Wolbachia in a Major African Crop Pest Increases Susceptibility to Viral Disease Rather than Protects

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Abstract

Wolbachia are common vertically-transmitted endosymbiotic bacteria found in < 70% of insect species. They have generated considerable recent interest due to the capacity of some strains to protect their insect hosts against viruses, and the potential for this to reduce vector competence of a range of human diseases, including dengue. In contrast, here we provide data from field populations of a major crop pest, African armyworm (*Spodoptera exempta*), which show that the prevalence and intensity of infection with a nucleopolydrovirus (SpexNPV) is positively associated with infection with three strains of *Wolbachia*. We also use laboratory bioassays to demonstrate that infection with one of these strains, a male-killer, increases host mortality due to SpexNPV by 6 - 14 times. These findings suggest that rather than protecting their lepidopteran host from viral infection, *Wolbachia* instead make them more susceptible. This observation potentially has implications for the biological control of other insect crop pests.

INTRODUCTION

Wolbachia pipientis is a maternally-transmitted, Gram-negative, obligate intracellular bacterium found in filarial nematodes, crustaceans, arachnids and insect species (Werren & Windsor 2000; Hilgenboecker *et al.* 2008). Many *Wolbachia* strains increase their prevalence in populations by manipulating host reproductive systems via cytoplasmic incompatibility, parthenogenesis, feminization or male-killing (Werren *et al.* 2008; Engelstaedter & Hurst 2009). Recent reports from a range of dipteran species demonstrate that some *Wolbachia* strains may also have the capacity to protect their host against a number of RNA viruses (e.g. Hedges *et al.* 2008; Teixeira *et al.* 2008; Moreira *et al.* 2009; Osborne *et al.* 2009; Bian *et al.* 2010; Glaser & Meola 2010; Jaenike *et al.* 2011). As a consequence, there has been growing interest in the potential for artificially enhancing *Wolbachia* prevalence in wild host populations of insect vectors of human diseases (e.g. mosquitoes), so as to limit their potential to transmit infections such as dengue (Hoffmann *et al.* 2011; Iturbe-Ormaetxe *et al.* 2011; Walker *et al.* 2011). As far as we are aware, no previous studies have assessed the potential for *Wolbachia* to protect lepidopteran host species against viruses, despite the fact that many moths and butterflies are highly susceptible to baculoviruses.

One of the most devastating migratory crop pests in sub-Saharan Africa is the larval stage of the African armyworm moth, *Spodoptera exempta* (Lepidoptera: Noctuidae), a major pest of staple cereals such as maize, wheat, sorghum, millet and rice, as well as pasture grasses (Rose *et al.* 2000). *S. exempta* moths are highly migratory, being capable of flying > 100 km per night over several nights (Rose *et al.* 1985). The seasonal pattern of outbreaks follows the movement of the inter-tropical convergence zone and the seasonal rains, such that early season outbreaks in central

Tanzania from October onwards act as source populations for subsequent outbreaks that occur downwind at one generation intervals (approximately monthly) at increasingly northerly latitudes in Tanzania and into neighbouring Kenya and beyond (Rose *et al.* 2000).

S. exempta is in the larval stage for approximately 16 – 20 days (Rose *et al.* 2000) and during this period it is susceptible to an endemic baculovirus, *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV). SpexNPV is a large double-stranded DNA virus of the baculovirus family that is highly host-specific, infecting only *S. exempta* at normal doses (Cherry 1992). Lethal baculovirus infections are characterized by systemic viral replication that results in the liquefaction and death of the insect within 4 - 7 days (Brown & Swaine 1965; Grzywacz *et al.* 2008) (Fig. 1). Larvae become infected with baculovirus when they feed on plants contaminated with viral occlusion bodies (OBs) liberated from infected cadavers at death. In late-season outbreaks, larval densities may reach 1000 per m^2 and more than 98% of larvae may die of SpexNPV infection, though much lower larval densities and viral prevalence are more common and there is considerable spatiotemporal variation (Brown & Swaine 1965; Rose *et al.* 2000). Current research is evaluating the potential of SpexNPV for use as a biological control agent against African armyworm in Tanzania and field trials show that when applied at 1×10^{12} OB per ha, mortalities in excess of 80% may be achieved (Grzywacz *et al.* 2008).

Wolbachia was recently detected in *S. exempta* larvae in natural outbreaks in Tanzania, providing an opportunity to test the hypothesis that a natural *Wolbachia* infection provides protection against an endemic baculovirus, both in the laboratory and in a natural field setting. These analyses revealed that in stark contrast to many previous

studies, *Wolbachia* infection in *S. exempta* substantially increases susceptibility to viral infection rather than providing protection. This novel finding has important implications for increasing our understanding of the interaction between symbiotic bacteria and viruses, and may have particular relevance to the integrated biological control of armyworm and other lepidopteran pests with insect pathogens.

MATERIALS AND METHODS

Field sampling

59 larval *S.exempta* outbreaks were visited across three field seasons (2007/08, n = 21; 2009/10, n = 20; and 2011/12, n = 18) covering *c.* 300,000 km^2 of Tanzania. At each outbreak, 30 standardized counts were conducted to determine the densities of live and SpexNPV-killed larvae. Where dead larvae were present, <30 cadavers were collected in individual micro-tubes and air-dried overnight before storage at -20^oC. In addition, 30+ live larvae were collected in individual micro-tubes and stored in 100% ethanol for determining *Wolbachia*-infection status using PCR; SpexNPV viral load using qPCR; and total bacterial community using DGGE. Larval stage was determined by measuring head-capsule width (Rose *et al.* 2000) - based on head-capsule data, most larvae were in the 4^{th} – 6^{th} instar (10 – 20 days old) at the time of sampling. Sample sizes vary between analyses where larval counts and/or sampling were not possible at some outbreaks. Adult males were caught throughout each season via a network of 14 pheromone traps across Tanzania. Trap operators collected specimens daily and stored moths in individual micro-tubes in 100% ethanol.

