Bull Math Biol (2014) 76:2646–2663
DOI 10.1007/s11538-014-0029-5

Society for Mathematical Biology

ORIGINAL ARTICLE

New Insight into *Wolbachia* Epidemiology: Its Varying Incidence During the Host Life Cycle Can Alter Bacteria Spread

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Received: 11 February 2014 / Accepted: 11 September 2014 / Published online: 24 September 2014

Abstract *Wolbachia* is an obligate endosymbiont whose spread depends mainly on its capacity to alter host reproduction by, for instance, cytoplasmic incompatibility. Several mathematical models have been developed to explain the dynamics of bacterial spread, because of its applied interest. However, some aspects of the host's and bacterium's biology have not been considered in modelling: for instance, changes in *Wolbachia* proportions during the host's life cycle have been observed in several species, including *Drosophila* sp., *Nasonia* sp. and *Aedes* sp. (Diptera), but also in the grasshopper *Chorthippus parallelus* (Orthoptera), the species studied in this article. These changes influence the proportion of incompatible crosses and, consequently, infection prevalence in subsequent generations. In this paper, we are interested in ascertaining whether these changes in the infection proportions during the host's life cycle can influence the dynamics of the spread of these bacteria. We have examined its consequences using a mathematical model to predict the evolution of *Wolbachia* infection frequencies. The simulations were validated by experimental field data from

Electronic supplementary material The online version of this article (doi:10.1007/s11538-014-0029-5) contains supplementary material, which is available to authorized users.

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C. parallelus. The main outcome is that those changes above mentioned might affect long-term infection spread, with possible consequences for the current distribution of *Wolbachia* and the way it affects its host's reproduction.

Keywords *Wolbachia* · Modelling · Life cycle infection proportion variation · *Chorthippus parallelus*

1 Introduction

Wolbachia (Rickettsiales) is one of the most widely studied endosymbionts. It is a widespread intracellular alpha-protobacterium that infects mainly the reproductive tissues of 20–76 % species of Arthropoda, including insects (Hilgenboecker et al. 2008; Zug and Hammerstein 2012). Several theoretical and applied studies focus on modelling the spread of *Wolbachia* infection in its host population. This can be useful in *disease control* strategies using *Wolbachia*, like malaria or dengue control programs (Hoffmann et al. 2011; Killen et al. 2013).

Mathematical models predict variation in *Wolbachia* infection frequencies from one generation to another based on transmission rate, female fecundity and the degree of cytoplasmic incompatibility (CI) induced (Dobson 2004; Egas et al. 2002; Engelstaedter et al. 2007; Frank 1998; Haygood and Turelli 2009; Turelli 1994; Vautrin et al. 2007; Vavre et al. 2003). *Wolbachia* are vertically transmitted from mother to descendants through the egg cytoplasm (maternal inheritance). However, transmission rates are typically lower in natural populations than in laboratory (Narita et al. 2007; Poinsot et al. 2000; Rasgon and Scott 2003). Host fitness (expressed as change in fecundity) may also be modified by this endosymbiont (Serbus et al. 2008).

Wolbachia induces reproductive alterations, including male feminisation, parthenogenesis, male-killing and the above-cited CI (Serbus et al. 2008; Werren 1997). CI is a form of conditional sterility resulting in embryonic lethality in crosses between infected males and uninfected females (unidirectional CI, expressed as H_{UNI} in this paper; Table 1) (Callaini et al. 1997; Atyame et al. 2014). If two or more strains of *Wolbachia* infect a host population, different incompatibility patterns may be expressed in crosses between infected females and males harbouring different strains of *Wolbachia* (bidirectional CI, expressed as H_{BI} in this paper; Table 1). (Serbus et al. 2008).

Infected females may have higher reproductive success than uninfected ones, since they are compatible with infected and uninfected males, facilitating *Wolbachia* spread under unidirectional CI. In addition, under bidirectional CI, females infected by all strains of *Wolbachia* that appear in a population have the highest reproductive success, since coinfection makes females presumably compatible with any male. The advantage is frequency dependent, whereby with a high coinfection frequency, a large proportion of crosses involving uninfected females will be incompatible. The frequency-dependent advantage determines a frequency threshold that *Wolbachia* must exceed in order to spread through a population. So, if this threshold is passed, the coinfected proportion will gradually increase towards a stable equilibrium (Dobson 2004; Frank 1998; Hoffmann and Turelli 1997; Perrot-Minnot et al. 1996).

Current models consider *Wolbachia* proportions to be constant during the host's life cycle. However, changes in *Wolbachia* proportions over all developmental stages have been barely reported in *Drosophila* sp., *Nasonia* sp. and *Aedes* sp. (Diptera) and other species (Tortosa et al. 2010). Several biological explanations have been advanced to account for this (Kittayapong et al. 2002; Noda et al. 2001; Tortosa et al. 2010).

