 2 days with an excess of females in order to remove potential artefacts associated with sperm storage. The statistical significance of the hatch rates for various crosses was determined using a Kruskal–Wallis test (STATVIEW 5.0), followed by comparisons using the simultaneous test procedure method (Sokal & Rohlf 1995).

(e) Real-time quantitative PCR

DNA was extracted as above. A real-time quantitative PCR was carried out using molecular beacon hybridization probes (Stratagene, La Jolla, CA, USA) (Tyagi & Kramer 1996) in a Lightcycler (Roche Diagnostics, Indianapolis, IN, USA). Primers were designed for amplifying 150–200 bp regions of the single copy *Wolbachia* *wsp* gene (440 F 5'-CTG GTG TTA GTT ATG ATG TAA C and 691R 5'-AAA AAT TAA ACG CTA CTC CA) (Braig *et al.* 1998), as well as a single copy gene that is found in both *D. simulans* and *D. melanogaster*, namely *su(fk)C* (5439F 5'-ACG TAA GGT CGC GCT ATC and 5596R 5'-ATG GGA CAG GTA GTC AAT G). We designed specific molecular beacon probes for the central regions of the PCR products for each amplicon (Tyagi & Kramer 1996). The beacons we used were as follows: *wsp* (5'-FAM CGG AGC GTT ATT TCG GTT CTT WTG GTG CTC CG-DABCYL) and *su(fk)C* (5'-FAM-CGG AGG CTG GGA TTG AAG CGT TTT GGT GCT CCG-DABCYL). The amount of fluorescence generated from bound beacons was measured during each cycle of annealing in a standard PCR regime (3 s denaturation at 94 °C followed by 40 cycles, 1 s at 94 °C, 30 s at 45 °C for *wsp* or 50 °C for *su(fk)C* and 10 s at 72 °C). The PCR chemistry was as above except that there was a reduction in the MgCl₂ concentration and addition of a beacon and bovine serum albumen (BSA) (50 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.1% Triton-X 100, 2.5 mM MgCl₂, 0.25 mM dNTPs and 0.5 µM primers, 2.0 mM MgCl₂, 0.3 µg µl⁻¹ BSA and 0.5 µM beacons). A standard curve was constructed using *wsp* and *su(fk)C* amplicons that had been cloned previously into pGEM-T (Promega, Madison, WI, USA) and linearized with *Pst*I and quantified as a template. Copy numbers for each gene within the DNA extractions from the flies were calculated using LIGHTCYCLER DATA ANALYSIS software, v. 3.1.02. The *su(fk)C* copy number was used for normalizing the *Wolbachia* *wsp* copy number for a given DNA extraction. Three replicates were run and averaged for each sample prior to the construction of density ratios. We collected measurements on 10 samples for each strain at each time-point.

(f) Fluorescence microscopy

Testes were dissected in and then exposed to fixative (4% formaldehyde in phosphate-buffered saline (PBS)) for a minimum of 20 min. The tissue was then given four 10 min rinses in PBS and Triton-X (PBT) and transferred to a 1 µg ml⁻¹ 4',6-diamidino-2-phenylindole-dihydroxychloride (DAPI) solution in 80% glycerol for 5 min. The testes were then removed to an 80% glycerol solution following four 10 min rinses in PBS. Some testes were teased apart in order to liberate sperm bundles. Others remained whole. The testes were examined after slide mounting using a Zeiss (Thorn Wood, NY, USA) Axioskop FS equipped with epifluorescence optics. Images were recorded with a Cohu (San Diego, CA, USA) grey-scale charged-coupled device camera, digitized with a Scion (Frederick, MD, USA) frame grabber under the control of SCION IMAGE 1.62 and compiled using PHOTOSHOP v. 5.5.

3. RESULTS

(a) Origin of the popcorn variant

The replication of the *popcorn* strain in somatic tissues represents a dead end for transmission. Given the severity of the host lifespan reduction that is associated with infection outside the gonads, it is likely that selection would in time operate in order to attenuate its effects. One explanation for the level of virulence seen in the *D. melanogaster* background is that the association between the insect host and the *popcorn* strain is of recent origin, which has been created through a horizontal transmission event in which there has not yet been enough time for the phenotype to attenuate. Alternatively, over-replication could be due to a recent mutation in the *popcorn* genome, the effects of which have not been attenuated by selection in a laboratory culture or because high densities in the soma confer parallel benefits for transmission in the gonads. We used sequences of the *wsp* gene for determining the phylogenetic relatedness of this infection to other *Wolbachia* strains, as *wsp* is the most rapidly evolving locus for this organism described to date and has been widely sequenced from a range of infections (Zhou *et al.* 1998). Fragments of the *wsp* gene were amplified using the primers 81F and 691R (Braig *et al.* 1998) and then directly sequenced from both Dmel *wMelPop* and Dsim *wMelPop* (GenBank accession no. AF338346). The latter was included in order to rule out a rebound of the *wRi* infection in our transinfected host. Comparisons revealed perfect identity between the *popcorn* strain and published sequences from other Dmel *Wolbachia*, e.g. *wMelCS* (GenBank accession no. AF020065) and *wMel* (GenBank accession no. AF020072). Barring a gene conversion event, this would indicate that the *popcorn* infection is native to the *D. melanogaster* host and that any changes in the *popcorn* genome responsible for its unique phenotype are of recent origin. In accordance with suggested strain nomenclature based on *wsp* sequences (Zhou *et al.* 1998), we propose that the *popcorn* variant be referred to as *wMelPop*.