Diagnosis of *Wolbachia*

Genomic DNA was extracted from individual armyworm using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA was PCR screened for presence of *Wolbachia* using the diagnostic *wsp* primer set 81F and 691R (Zhou *et al.* 1998). Further analysis of *Wolbachia* strains was undertaken using MLST gene sequencing (Baldo *et al.* 2006). Reaction mixtures (50 µl) contained PCR buffers (10 mM Tris-HCl pH 8.3 at 25 °C; 50 mM KCl; 1.5 mM MgCl; 0.001% gelatin), 5 mM each of dATP, dTTP, dCTP and dGTP, 10 mM of the relevant primers, 1 unit Taq polymerase (Qiagen) and approximately 5 ng DNA template. PCR was carried out in a Techne TC-512 thermal cycler (Bibby Scientific Ltd., Stone, UK), using the following reaction conditions: (i) 94 °C for 5 min, 1 cycle; (ii) 94 °C for 30s, 52 °C for 30s, 72 °C for 30s, 40 cycles; and (iii) 72 °C for 5 min, 1 cycle. PCR amplicons were purified (PCR Purification Kit, Qiagen), and sequenced (Source Bioscience, UK). Sequence data are archived at the following databases: Genbank, accession numbers JN656938 to JN656955; Wolbachia MLST Database, isolate identity no. 322, 323 and 324.

Screen for correlating microorganisms

To test for the presence of other microorganisms, PCR and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was undertaken on a sub-sample of field-collected *S. exempta* larvae, including *Wolbachia*-infected samples. Results indicated near identical banding patterns in all larvae, suggesting similar bacterial communities in *Wolbachia*-free and –infected larvae (Table S8).

Quantifying covert virus infection

Taqman qPCR was used to quantify covert NPV infection within individual samples of field-collected larvae. Briefly, a 62 bp portion of the *polyhedrin* protein gene was amplified, whereby 5µl of genomic DNA was used as template in 25 µl reactions containing 12.5 ul 2 x TaqMan® Universal PCR Master Mix, 0.4 µM each of primers P1 and P2, and probe (Applied Biosystems). The forward primer CCC GTG TAC GTA GGA AAC AAC A, reverse primer CAA CCG CCG CCC TTC T and probe 6FAM-CGA GTA CCG CAT CAG CCT GGC C-TAMRA. Reactions were run on a Prism 7000 Real-Time Cycler (Applied Biosystems) in triplicate, using a 2 step PCR method consisting of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. For each qPCR assay, a standard curve was constructed and negative controls (water instead of template DNA) were included in all reactions. 10-fold serial dilutions of viral genomes were made in water, and each dilution was processed in triplicate on the same 96-well PCR plate with the samples. Data were analyzed using Sequence Detection Software (1.2.3) 7000 (Applied Biosystems). SpexNPV viral loads are reported as number of viral genomes per µg of total DNA.

Laboratory insect and virus cultures

An *S. exempta* culture was established from larvae collected in Tanzania in January 2011. All larvae were maintained on standard artificial diet (Reeson *et al.* 1998; Vilaplana *et al.* 2009) at 25°C and 12/12 light/dark cycle. *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) was collected from a single larval cadaver in central Tanzania in 2008 and isolated using standard centrifugation techniques, as previously

described (Hunter-Fujita *et al.* 1998). The concentration of SpexNPV occlusion bodies (OBs) of each virus preparation was measured using a Neubauer Improved haemocytometer, with replicated samples taken at two dilutions.

Tetracycline treatment

A proportion of all *Wolbachia*-infected larvae were treated with 0.03% tetracycline (10 mg/ml) to generate uninfected lines (Kageyama *et al.* 2002). Following treatment, larvae were maintained for at least a further generation to recover before experimental use. All tetracycline-treated lines were tested with *Wolbachia*-specific PCR to identify *Wolbachia* infection. Only clean uninfected lines were used in subsequent experiments, and labelled $wExe1_{tet}$.

Baculovirus bioassays

For virus survival assays, small diet-plugs (1 mm^3) were inoculated with 0, 100, 500, 1000 and (for Bioassay 2 only) 5000 SpexNPV OB. Newly-moulted 4^{th}-instar larvae were allowed to ingest the diet-plug (n = 25 larvae per OB concentration). After 24h, larvae that had eaten all the diet-plug and virus were transferred to individual polypots (30 ml) containing artificial diet. Larvae were kept at 24°C and checked every 12h thereafter for viral mortality, until death or pupation, excluding larvae that died during the first 24h due to handling mortality. Cadavers were transferred to 1.5 ml micro-tubes and kept at -20°C for later yield analysis. There is no evidence for sex-biased NPV mortality in this species ($X^2_1 = 0.85$, P = 0.36; Vilaplana *et al.* 2009). For Bioassays 1 and 3, *wExe1*-infected and $wExe1_{tet}$ lines from the same genetic stock were used, at generations 2 and 3, respectively, following tetracycline treatment. Insects used in

Bioassay 2 were derived from a genetically-different stock and the *Wolbachia*-free insects were naturally free of infection.

Statistical analysis

All statistical analyses for conducted using S-PLUS® 8.0. For most analyses, we used generalized linear models (GLM) with appropriate error distributions and link functions (see Supplementary Tables for details; Aitkin *et al.* 1989). For the survival analysis, we used a Cox's proportional hazards model, and survival curves were visualized used Kaplan-Meier. Reported statistics are for minimal models following model simplification, such that all terms in the model caused a significant reduction in model residual deviance and no additional terms significantly improved the fit of the model.

RESULTS

Baculovirus prevalence and intensity within field populations

Larval densities at outbreaks ranged between 5.6 and 234.9 larvae per m^2 (mean $\pm$ s.d. = 77.2 $\pm$ 63.4, n = 57 sites) and overt baculovirus disease was observed in 21% (12/58) of outbreaks visited, with up to 16.5% of larvae exhibiting symptoms of lethal baculovirus infection (later verified by PCR). In contrast, SpexNPV-specific qPCR revealed that apparently healthy larvae carrying sub-lethal or 'covert' SpexNPV infections were present at all outbreaks (n = 59), with 97% of 1,105 larvae testing positive for SpexNPV. RT-PCR on a random sub-sample of larvae (n = 96) showed that in each case the virus was actively transcribing (see also Vilaplana *et al.* 2009). Moreover, PCR

revealed that 100% of adult males from pheromone traps were infected with SpexNPV (n = 334).