In this study, we consider whether the decrease in the number of infected individuals during the host's life cycle in a given population could produce a different pattern of coinfection spread from those predicted by previous theoretical studies. To this end, we develop a new model, based on the modified Turelli-like matrix model proposed by Vautrin et al. (2007), to investigate the possible effect of variation in infection proportions on the spread of *Wolbachia*.

We validate our theoretical model with new field data about *Wolbachia* infection in *Chorthippus parallelus* (Orthoptera). This organism is a good candidate with which to validate the model because our current experimental data show that coinfection in males decreases during the insect's life cycle. These organisms have an annual life cycle, appearing above ground at the beginning of the summer and dying by its end. Our results show that the dynamics of *Wolbachia* spread predicted by this modified model (considering the variation in proportions of *Wolbachia*) coincide with the observed proportions of F, B and coinfected individuals in the Iberian populations of *C. parallelus* (Bella et al. 2010; Dillon et al. 2008; Martínez et al. 2009; Zabal-Aguirre et al. 2010) in contrast to other European populations.

Finally, we briefly propose some biological explanations for the variation in infection proportions, including genetic factors and the influence of high temperatures detected in some field populations.

2 The Model

2.1 Differences from and Similarities to Previous Studies

Vautrin et al. (2007) re-wrote a previous general model, applicable to multiple species, to study the consequences of *Wolbachia* coinfections, including CI type influence and invasion threshold. Here, we explored the dynamics of *Wolbachia* in a modified "Vautrin-like model" (see details in Vautrin et al. 2007), including different incompatibility scenarios in diploid species, and a new factor, the variation in infection proportions (see Table 1 for parameter definitions).

2.2 Basic Model

As Vautrin's model suggests, initial *Wolbachia* proportions were expressed as a frequency vector (P_t). A simplified model was employed to simulate single infection, Eq. (1).

$$P_t = \left[P_{f,\emptyset},\, P_{f,F},\, P_{f,B},\, P_{f,FB},\, P_{m,\emptyset},\, P_{m,F},\, P_{m,B},\, P_{m,FB}\right]^t \tag{1}$$

where P stands for each frequency, the first subscript indicates sex (f, female; m, male), $[]^t$ indicates transposition and the second subscript indicates infection status

Table 1 Definitions of variable symbols

Parameter	Definition
$x = F$	Individuals infected by F strain
$x = B$	Individuals infected by B strain
$x = FB$	Individuals coinfected by B and F strains
$x = \emptyset$	Uninfected individuals
F_x	Fecundity of X-infected females relative to uninfected females, regardless of strain
μ_x	Proportion of uninfected eggs from an infected mother, regardless of strain X
H_x	Proportion of viable eggs in an incompatible cross affected by X strain
H_{UNI}	Proportion of viable eggs in a unidirectional incompatible cross
H_{BI}	Proportion of viable eggs in a bidirectional incompatible cross
ρ	CI type

(in our model, Ø, uninfected; F or B, infected by F or B strains; FB, coinfected individual).

As we considered only vertical transmission (from mothers to offspring) assuming a single, panmictic population, *Wolbachia* proportions in the next generation depend on two effects: the maternal effect, expressed in the M_{ct} matrix, and the paternal effect, expressed in the M_{ci} matrix.

The model had the following form (Eq. (2)):

$$P_{t+1} = (M_{\text{ct}} P_t) \cdot *(M_{\text{ci}} P_t) \cdot *W_t \tag{2}$$

where "·*" means component-wise multiplication, this is known as Hadamard's product of matrices. W_t serves to normalise the proportions.

Since *C. parallelus* is a diploid species, we take $\rho = -1$ and $Mmf = Mmm$ from the model by Vautrin et al. (2007). Consequently, the matrices Mct and Mci were written in terms of Eqs. (3, 4 and 5):

$$M_{\text{ct}} = \begin{bmatrix} M_f & 0 \\ M_f & 0 \end{bmatrix} \tag{3}$$

and

$$M_{\text{ci}} = \begin{bmatrix} 0 & M_{mf} \\ 0 & M_{mf} \end{bmatrix} \tag{4}$$

where

$$M_f = \begin{bmatrix} 1 & F_F\mu_F & F_B\mu_B & F_F F_B \mu_F \\ 0 & F_F(1-\mu_F) & 0 & F_F F_B (1-\mu_F)\mu_B \\ 0 & 0 & F_B(1-\mu_B) & F_F F_B \mu_F (1-\mu_B) \\ 0 & 0 & 0 & F_F F_B (1-\mu_F)(1-\mu_B) \end{bmatrix} \tag{5}$$

Mmf is given below depending on the incompatibility settings considered. Transmission and fecundity were assumed to be independent (i.e. $\mu_{FB} = \mu_F * \mu_B$) in