(b) Modification status

We measured the ability of *wMelPop* to induce sperm modification in Dmel and Dsim males using crosses to uninfected control females (figure 1). Our data confirm the results of Min & Benzer (1997) that *wMelPop* in the *D. melanogaster* background does not induce CI ($p > 0.05$), as evidenced by the high percentage of eggs hatched from the test (95.4%) (s.e. = 1.3 and $n = 75$ single pairs) in comparison to the control cross (98.3%) (s.e. = 0.40 and $n = 75$ single pairs). Given our working hypothesis that modification status is under bacterial genomic control, we expected the crossing results from the transinfected line Dsim *wMelPop* to parallel those for *wMelPop* in its native host. Surprisingly, moving the popcorn infection into the *D. simulans* background leads to strong CI expression ($p < 0.01$). The level of egg hatch was severely reduced in the test cross to only 6.28% (s.e. = 2.1 and $n = 75$ single pairs) when compared with a mean hatch percentage from controls of 97.9% (s.e. = 0.40 and $n = 75$ single pairs).

Figure 1. The egg hatch percentage of crosses for testing the *wMelPop* infection's ability to modify sperm in *D. melanogaster* and *D. simulans* hosts compared with uninfected (T) control crosses. Each mean is based on data from 75 individual mating pairs. The crosses are listed female × male crosses and the error bars represent s.e.m.

Figure 2. The normalized *wsp* copy numbers from the testes of adults collected for three ages post-emergence. Each data point on the graph represents a mean based on 10 testes. Three estimates of the copy numbers for *wsp* and *su(fk)C* were obtained and averaged before ratios representing normalized densities were calculated. The error bars represent s.e.m.

(c) Testes density

We used a real-time quantitative PCR assay for comparing the levels of infection in whole testes of Dmel *wMelPop*, Dsim *wRi* and the transinfected line Dsim *wMelPop* in order to determine whether *Wolbachia* densities in the transinfected strain might correlate with the sperm modification phenotype. Several time-points were included in order to capture any age-dependent density increases (figure 2). As predicted, the density of the *wMelPop* infection in both hosts dramatically increased with age. The mean densities (normalized *wsp* gene numbers) for *Wolbachia* in testes of 28 day old males demonstrated wide variability, regardless of host species, and contributed to an inability to detect significant differences (Mann–Whitney *U*-test, $p > 0.05$) between the

Figure 3. The *Wolbachia* distribution in the testes and testis sheaths of *D. melanogaster* and *D. simulans*. Single representative sperm bundles from the testes of (a) a male *D. melanogaster* and (c) a male *D. simulans*, both infected with *wMelPop*, are shown. The staining in *D. melanogaster* is only present in the elongate sperm nuclei (thick arrows), whereas in *D. simulans* *wMelPop* are present in the cytoplasm of the sperm bundle (asterisks). (b, d) The testis sheaths of *D. melanogaster* and *D. simulans*. The *Wolbachia* are concentrated in the cytoplasm of the sheath cells in both species (asterisks). Both large (vertical arrow) and small (horizontal arrow) nuclei are present in sheath cells.

wMelPop infection in *D. melanogaster* (42.4) (s.e. = 8.5 and $n = 10$) versus *D. simulans* (120.4) (s.e. = 38.2 and $n = 10$). However, the density of *wMelPop* was significantly higher ($p < 0.01$) than *wRi* (5.2) (s.e. = 0.93 and $n = 10$) in *D. simulans* testes, thereby indicating that the over-replication phenotype, which is characteristic of *wMelPop*, was still expressed in the new host background.

All crosses to test for sperm modification (figure 1) were carried out with 3 day old males. Although the densities in young flies are high enough to cause strong CI in the *D. simulans* background, this does not rule out the possibility that the required density threshold for CI expression could be higher in *D. melanogaster*. In order to address this question, we repeated tests for modification using aged Dmel *wMelPop* males containing high densities of *Wolbachia*. We crossed previously mated 14 day old males with uninfected females and again found no evidence for sperm modification ($p > 0.05$) as measured by high egg-hatch rates (96.2%) (s.e. = 0.60 and $n = 50$) in comparison to controls. As such, testes density *per se* is insufficient to explain the differences in CI expression caused by *wMelPop* in different host backgrounds.