Wolbachia identification and prevalence within field populations

Wolbachia infection was observed in nearly three-quarters of all sites visited (43/59, 72.9%), with up to 56% of larvae in each outbreak being infected (mean % $\pm$ s.d. = 11.3 $\pm$ 12.0). Three strains of *Wolbachia* were identified by PCR, which we refer to as strains *wExe1*, *wExe2* and *wExe3*. Based on MLST classification, strain *wExe1* shows an exact match at all loci to ST-125 (*Wolbachia* supergroup B), a male-killing phenotype found in the Nymphalid blue moon butterfly, *Hypolimnas bolina* (Dyson *et al.* 2002). The other two strains of *Wolbachia* are new sequence-types, assigned ST-222 (*wExe2* – supergroup B) and ST-223 (*wExe3* – supergroup A) (Graham & Wilson, submitted). Strains *wExe1*, *wExe2* and *wExe3* were observed in 23, 18 and 19 of the 59 outbreaks (39%, 31% and 32%), respectively, and there was no evidence for non-random association of *Wolbachia* strains (X^2_{58} = 48.25, P = 0.82). *Wolbachia* was not detected in any of the male moths tested (n = 334).

Covariation of *Wolbachia* and baculovirus within field populations

The prevalence of overt viral deaths across outbreaks varied significantly between seasons and increased with both mean larval stage and *Wolbachia* prevalence (GLM: Season: $F_{1,47}$ = 13.01, P = 0.0007; Head-capsule width (larval stage): $F_{1,47}$ = 14.23, P = 0.0004; *Wolbachia*: $F_{1,47}$ = 12.01, P = 0.0011; $\Delta\text{dev}_{\text{full model}}$ = 63.2%, $\Delta\text{dev}_{\textit{Wolbachia}}$ = 13.8%; Fig. 2a and Table S1).

To determine whether all three strains of *Wolbachia* had similar relationships with viral prevalence, we tested each strain separately and in combination. After

accounting for the effects of mean larval stage and season, two of the three strains exhibited significant positive associations with the prevalence of virus deaths when tested separately (GLM: *wExe1*: $F_{1,47}$ = 7.77, P = 0.0076; *wExe2*: $F_{1,47}$ = 8.08, P = 0.0066; *wExe3*: $F_{1,47}$ = 0.01, P = 0.91; Table S2a). Moreover, consistent with all three *Wolbachia* strains having independent positive influences on host susceptibility to baculovirus mortality, when all three strains were included in the same model, *Wolbachia* accounted for 40% of the explainable variation in SpexNPV prevalence across outbreaks (GLM: $\Delta dev_{\text{full model}}$ = 82.1%, $\Delta dev_{Wolbachia}$ = 32.8%; *wExe1*: $F_{1,45}$ = 15.84, P = 0.0002; *wExe2*: $F_{1,45}$ = 9.45, P = 0.0036; *wExe3*: $F_{1,45}$ = 4.10, P = 0.049; Table S2b). The additive effect of the three strains is reflected in a positive relationship between the number of *Wolbachia* strains within an armyworm outbreak and the prevalence of SpexNPV deaths (GLM: $F_{1,47}$ = 60.61, $P < 0.0001$; $\Delta dev_{\text{full model}}$ = 83.4%, $\Delta dev_{Wolbachia}$ = 34.0%; Table S2c), probably because the number of *Wolbachia* strains at an outbreak reflects the overall prevalence of *Wolbachia* infection (GLM: $F_{1,57}$ = 26.11, $p < 0.0001$).

The prevalence of viral deaths is a relatively crude measure of the amount of virus present in a population. To determine if *Wolbachia* affects the *intensity* of viral infections in larvae carrying sub-lethal infections or those that have yet to die of a lethal infection, we examined variation in mean viral load of apparently healthy larvae, using SpexNPV-specific quantitative PCR. Mean viral load increased as the number of armyworm outbreaks increased during the season and decreased with increasing larval density (GLM: Outbreak number: $F_{1,52}$ = 12.97, P = 0.0021; Larval density: $F_{1,52}$ = 6.49, P = 0.014; Table S3). In addition to these significant main effects, there was also a highly significant interaction between *Wolbachia* prevalence and outbreak number,

i.e. the number of previous outbreaks reported that season (GLM: Outbreak number x *Wolbachia* interaction: $F_{1,52} = 9.66$, $P = 0.0024$; *Wolbachia* main effect: $F_{1,52} = 6.10$, $P = 0.031$; *Wolbachia* main effect and interaction terms combined: $F_{2,54} = 7.31$, $P = 0.0016$; $\Delta\text{dev}_{\text{full model}} = 39.0\%$, $\Delta\text{dev}_{\textit{Wolbachia}} = 19.1\%$). Thus, late-season outbreaks with a high prevalence of *Wolbachia* had highest mean viral loads (Fig. 3).

Laboratory baculovirus bioassays

Field data provide strong circumstantial evidence that *Wolbachia* infection increases the susceptibility of *S. exempta* to baculovirus infection. To test this experimentally, we established two lines of *wExe1*-infected *S. exempta* and two *wExe1*-free lines, one generated by tetracycline treatment (*wExe1*$_{tet}$) and one that was naturally free of *wExe1*. Breeding studies established that, like ST-125 isolated from *H. bolina*, ST-125 (*wExe1*) from *S. exempta* is also a male-killer: sex ratios - *wExe1*♀ x *wExe1*$_{tet}$♂ = 100% female offspring (n = 41 pairs); *wExe1*$_{tet}$ ♀ x *wExe1*$_{tet}$ ♂ = 51% female (n = 22 pairs).