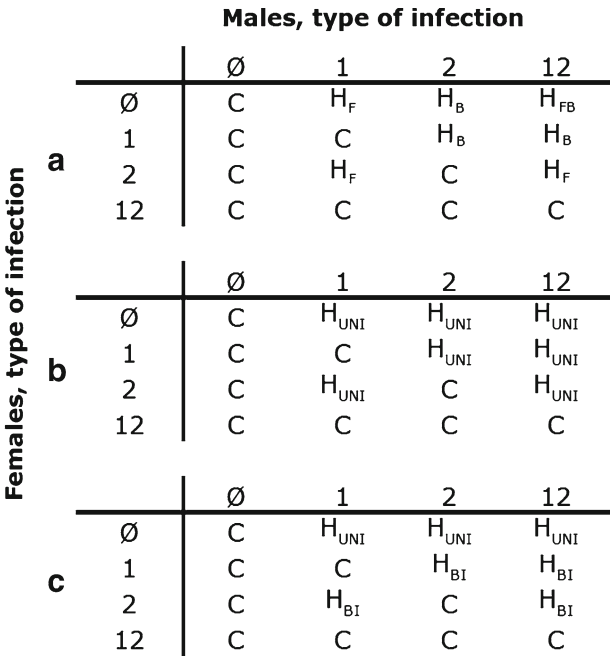

Fig. 1 Modifications affecting the M_{ci} matrix. C signifies a compatible cross. H_{UNI} represents the proportion of viable eggs in a unidirectional incompatible cross. H_{BI} represents the proportion of viable eggs in a bidirectional incompatible cross. Ø indicates uninfected individuals. F and B represent individuals infected by strain F or B, respectively. FB indicates individuals coinfected by the F and B strains. **a** M_{mf} following (Vautrin et al. 2007), **b** M^1_{mf}, **c** M^2_{mf}

all simulations. In addition, to simplify calculations, all simulations assumed a fixed value of $F_F = F_B = 1$.

2.2.1 Unidirectional Incompatibility

The CI level is usually defined as $(1 - H)$, where H is the proportion of viable eggs in an incompatible cross affected by *Wolbachia*. The simultaneous use of CI level and H terminology may be confusing, so we have opted to refer to "H" throughout this paper, on the assumption that the reader is familiar with the terminology.

At this point, we explore the different incompatibility settings. These modifications mostly affected the M_{ci} matrix (Fig. 1), so the Mmf matrix depended on the scenario under consideration.

The first model is a simplified one in which we assumed that H in all incompatible crosses was similar ($H_{UNI} = H_F = H_B = H_{FB}$) and that no partial rescue occurred between strains of *Wolbachia*. This means, for instance, that the F strain could not rescue the sperm modification caused by the B strain, in terms of the modified rescue model proposed for *Wolbachia* (Werren 1997).

The M_{ci} was modified in terms of Eq. (6).

$$M_{mf}^{1} = \begin{bmatrix} 1 & H_{\mathrm{UNI}} & H_{\mathrm{UNI}} & H_{\mathrm{UNI}} \\ 1 & 1 & H_{\mathrm{UNI}} & H_{\mathrm{UNI}} \\ 1 & H_{\mathrm{UNI}} & 1 & H_{\mathrm{UNI}} \\ 1 & 1 & 1 & 1 \end{bmatrix} \tag{6}$$

We do not know whether the interactions between strains in our host affect transmission or fecundity, so we maintained the M_{ct} matrix proposed by Vautrin et al. (2007).

2.2.2 Uni–Bidirectional Incompatibility

Experimental data from *C. parallelus* suggest that partial rescue happens between strains of *Wolbachia*, since the value of H_{BI} (0.83) is greater than that of H_{UNI} (0.67) (Bella et al. 2010; Zabal-Aguirre et al. 2014). This partial rescue can result in, for instance, more viable eggs from a cross between a coinfected male and an F-infected female than from one between a coinfected male and an uninfected female. This prompted us to test another model.

This second model assumes that $H_{\mathrm{UNI}} = H_F = H_B$. H_{UNI} was considered in crosses between single- or double-infected males and uninfected females. All experimental crosses involving different strains of *Wolbachia* were considered to be affected by H_{BI} (Fig. 1c).

Thus, the matrix M_{ci} is written in terms of Eq. (7).

$$M_{mf}^{2} = \begin{bmatrix} 1 & H_{\mathrm{UNI}} & H_{\mathrm{UNI}} & H_{\mathrm{UNI}} \\ 1 & 1 & H_{\mathrm{BI}} & H_{\mathrm{BI}} \\ 1 & H_{\mathrm{BI}} & 1 & H_{\mathrm{BI}} \\ 1 & 1 & 1 & 1 \end{bmatrix} \tag{7}$$

2.2.3 Evolution of Wolbachia Spread in Populations with Variation in the Proportions of Infection During the Host's Life cycle

We defined infection proportion variation as *infection frequency changes during the life cycle of the host*, in other words, during the same generation.