(d) Tissue tropism

In order to investigate *Wolbachia* distribution within testes we stained dissected testes tissue with DAPI and examined sperm bundles by epifluorescence microscopy (Bressac & Rousset 1993). Although *wRi* in *D. simulans* infected both sheath cells and sperm bundles, *wMelPop* in *D. melanogaster* could be found in very high numbers in the sheath, but not at all in the bundles (figure 3). The transinfected line demonstrated a blending of the two phenotypes, with a strong bundle and sheath cell infection (figure 3). These qualitative assessments present a possible explanation for the species-specific modification associated with the *wMelPop* infection. In order to determine

whether this pattern could be generalized to other *mod*⁻ strains, we dissected and stained testes from Dmel *wMelCS* (Holden *et al.* 1993) and Dsim *wCof* (Hoffmann *et al.* 1996), both of which are *mod*⁻, with DAPI. *wMelCS* also showed no infection of bundles, whereas the *wCof* strain did (data not shown), thereby indicating that not all *mod*⁻ strains are incapable of infecting sperm cells.

4. DISCUSSION

The strong CI demonstrated by *wMelPop* once transinfected into *D. simulans* proves that this bacterium has the genetic machinery required for sperm modification and that there are distinct host species responses to this infection. The basis for the *mod*⁻ phenotype of *wMelPop* and infection of *D. melanogaster* appears to be their lack of contact with developing sperm in the testes. The most likely explanation for the *mod*⁻ behaviour of the *wCof* strain is that it has a defect in the genetic pathway that is responsible for sperm modification. Our data indicate there are at least two distinct pathways by which *Wolbachia* can become *mod*⁻ in a given host. The first is through a change in *Wolbachia* tissue tropism and the second is presumably through a direct disruption in the *Wolbachia* loci involved with sperm modification. In keeping with results from past transinfection studies (Boyle *et al.* 1993; Giordano *et al.* 1995; Clancy & Hoffmann 1997; Poinot *et al.* 1998), our results reflect the complexity of the interaction between bacterial and host genomes in determining the CI phenotype.

The means by which *mod*⁻ strains compete with *mod*⁺ strains and rise to high frequencies in populations are not entirely clear. Although empirical data are lacking, several theoretical studies have generated scenarios explaining how *mod*⁻ strains could arise and sweep through populations depending on their compatibility

with other strains present in the population, the genetic cost of maintaining CI status, maternal transmission rates, the fitness costs to hosts and other parameters (Turelli 1994; Hurst & Mcvean 1996). Because *Wolbachia* is maternally transmitted, direct selection pressures are likely to drive adaptation to female tissues, so much so that selection may act to increase transmission even at the cost of reduced CI expression (Turelli 1994). Mod⁻ isolates in *D. melanogaster* may therefore be competitive in populations if they have very high maternal transmission efficiencies and/or are capable of rescuing the modification of sperm by other co-occurring strains (Turelli 1994). The *popcorn* infection in both *D. melanogaster* and *D. simulans* hosts is transmitted at near 100% in the laboratory (E. A. McGraw, unpublished data).

A second hypothesis is that *D. melanogaster* may have evolved a mechanism for controlling tissue tropism in response to the pathology induced by certain *Wolbachia* strains, although the effects of heavy infections in the testes on male fitness are unknown. The rampant replication of *wMelPop* in somatic tissues seems at odds with the inability for infecting sperm bundles. An examination of the fitness costs for males with no-bundle versus heavy-bundle infections would be useful.

The normalized *Wolbachia* densities in testes, although uninformative about CI expression, were revealing about the behaviour of this infection over the lifespan of a fly in two different hosts. Demonstration of over-proliferation in the transinfected *D. simulans* indicates that the phenotype is due to the *Wolbachia* genome and the *wsp* sequence indicates that the responsible genetic changes are likely to be a recent event in the evolutionary history of *wMel* infections. As bacterial densities increased in an age-dependent manner, so did the variability in our density estimates, which indicates that there might be increasing stochasticity associated with replication rates and possibly the physiology of ageing testes tissue. Measures from older males are probably skewed, as individuals with the highest densities may have earlier succumbed to death associated with nervous tissue infection (Min & Benzer 1997).

It may be possible to use the biology of *Wolbachia* as a means of vector-borne disease control through two methods, both of which require the driving force of CI for aiding in the spread of the bacteria into target populations (Sinkins *et al.* 1997). The first method is to use the symbiont as a gene expression platform for products that render hosts refractory to parasites, such as those that cause malaria, African sleeping sickness or dengue fever. The second method is to alter the age structure of vector populations by taking advantage of the early death effects associated with strains such as *wMelPop* (Sinkins & O'Neill 2000). Reducing the number of individuals in the oldest age class should dramatically reduce parasite transmission for many vector-borne diseases. The switch in CI expression following transinfection of *wMelPop* from *D. melanogaster* to *D. simulans* demonstrates that the phenotype is not entirely under bacterial genomic control and it is possible that transinfected insect vectors might respond similarly. The *wMelPop* infection may offer the right combination of phenotype and delivery system. However, the ability to induce CI will have to be determined individually for each host of interest.

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