Mortality of 4th-instar *S. exempta* larvae from *Wolbachia*-infected (Wol+) and *Wolbachia*-free (Wol-) lines was determined in three separate viral bioassays following oral inoculation with a range of doses of SpexNPV. Virus-induced mortality varied between the three bioassays and model simplification indicated that mortality profiles in Bioassays 1 and 3 were significantly different from that in Bioassay 2 (GLM: Bioassay: $\chi^2_1 = 24.22$, $P < 0.0001$), but the effects of viral dose and *Wolbachia* status were consistent across bioassays, as indicated by a non-significant interaction term ($\chi^2_1 < 1.37$, $P > 0.31$). After accounting for bioassay variation, mortality was found to be positively dose-dependent ($\chi^2_1 = 133.34$, $P < 0.0001$) and markedly higher for insects from *Wolbachia*-infected lines than those from lines that were *Wolbachia*-free ($\chi^2_1 = 58.81$, $P < 0.0001$; $\Delta\text{dev}_{\text{full model}} = 31.4\%$, $\Delta\text{dev}_{\textit{Wolbachia}} = 8.2\%$; Table S4). Across the

three bioassays, LD_{50} values for the *Wolbachia*-free insects were 6 - 14 times greater than those that were infected with *Wolbachia* (Fig. 4a, Table S5).

Survival analysis indicated that after accounting for significant variation between bioassays and due to NPV dose (Cox's proportional hazards model: Bioassay: $\chi^2_1 = 26.0$, $P < 0.0001$, Virus dose: $\chi^2_1 = 71.67$, $P < 0.0001$), larvae from Wol- lines had significantly higher survival rates than those from Wol+ lines (*Wolbachia*: $\chi^2_1 = 61.31$, $P < 0.0001$; full model: $\chi^2_3 = 173$, $P < 0.0001$; $r^2 = 0.29$; Fig. 4b; Table S6). The effect of *Wolbachia* on susceptibility to NPV is such that the average daily mortality risk for Wol+ larvae was on average 3.4 times greater than that for Wol- larvae (95% confidence interval = 2.5 – 4.7), and the survival curves of Wol+ larvae were comparable to those of Wol- larvae dosed with approximately 5 times the number of viral occlusion bodies (Fig. S1).

For SpexNPV-killed larvae, the number of occlusion bodies (OB) produced at death (virus yield) was significantly negatively correlated with the speed of kill (GLM: $F_{1,177} = 16.16$, $P < 0.0001$; Table S7), consistent with a virus trade-off between virulence (speed of kill) and reproductive rate (OB yield), as observed in some other baculovirus systems. Interestingly, virus yield was significantly lower (by 15 – 31%) in larvae from *Wolbachia*-infected lines than in those that were *Wolbachia*-free (GLM: *Wolbachia:* $F_{1,177} = 8.10$, $P = 0.005$; $\Delta\text{dev}_{\text{full model}} = 13.2\%$, $\Delta\text{dev}_{\textit{Wolbachia}} = 4.2\%$; Table S7). However, there was no significant interaction between these two main effects, indicating that for a given speed of kill, there was a relatively constant reduction in virus yield for *Wolbachia*-infected insects of approximately 1.73 million OB per larva. Combining the effects of *Wolbachia* infection on SpexNPV mortality rate and OB yield indicated that infection with the symbiont increased the (laboratory) basic reproductive

rate of the virus, R_0, by 1.05 – 4.40 times, with the magnitude of the *Wolbachia* effect appearing to decline with increasing virus dose (see legend to Table S7).

DISCUSSION

There is a growing literature on the capacity of *Wolbachia* to protect host insects from viral infections (e.g. Hedges *et al.* 2008; Teixeira *et al.* 2008; Moreira *et al.* 2009; Osborne *et al.* 2009; Bian *et al.* 2010; Glaser & Meola 2010; Jaenike *et al.* 2011). In contrast, our field and laboratory analyses suggest that *Wolbachia* in the African armyworm, *S. exempta*, greatly *increases* its susceptibility to baculovirus infection. To our knowledge, this is the first time that an increase in viral susceptibility in *Wolbachia*-infected insects has been reported. As yet, the reason for these contrary results is not known but there several possibilities. First, although the virus protective effect of some *Wolbachia* strains (including *w*Mel, *w*MelCS, *w*Melpop, *w*Au, *w*Ri, *w*Inn and *w*AlbB) is reasonably well established, the evidence base is limited to a rather restricted pool of dipteran host species (including *Drosophila melanogaster*, *D. simulans, D. innubila*, *Aedes aegypti* and *Culex quinquefasciatus*), viral species (mostly positive sense ssRNA viruses, including dengue virus, *Drosophila* C virus, West Nile virus, Chikungunya virus, cricket paralysis virus, flock house virus and Nora virus), and *Wolbachia* strains (principally those expressing cytoplasmic incompatibility, CI, phenotypes). Conversely, our study involved a lepidopteran, a dsDNA virus and a male-killing *Wolbachia* strain. Thus, it is possible that across a wider spectrum of species interactions, a broader range of outcomes will emerge, from *Wolbachia* being protective through neutral to antagonistic, as is seen for other multi-species interactions (e.g. Begon *et al.* 2005).

However, it is worth noting that similar protective effects to those seen in *Wolbachia* studies have also been observed in interactions involving other symbionts (e.g. *Spiroplasma spp.*, *Regella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica*) and other natural enemies (including fungi, bacteria, filarial nematodes, protozoa, predators and parasitoids) (e.g. Oliver *et al.* 2003; Scarborough *et al.* 2005; Moreira *et al.* 2009; Vorburger *et al.* 2009; Kambris *et al.* 2010; Jaenike *et al.* 2011).