To simulate the monthly infection proportion variation and its consequences (see Fig. 2), we split a given generation, t, into three reproductive periods, corresponding to the three months July, August and September in accordance with the life cycle of *C. parallelus*. In each reproductive period, males were able to display a different infection frequency, as we know to be the case from experimental observations in several species (Bordenstein and Bordenstein 2011; Hurst et al. 2001; McGraw et al. 2002; Tortosa et al. 2010), including *C. parallelus* (see Supplemental Material). In the next generation, $t + 1$, individuals would be infected by *Wolbachia* to an extent related to the proportions of *Wolbachia* during each reproductive period and the proportion of incompatible crosses that occurred as a consequence.

We assumed an initial *Wolbachia* frequency for generation t of $P_{0,t}$ =July proportions. These individuals reproduced according to the model under consideration (see (1) and the modified M_{ci} matrices in the previous section). We saved the outcome of this reproduction in a new frequency vector, $P'_{0,t}$ (Eq. (8)).

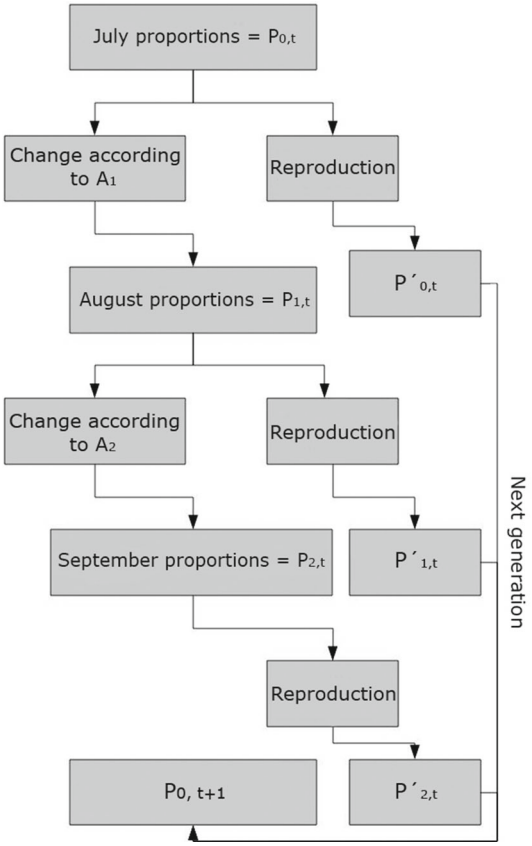

Fig. 2 Diagram of the modified model, including the variation in monthly infection proportions

$$P'_{0,t} = \left(M_{\mathrm{ct}} P_{0,t}\right) . * \left(M_{\mathrm{ci}} P_{0,t}\right) . * W_t \tag{8}$$

To simulate the observed reduction of coinfection frequency in males and the increase of uninfected ones, we introduced a Malthusian rule (Eqs. (9) and (10)). Different rates of change, expressed as a_0 and a_1, were tested and were in accordance with the experimental results obtained in this study.

$$P_{i+1,t} = A_i * P_{i,t} \tag{9}$$

where

$$A_i = \begin{bmatrix} 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 0 & 1 - a_i \\ 0 & 0 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & a_i \end{bmatrix} \tag{10}$$

This gave the proportions for August, $P_{1,t}$. These individuals reproduced following Eq. (8), giving rise to $P'_{1,t}$. Later, $P_{1,t}$ changes according to Eq. (9). In this way, we derived $P_{2,t}$, the proportions corresponding to September. These individuals reproduced and gave a third value, $P'_{2,t}$, which we used to obtain three frequency vectors, one for each month, for generation t. The *Wolbachia* infection frequency vector for the population in July of the subsequent year is given by Eq. (11):

$$P_{0,t+1} = \frac{1}{3}\sum_{i=0}^{2} P'_{i,t} \tag{11}$$

All simulations were carried out with MATLAB (2010; The MathWorks, Natick, MA). We simulate 4,000 generations, as suggested by previous studies.

2.2.4 *Progression of Infection Frequencies: Single Strain, with and without Intra-generational Variation*

Before we address the main question with two strains of *Wolbachia* and monthly infection proportions, we studied the (more academic) case for a single strain.

2.3 Progression of Infection Frequencies: Two Strains of *Wolbachia* and Coinfection, with and without Intra-generational Variation

Vautrin's original model assumed the two strains of *Wolbachia* to be independent, in the sense that they coexist within the host but do not interact (i.e. $H_{FB} = H_F * H_B$). The current data suggested that the CI expressed by a double-infected male and an uninfected female of *C. parallelus* could not be considered to be a product of the values of H (i.e. $H_{FB} \neq H_F * H_B$). We adapted the model to incorporate the estimate of H_{UNI} or H_{BI} obtained from field experiments in this species (Zabal-Aguirre et al. 2014).