A second reason for our contrary results could be due the natural history of the interaction between *S. exempta*, SpexNPV and *Wolbachia wExe*, which is very different from most of the host-virus-symbiont interactions studied previously, both theoretically and empirically, in that SpexNPV is naturally found in a covert form in nearly all *S. exempta* larvae (97%, n = 1105) and all adult males (100%, n = 334) tested (see also Vilaplana *et al.* 2009). This complicates the nature of the interaction between the *Wolbachia* and the virus because although both microbes are obligate intra-cellular residents of their hosts, within host species the bacterium is almost exclusively vertically-transmitted between females and their offspring, whilst the virus may be transmitted between hosts via both horizontal transmission between larvae of the same cohort via ingestion of occluded virus (Brown & Swaine 1965; Vilaplana *et al.* 2008) and vertical transmission between parents of both sexes and their offspring (e.g. Vilaplana *et al.* 2008). The conditions favouring the evolution of symbiont-mediated host protection from natural enemies has been modelled in a number of recent studies (Fenton *et al.* 2011; Jones *et al.* 2011). Fenton *et al.* (2011) argue that invasion of (CI phenotype) *Wolbachia* may be *increased* by their protective effects against rapidly transmitted, highly pathogenic natural enemies, whereas asymptomatic covert viral infections may *reduce* the occurrence of antiviral *Wolbachia* strains. Importantly, at

different times in its seasonal cycle, SpexNPV may manifest both of these key features (i.e. highly virulent horizontal transmission and asymptomatic covert infections), suggesting that there may be oscillating selection pressures depending on the dominant form of baculovirus transmission, which is itself a function of larval density and the capacity for horizontal transmission of virus. Tellingly, Fenton *et al.* (2011) suggest that 'the precise outcome of interaction between a given pathogen and a given *Wolbachia* strain over both ecological and evolutionary time scales will be highly dependent on the specific life-history traits of the species involved'. None of the models to date have explicitly considered a scenario in which *Wolbachia* enhances viral mortality, the virus is both vertically and horizontally transmitted, and the bacterium exhibits a male-killing phenotype. Thus, the eco-evolutionary dynamics of the interaction remain to be explored.

A third possible explanation for why our results differ from most previous studies is simply that they are due to some sampling bias or artefact. For example, our field analyses are based on samples collected from outbreaks at a single point in time and so could be open to systematic temporal biases. Whilst it is true that cross-sectional sampling of disease epizootics provides only a snapshot of the epidemiological picture, it is most likely that rather than generate spurious trends, variation in point of sampling will *mask* associations between *Wolbachia* and SpexNPV infections by adding demographic 'noise'. By measuring head-capsule width of a random sample of larvae at every outbreak, we were able to generate an age-profile for each population at the time of sampling, which could then be included in the statistical analyses to partially control for the temporal dynamics of the epizootic. Moreover, by analysing both 'overt' viral mortality and 'covert' viral intensity, we were able to account for both lethal and sub-

lethal variation in the population viral load. We therefore think it is unlikely that our novel field results are due to sampling biases. In addition, the laboratory bioassays of *Wolbachia*-infected insects, *Wolbachia*-free insect lines, and lines experimentally cleared of infection with antibiotics verified the results from the field; it is highly unlikely that the magnitude of the effects seen (6 – 14 fold increase in viral susceptibility) could be due to experimental or sampling error. We therefore believe that these results are robust.

An unusual feature of the SpexNPV - *S. exempta* - *Wolbachia* interaction is that it involves the coexistence of at least three strains of *Wolbachia*. The coexistence of multiple strains of *Wolbachia* in a single population has been reported previously (e.g. Duron *et al.* 2011) but not, as far as we are aware, in the context of symbiont-mediated interactions with natural enemies. We do not yet know the phenotypes of all three *Wolbachia* strains present in armyworm populations, however one of them has been identified as a male-killer, and the absence of *Wolbachia* from male moths collected from a network of pheromone traps in Tanzania would seem to suggest that CI is probably not relevant in this system. We found no evidence for non-random association of the three *Wolbachia* strains across populations and all three appear to have independent and additive effects on host susceptibility to baculovirus. The mechanisms maintaining multiple *Wolbachia* strains in this population are not yet known.

Field data indicated that the prevalence of baculovirus deaths in *S. exempta* increased from less than 1% in *Wolbachia*-free outbreaks to around 13% in the outbreak with the highest prevalence of *Wolbachia*, with the bacterium accounting for more than one-fifth of the explainable variation in SpexNPV overt disease. Thus, even in populations in which the symbiont was prevalent, viral mortality was not high, though it

was at a level that is consistent with previous longitudinal-sampling studies in Tanzania (Rose *et al.* 2000). In contrast, some previous studies have found that armyworm mortality due to baculovirus is often considerably greater, sometimes resulting in > 98% larval mortality (Brown & Swaine 1965; Rose *et al.* 2000). The difference is most likely because these other studies sampled outbreaks later in the armyworm seasonal cycle, towards the end of the rainy season in neighbouring Kenya, after there had been more time for horizontal transmission of virus to occur. Consistent with this hypothesis, the qPCR results show a seasonal increase in the intensity of covert virus infection in Tanzania during the generations preceding their migration northwards into Kenya, and this increase is modulated by the presence of *Wolbachia*. Indeed, *Wolbachia* prevalence accounted for about half of the explainable variation in SpexNPV intensity across outbreaks. Thus, had we been able to follow the seasonal progression of outbreaks beyond Tanzania into Kenya, we would probably have seen much higher prevalence of viral disease.