The great complexity of the model and the large number of parameters estimated required us to calculate the final stable equilibrium, for females and males over a range of transmission rates and incompatibility levels. We examined a range between $\mu_F = \mu_B = 0.1$ and $\mu_F = \mu_B = 0$ (where $\mu = 0$ indicates perfect transmission, i.e. 100 % of offspring are infected) in accordance with values estimated from experimental data from several species (Turelli and Hoffmann 1995). These restricted values were also consistent with results from other current studies in *C. parallelus* (data not shown). Values of $H_F = H_B$ from 0 to 1 were tested, where $H_F = H_B = 0$ represents total incompatibility and $H_F = H_B = 1$ indicates no incompatibility.

We also tested different values for a_i of Eq. (10) to explore the influence of the rate of decrease of coinfection on the final outcome.

3 Modelling, Verification and Field Validation of Simulation Models

3.1 Field Studies: *Wolbachia* Infection Variation in *C. parallelus*

Navafria (NAV) and Sallent de Gállego (SAL) populations of *C. parallelus* (both sexes) (see Supplemental Material for methodological details, Fig. S1) are heavily infected with *Wolbachia* (Table S-1). Both populations have been monitored in recent years, and the prevalent infections (coinfection in SAL; *F* infection in NAV) are known to be stable (Martínez 2013; Zabal-Aguirre et al. 2010). Sequencing and phylogenetic analyses (Figs. S2 and S3) indicated that both populations of grasshoppers were infected by the same *F* and *B* strains.

We analysed the monthly infection dynamics of both populations and found no significant differences ($p > 0.01$) within each month (July, August and September) in the infection proportions estimated from individuals collected from SAL (see Supplemental Materials). Infection proportions (regardless of sex) were constant throughout the summer: $\chi 2 = 10.31, d.f. = 6, p = 0.112$ (Table S-2): Infection proportions remain constant when sex is taken into account (Females: $\chi^2 = 11.32, d.f. = 6, p = 0.790$; Males: $\chi^2 = 11.18, d.f. = 6, p = 0.830$). However, when the individuals are collected from NAV, we obtain different infection proportions over the summer (Table S-3). Differences in the infection proportions during the season were significant for males and females combined ($\chi^2 = 29.33, d.f. = 6, p \approx 0.000$) and males separately ($\chi^2 = 31.44, d.f. = 6, p \approx 0.000$), but not among infected females (females: $\chi^2 = 5.44, d.f. = 6, p = 0.488$) (Table S-3). Analysis of the adjusted residuals indicated that the differences in the infection proportions of males between July and September infection proportions was due to the decrease in the number of coinfected males (Fig. S3), whilst, conversely, there was an increase in the frequency of uninfected males over the summer. Inspection of the adjusted residuals did not reveal any variation between *F* and *B* single-infected male proportions. These results suggest that *Wolbachia* infection disappears in some individuals or at least that the *Wolbachia* titre falls below detection limit (even when employing NESTED-PCR). Notice that these extremely low bacterial densities correlate with the cost of the infection and the induced phenotypes (Serbus et al. 2008).

Field data confirm that infection proportions are not constant in *C. parallelus* during its life cycle. We discuss the outcomes of the simulations with respect to these experimental findings in the next section.

3.2 Progression of Single Infection

First, we analysed the theoretical predictions of Turelli-like models, considering a single *Wolbachia* strain (without considering the decrease in infection proportions, a_i). We examined the different dynamics of infected males and females based on transmission (μ) as a function of the initial uninfected male frequency ($P_{m,i}$) when the initial female frequency ($P_{f,i}$) was 0.5 and *vice versa* (Fig. S5A and S5B). Secondly, we checked the influence of the decrease in infection rate on this final equilibrium (Fig. 3a–c).

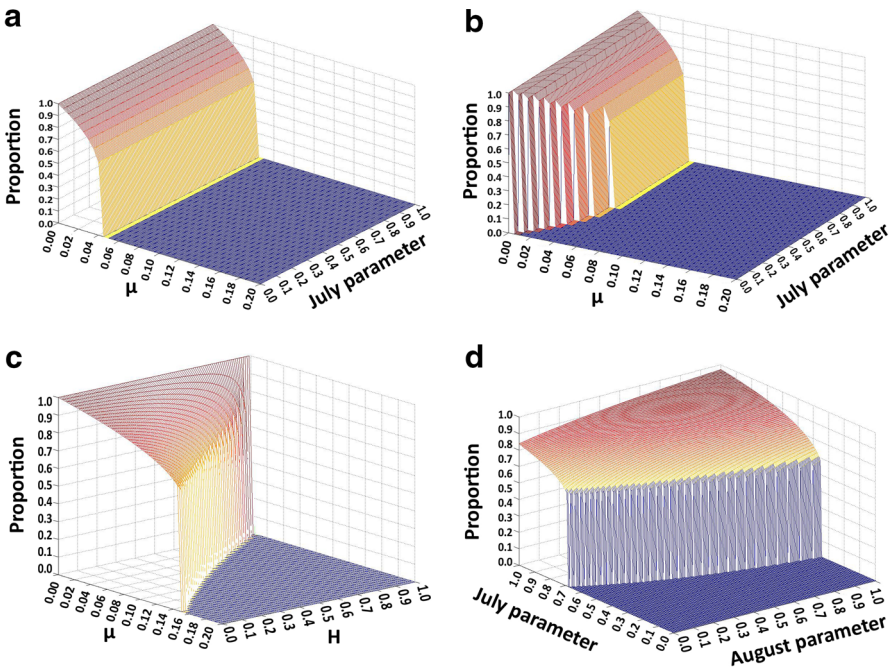

Fig. 3 Progression of single infection in *C. parallelus* as a function of the decrease in the rate of infection (a_i). **a** Change in the proportions of infected males after 4,000 generations (stable equilibrium) as a function of the decrease in the rate of infection (a_i), the initial frequency of infected males ($P_{m,i}$) and maternal transmission (where $\mu = 0$ represents perfect transmission). The initial female frequency was $P_{f,i} = 0.5$. **b** Change in the proportions of infected females as a function of initial infected female frequency (P_{f0}), and maternal transmission. The initial male frequency was $P_{m,i} = 0.5$. **c** Dependence of threshold value on H and μ (where $H = 0$ represents total incompatibility and $\mu = 0$, perfect transmission). Initial male and female proportions were $P_{m,i} = 0.5$ and $P_{f,i} = 0.5$. Note the discontinuity in the predicted infection proportions over the range of parameter values. This discontinuity appeared irrespective of the initial infection proportions. **d** Dependence of threshold value on rate of decrease (a_0 and a_1) in July and August, $P_{m,i} = 0.5$ and $P_{f,i} = 0.5$, $H = 0.67$, $\mu = 0.05$

Both simulations showed that the spread and maintenance of the infection mainly depend on the initial infection frequency, for a fixed μ and H (Figs. 3c, S5A, S5B and S5C). These predictions were in agreement with those of previous studies that required a minimum threshold infection frequency for the infection to spread (Frank 1998; Turelli 1994; Vautrin et al. 2007). However, this threshold was only observed in females, in which a minimum infection frequency is required to guarantee the spread of the infection (Fig. S5B), possibly reflecting the different role of females and males in *Wolbachia* infection (see above). In males (Fig. S5A), the evolution of infection proportions did not depend on their initial values, i.e. fixed μ and H; the same final proportions are attained regardless the initial proportions. Simulations also showed that a high transmission rate (expressed as $\mu < 0.1$) is needed to maintain the single infection of *Wolbachia* assuming the experimental value of $H_{\mathrm{UNI}} = 0.67$. A lower μ was required if H_{UNI} increased (Fig. S5).

Furthermore, the infection proportion turned out to be discontinuous (Figs. 3c, S5C, S5D), with the discontinuity limits depending on H and μ. These effects are illustrated

in Fig. 3c, in which, for a fixed initial uninfected frequency of 0.5 for $P_{f,i}$ and $P_{m,i}$, the discontinuity range varied with H and μ. This discontinuity appeared whatever the value of H, meaning that some frequency values were unattainable under this model. The simulations suggested that at least 60 % of individuals should be infected by *Wolbachia* in a stable population. This minimum proportion could be modified if any factor would reduce the degree of male infection during the life cycle.

The outcome of this scenario was consistent with the proportions reported in some populations of *C. parallelus*, like that of Bubión, in which only the F strain was detected (Zabal-Aguirre et al. 2010). However, we have found populations of this grasshopper infected by the *B* strain with lower infection proportions. The discrepancy could be due to these European populations, which are mainly infected by the *B* strain, not yet being at equilibrium because of a relatively recent infection (Bella et al. 2010; Martínez 2013). In addition, single infection is rare in *C. parallelus*. For this reason, models including two strains of *Wolbachia* are more appropriate.

Simulations also show that the decrease in the rate of infection (a_i) can modulate infection spread (Fig. 3a, b, d). So, if any factor would reduce the degree of infection in males (as field studies indicate to be the case), an infection/non-infection polymorphism would prevail under a wider range of parameters (Fig. 3d).