Over the last decade, we have been exploring the potential for SpexNPV to be used as a microbial biopesticide in eastern Africa (Parnell & Dewhurst 1999; Grzywacz et al. 2008). Laboratory and large-scale field trials have demonstrated its efficacy and safety, and a large biopesticide production facility was constructed in Arusha, northern Tanzania, in 2011. Thus, the observation that *Wolbachia* enhances the susceptibility of *S. exempta* to SpexNPV has a number of potentially important implications for both our understanding and exploitation of SpexNPV as a microbial biopesticide. First, it may explain some of the inconsistent results from field trials using low doses of SpexNPV (i.e. 1×10^{11} OB per ha), in which mortality was just 22% in a large-scale field trial in M'ringa, northern Tanzania, yet was around 90% in a replicated field trial conducted

just 15 km away in Ikiriguru (Parnell & Dewhurst 1999). It is possible that at least some of this difference in mortality rates was due to variation between outbreaks in the prevalence of *Wolbachia* infection. Second, it means that if there are predictable spatiotemporal patterns in the prevalence of *Wolbachia* across the range of armyworm outbreaks then this could be taken into account in their biological control using SpexNPV, with lower doses being used at times or places where *Wolbachia* levels are highest. Finally, it may be possible to manipulate *Wolbachia* levels in the wild so as to amplify the negative effects of SpexNPV on armyworms. The anti-viral protection (reduced susceptibility) associated with other *Wolbachia*-infection systems is being explored for future strategies to reduce insect-vectored human diseases, such as dengue, via the mass release of symbiont-infected insects (Hoffmann *et al.* 2011; Walker *et al.* 2011). A similar mass release programme for *Wolbachia*-infected armyworms could enhance efforts to use SpexNPV for their strategic biological control across Africa. However, considerably more research is required to explore the virus-endosymbiont-host interactions in a controlled environment before this could be considered.

In conclusion, our laboratory and field studies indicate that *Wolbachia* may increase the susceptibility of African armyworms to its endemic baculovirus by 6 - 14 times. It remains to be seen whether this phenomenon is observed in other Lepidoptera-baculovirus-*Wolbachia* interactions. However, baculoviruses are currently being used, or developed, for the integrated biological control of a number of globally important lepidopteran pests of major economic importance, including cotton bollworm (*Helicoverpa armigera*), diamond-back moth (*Plutella xylostella*), Egyptian cotton leafworm (*S. littoralis*) and beet armyworm (*S. exigua*). Thus, our findings suggest a possible novel approach for exploiting bacterial endosymbionts to enhance the impact

of both endemic baculoviruses and microbial biopesticides so as to achieve more effective integrated pest control. A number of these species are already known to harbour *Wolbachia* infections (e.g. *S. littoralis* and *P. xylostella*), but the impact of the symbionts on susceptibility to baculoviruses is not yet known. For those species that do not naturally harbour *Wolbachia* infections, transinfection of *wExe1* or other suitable strains is a possibility, as has been achieved in mosquitoes (Walker *et al.* 2011).

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REFERENCES

1.

Aitkin M., Anderson D., Francis B. & Hinde J. (1989). *Statistical Modelling in GLIM*. Oxford University Press, Oxford, UK.

2.

Baldo L., Hotopp J.C.D., Jolley K.A., Bordenstein S.R., Biber S.A., Choudhury R.R., *et al.* (2006). Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Appl. Environ. Microbiol.*, **72**, 7098-7110.

3.

Begon M., Townsend C.R. & Harper J.L. (2005). *Ecology: From Individuals to Ecosystems*. Wiley-Blackwell, Oxford, UK.

4.

Bian G., Xu Y., Lu P., Xie Y. & Xi Z. (2010). The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Path.*, **6**.

5.

Brown E.S. & Swaine G. (1965). Virus disease of African armyworm *Spodoptera exempta* (Wlk). *Bull. Entomol. Res.*, **56**, 95-116.

6.

Cherry A.J. (1992). Cross-infectivity of *Spodoptera exempta* nuclear polyhedrosis virus (SeNPV) and the infectivity of foreign viruses in *S. exempta*. In: *Project Technical Report. Project A0047*. Natural Resources Institute Chatham UK, pp. 1-6.

7.

Duron O., Raymond M. & Weill M. (2011). Many compatible *Wolbachia* strains coexist within natural populations of *Culex pipiens* mosquito. *Heredity*, **106**, 986-993.

8.

Dyson E.A., Kamath M.K. & Hurst G.D.D. (2002). *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity*, **88**, 166-171.

9.

Engelstaedter J. & Hurst G.D.D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. S.*, **40**, 127-149.

10.

Fenton A., Johnson K.N., Brownlie J.C. & Hurst G.D.D. (2011). Solving the *Wolbachia* paradox: Modeling the tripartite interaction between host, *Wolbachia*, and a natural enemy. *Am. Nat.*, **178**, 333-342.

11.

Fytrou A., Schofield P.G., Kraaijeveld A.R. & Hubbard S.F. (2006). *Wolbachia* infection suppresses both host defence and parasitoid counter-defence. *Proc. R. Soc. Lond. B*, **273**, 791-796.

12.

Glaser R.L. & Meola M.A. (2010). The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile Virus infection. *Plos One*, **5**.

13.

Graham, R.I. & Wilson, K. (submitted). Male-killing *Wolbachia* and mitochondrial selective sweep in a migratory African insect. *BMC Evolutionary Biology*.

14.

Grzywacz D., Mushobozi W.L., Parnell M., Jolliffe F. & Wilson K. (2008). Evaluation of *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) for the field control of African armyworm (*Spodoptera exempta*) in Tanzania. *Crop Protect.*, **27**, 17-24.

15.

Hedges L.M., Brownlie J.C., O'Neill S.L. & Johnson K.N. (2008). *Wolbachia* and virus protection in insects. *Science*, **322**, 702-702.

16.

Hilgenboecker K., Hammerstein P., Schlattmann P., Telschow A. & Werren J.H. (2008). How many species are infected with *Wolbachia*? - a statistical analysis of current data. *FEMS Microbiol. Lett.*, **281**, 215-220.

17.

Hoffmann A.A., Montgomery B.L., Popovici J., Iturbe-Ormaetxe I., Johnson P.H., Muzzi F., *et al.* (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, **476**, 454-U107.

18.

Hunter-Fujita F.R., Entwhistle P.F., Evans H.F. & Crook N.E. (1998). *Insect viruses and pest management* Wiley & Sons, Chichester, UK.

19.

Iturbe-Ormaetxe I., Walker T. & O'Neill S.L. (2011). *Wolbachia* and the biological control of mosquito-borne disease. *Embo Rep.*, **12**, 508-518.

20.

Jaenike J., Unckless R., Cockburn S.N., Boelio L.M. & Perlman S.J. (2011). Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science*, **329**, 212-215.

21.

Jones E.O., White A. & Boots M. (2011). The evolution of host protection by vertically transmitted parasites. *Proc. R. Soc. Lond. B*, **278**, 863-870.

22.

Kageyama D., Nishimura G., Hoshizaki S. & Ishikawa Y. (2002). Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (Lepidoptera : Crambidae). *Heredity*, **88**, 444-449.

23.

Kaltenpoth M., Gottler W., Herzner G. & Strohm E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Curr. Biol.*, **15**, 475-479.

24.

Kambris Z., Blagborough A.M., Pinto S.B., Blagrove M.S.C., Godfray H.C.J., Sinden R.E. & *et al.* (2010). *Wolbachia* stimulates immune gene expression and inhibits plasmodium development in *Anopheles gambiae*. *PLoS Path.*, **6**.

25.

Longdon B., Fabian D.K., Hurst G.D.D. & Jiggins F.M. (2012). Male-killing *Wolbachia* do not protect *Drosophila bifasciata* against viral infection. *BMC Microbiol.*, **12**, S8.

26.

Moreira L.A., Iturbe-Ormaetxe I., Jeffery J.A., Lu G., Pyke A.T., Hedges L.M., *et al.* (2009). A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and *Plasmodium*. *Cell*, **139**, 1268-1278.

27.

Oliver K.M., Russell J.A., Moran N.A. & Hunter M.S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100**, 1803-1807.

28.

Osborne S.E., Leong Y.S., O'Neill S.L. & Johnson K.N. (2009). Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. *PLoS Path.*, **5**, e1000656, DOI: 10.1371/journal.ppat.1000656.

29.

Parnell, M., & Dewhurst, C. (1999). Entomopathogenic baculoviruses for control of East African armyworm *Spodoptera exempta*, in Tanzania: field trials of *Spodoptera exempta* nucleopolyhedrovirus carried out against armyworm outbreaks on rangeland in Tanzania. In: *NRI Report No. 2457*. NRI, Chatham, UK. pp 32.

30.

Reeson A.F., Wilson K., Gunn A., Hails R.S. & Goulson D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proc. R. Soc. Lond. B*, **265**, 1787-1791.

31.

Rose D.J.W., Page W.W., Dewhurst C.F., Riley J.R., Reynolds D.T., Pedgley D.E. *et al.* (1985). Down-wind migration of the African armyworm moth, *Spodoptera exempta*, studied by mark-and capture and by radar. *Ecol. Entomol.*, **10**, 299-313.

32.

Rose D.J.W., Dewhurst C.F. & Page W.W. (2000). *The African Armyworm Handbook (2nd Edn)*. NRI, Chatham, UK.

33.

Scarborough C.L., Ferrari J. & Godfray H.C.J. (2005). Aphid protected from pathogen by endosymbiont. *Science*, **310**, 1781.

34.

Teixeira L., Ferreira A. & Ashburner M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.*, **6**, 2753-2763.

35.

Unckless R.L. & Jaenike J. (2012). Maintenance of a male-killing *Wolbachia* in *Drosophilla innubila* by malekilling dependent and male-killing independent mechanisms. *Evolution*, **66**, 678-689.

36.

Vilaplana L., Redman E.M., Wilson K. & Cory J.S. (2008). Density-related variation in vertical transmission of a virus in the African armyworm. *Oecologia*, **155**, 237-246.

37.

Vilaplana L., Wilson K., Redman E.M. & Cory J.S. (2009). Pathogen persistence in migratory insects: high levels of vertically-transmitted virus infection in field populations of the African armyworm. *Evol. Ecol.*, **24**, 147-160.

38.

Vorburger C., Sandrock C., Gouskov A., Castaneda L.E. & Ferrari J. (2009). Genotypic variation and the role of defensive endosymbionts in an all parthogenetic host-parasitoid interaction. *Evolution*, **63**, 1439-1450.

39.

Walker T., Johnson P.H., Moreira L.A., Iturbe-Ormaetxe I., Frentiu F.D., McMeniman C.J., *et al.* (2011). The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, **476**, 450-U101.

40.

Werren J.H. & Windsor D.M. (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B*, **267**, 1277-1285.

41.

Werren J.H., Baldo L. & Clark M.E. (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.*, **6**, 741-751.

42.

Zhou W.G., Rousset F. & O'Neill S. (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond. B.*, **265**, 509-515.

FIGURE LEGENDS

Figure 1. African armyworm and its baculovirus. (a) High-density outbreak of *S. exempta* larvae on *Cynodon dactylon* (photo W.L. Mushobozi). (b) A SpexNPV-infected larva exhibiting typical symptoms of baculovirus infection, historically referred to as 'wilting disease' (photo K. Wilson).

Figure 2. Relationship between SpexNPV disease and *Wolbachia* prevalence. Symbols are the fitted values for SpexNPV prevalence at each outbreak (Supplementary Table 1), with symbol size proportional to sampling effort. Solid black line is a smoothing spline through the fitted values. Note that this relationship remains highly significant even if the high-leverage data point on the right of the plot is omitted ($F_{1,46} = 8.55$, $P = 0.0053$).

Figure 3. Relationship between *Wolbachia* infection and SpexNPV viral load across the armyworm outbreak season. The contour plot illustrates how SpexNPV viral load increases as both *Wolbachia* prevalence increases and as the outbreak season progresses (Outbreak number). The contours and associated numbers show the fitted values from the model (Supplementary Table 3). As viral loads increase, so the colour of the contour shifts from yellow (low viral load) to red (high viral load). Crosses indicate the position of outbreaks in *Wolbachia* prevalence – Outbreak number space.

Figure 4. Relationship between *Wolbachia* infection and resistance to SpexNPV. (a) LD_{50} estimates (means $\pm$ s.e.m.) for three independent bioassays. Solid black bars are for larvae from *Wolbachia*-free (Wol-) lines and the red striped bars are for larvae from

Wolbachia-infected (Wol+) lines. Note that the y-axis is on the log scale. (b) Kaplan-Meier survivorship plot. Black lines are for larvae from Wol- lines and red lines are for larvae from Wol- lines, averaged across the three bioassays standardized for a viral dose of 1000 OB per larva. Thick lines are the mean survival curves and the thin lines are the 95% confidence intervals.