3.3 Coinfection Progression: Effect of the Decrease in Coinfection Frequency on *Wolbachia* Dynamics

We developed two models based on four distinct scenarios (including unidirectional and uni–bidirectional effects) incorporating or excluding monthly variation in infection proportions (see Methods) and explored the progression of the coinfection with respect to fecundity (F), transmission (μ) incompatibility (H) and the decrease rate (a_i), when the host is infected by two strains of *Wolbachia*.

We analysed the stable equilibrium obtained in a population whose initial infection proportions were equivalent to those in the NAV population in order to predict the change in frequency over a range of parameter values. Previous studies predicted a final coinfection equilibrium because of the frequency-dependent advantage of coinfected females (Frank 1998; Turelli 1994). Two models were simulated to describe coinfection progression in a *C. parallelus* population with initial proportions like those in NAV. The two models differed in their M_{ci} matrix, reflecting the paternal effect on infection progression.

In addition, we included monthly variation in infection proportions to test whether this new phenomenon influenced the progression of infection. We were also interested to learn which model better explained the empirical data from the Iberian populations.

3.3.1 Unidirectional Incompatibility Model (Strain 1 Does Not Rescue Strain 2 Modifications and Vice Versa) with and without Monthly Variation in Infection Proportions

Simulations are summarised in Fig. 4a, c, indicating the infection prevailing at equilibrium. These feature different outcomes, with a particular prevalent infection status (i.e.

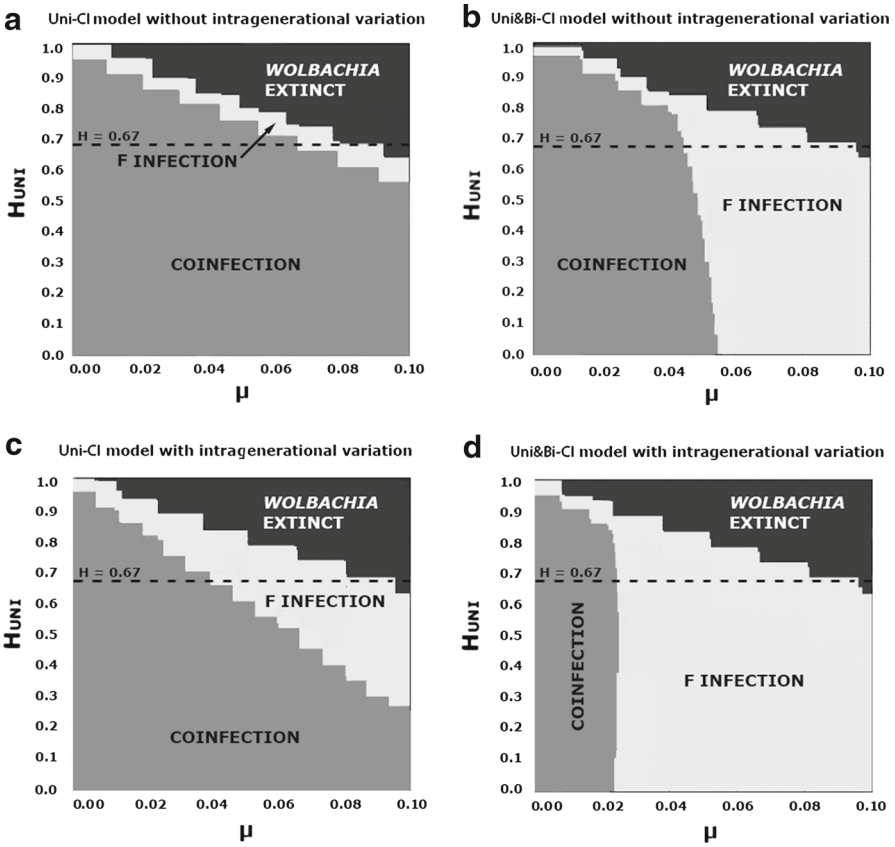

Fig. 4 Progression of infection proportions considering (or not) monthly fluctuations in infection proportions. Simulations based on unidirectional and bidirectional CI models without monthly variation in infection proportions are summarised in **a** and **b**, indicating the infection at equilibrium **c** and **d** illustrates monthly variation in infection proportions. The *light-grey area* indicates coinfection by B and F strains, the *dark-grey area* corresponds to infection by F, and the *black area* corresponds to *Wolbachia* extinction. The empirical estimate of $H_{\mathrm{UNI}} = 0.67$ in *C. parallelus* is indicated by a *broken line*

Wolbachia extinct, infection by one of the two strains of *Wolbachia*, or coinfection) in conjunction with low proportions of other *Wolbachia* strains.