Figure 1.

(a)

(b)

Figure 2.

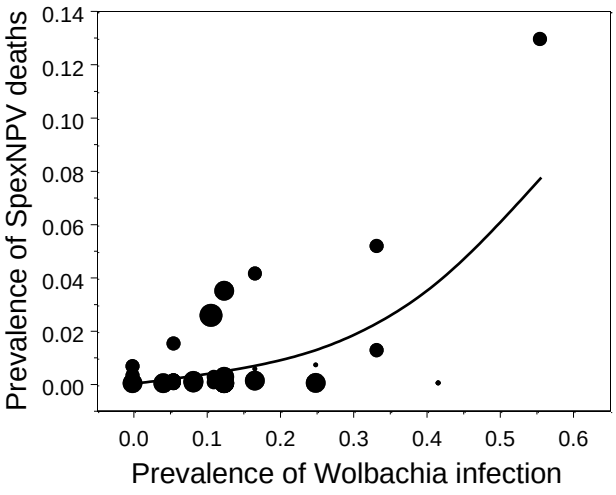

Prevalence of SpexNPV deaths
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Prevalence of Wolbachia infection

Figure 3.

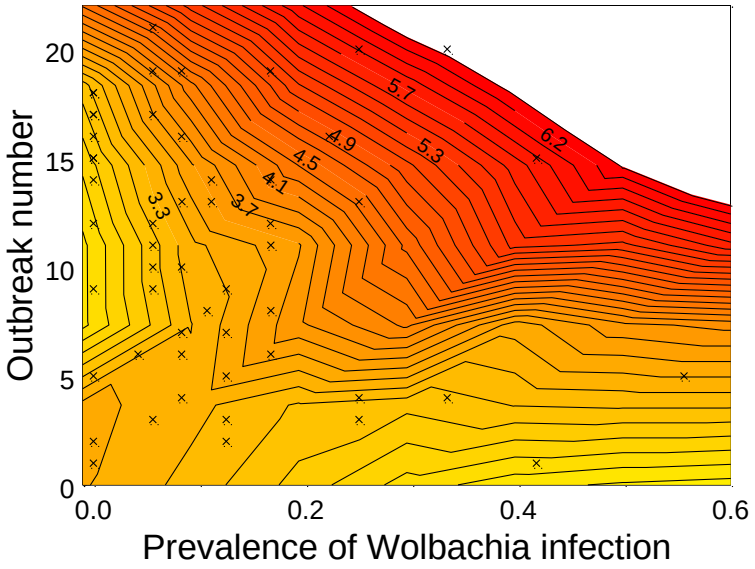
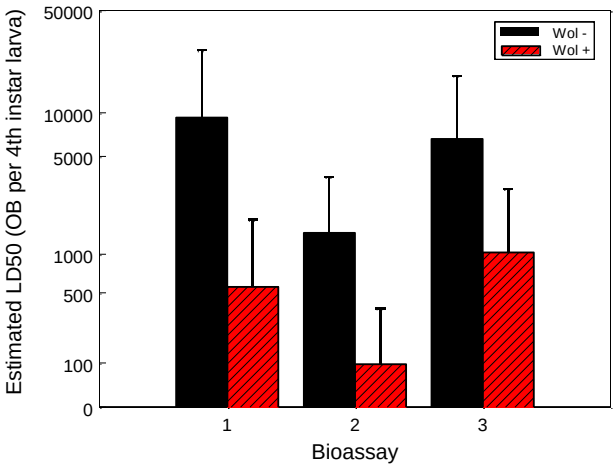
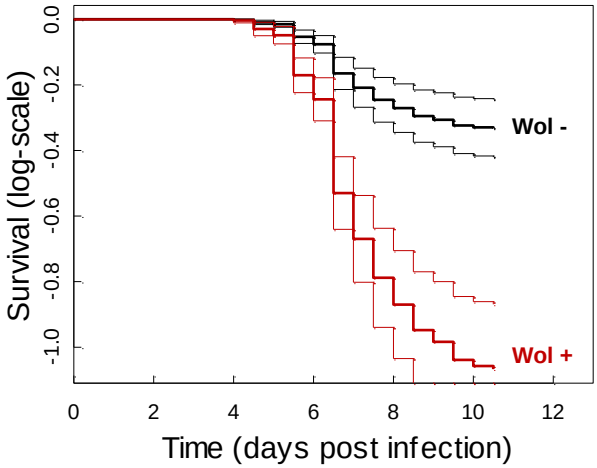

Outbreak number
0
5
10
15
20
0.0
0.2
0.4
0.6
Prevalence of Wolbachia infection
3.3
3.7
4.1
4.5
4.9
5.3
5.7
6.2

Figure 4.

(a)


Estimated LD50 (OB per 4th instar larva)
50000
10000
5000
1000
500
100
0
Wol -
Wol +
1
2
3
Bioassay

(b)


Survival (log-scale)
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Wol -
Wol +
0
2
4
6
8
10
12
Time (days post infection)

SUPPORTING INFORMATION

Additional Supporting Information may be downloaded via the online version of this article at Wiley Online Library (www.ecologyletters.com).

Table S1. Determinants of overt viral deaths in *S. exempta* across outbreaks.

Table S2. Determinants of overt viral deaths in *S. exempta* across outbreaks.

Table S3. Determinants of viral load in *S. exempta* across outbreaks.

Table S4. SpexNPV bioassay mortality analysis.

Table S5. SpexNPV bioassay summary data.

Table S6. SpexNPV bioassay survival analysis.

Table S7. Determinants of viral loads in bioassay experiments.

Table S8. Microbial species infecting *Spodoptera exempta* in outbreak populations.

Figure S1. Survival of *S. exempta* larvae from *Wolbachia*-infected (Wol+) and *Wolbachia*-free (Wol–) lines following inoculation with SpexNPV.