Under the unidirectional model, without monthly variation in infection proportions, we found two main stable equilibria taking empirical NAV infection proportions as the initial proportions: coinfection or infection extinction. A third equilibrium, infection by strain F, was also noted, although the range of parameters required to produce this was restricted (Fig. 4a). The unidirectional CI model resulted in the maintenance of a coinfection with a low value of μ (highly efficient transmission), even when the host experienced weak incompatibility, with a large number of eggs surviving from incompatible crosses (high values of H). Conversely, when μ and H were high (inefficient transmission and weak incompatibility), the model predicted infection extinction.

The simulations considering the unidirectional model with monthly variation in infection proportions are summarised in Fig. 4c. They show that if male coinfection proportions decreased during the life cycle of this species, a wider range of parameters could give rise to *F* infection. The spread of coinfection was apparently slowed, which might favour the maintenance of a single infection.

The unidirectional model showed restricted ranges of parameters under which the *F* strain could progress in a NAV-like population. These results do not reflect those observed in most Iberian populations, in which the *Wolbachia* infection is mainly by the *F* supergroup. We wondered whether the observed monthly coinfection proportion reduction in males affects coinfection progression, for which reason we included this factor in the previously tested unidirectional and uni–bidirectional CI models.

Simulations showed that variation in infection proportions altered the outcome of the previous model. So, this mechanism could alter *Wolbachia* progression and should be considered in our attempts to understand how the proportions in natural populations change. In addition, under the unidirectional CI model, considering variation in infection proportions, a wider range of parameter values gave rise to the strain *F* equilibrium. This better explains the experimental data from Iberian populations, in which a stable predominant *F* strain infection appeared, than the unidirectional CI model, which did not consider the variation in coinfection frequency. A single *F* infection equilibrium could easily be achieved in a population-like NAV, in which some factors induce the reduction in male coinfection frequency, under transmission rates and the incompatibility level that gave rise to the spread of coinfection in the previous model that did not take this phenomenon into account.

It is also clear that efficient transmission, low $\mu_F = \mu_B < 0.065$, is essential to guarantee the spread of coinfection in an initial NAV-like population, assuming the experimental estimate of $H_{\mathrm{UNI}} = 0.67$ (Fig. 4a, broken line). The simulations showed that infection by strain F could only be maintained in the initial NAV proportions over a narrow range of parameter values. According to the infection proportions for each month, incompatibility crosses and offspring infection proportions may vary. We examined whether this pattern could influence the steady state achieved in the previous simulations.

3.3.2 Uni–Bidirectional Incompatibility Model: Strain 1 Partially Rescues Strain 2 Modifications and Vice Versa, with and without Monthly Variation in Infection Proportions

Uni–bidirectional models that did not consider infection proportion variation showed the three stable equilibria found in the previous simulations based on the initial infection proportions of NAV (Fig. 4b). Furthermore, simulations considering infection proportion variation indicated significant changes in the final stable equilibria (Fig. 4d). We observed that coinfection predominance in the final equilibrium was limited to conditions of high transmission rates ($\mu < 0.025$). A single *F* supergroup infection may be maintained over a wider range of values, even if transmission is nearly perfect, including some values for which coinfection was the outcome under the assumptions of other models. This model (considering infection proportion variation) extended the range of parameters that yield a prevalence of *F* infection. Without considering any

other factors, a uni–bidirectional CI model may better explain the experimental data than does the standard model of how the infection by the F supergroup is maintained in Iberian populations of *C. parallelus*, even when coinfection is present.

In fact, experimental data suggest that partial rescue between strains of *Wolbachia* is possible in *C. parallelus*, thereby supporting this model (Bella et al. 2010; Zabal-Aguirre et al. 2014). The slight incompatibility (expressed as $H_{BI} > H_{UNI}$) between strains of *Wolbachia* applied in some crosses in the bidirectional CI model had a negative effect on the invasiveness and maintenance of coinfected individuals, whose invasion threshold increased. When H_{UNI}/H_{BI} increased, the advantage of coinfected females decreased, so we did not expect coinfected individuals to increase with respect to other types of infection. This mechanism favours the strain that is initially most frequent in the population (F strain).

Under the uni–bidirectional CI models (with monthly infection proportions variation), the equilibrium resulting in a stable F infection could be achieved for the widest range of values compared with previously tested models (compare Fig. 4a, b or c, d). Simulations assuming $H_{UNI} = 0.67$ (broken line, Fig. 4) suggested that the maintenance of a predominant F infection in a population-like NAV could be ensured over a wider range of transmission ($0.095 > \mu > 0.045$), and even when H was lower than this value (implying higher incompatibility), a stable F strain infection equilibrium was possible if transmission was not perfect ($\mu > 0.045$). As we reported, monthly infection proportion variation significantly modified the outcome: under this model, F prevalence was easily achieved over a wider range of parameters, including values that produced a coinfection equilibrium in previous models.

These simulations showed that a decrease in male coinfection proportions could act as a barrier to the spread of coinfection, independent of the factors causing the coinfection frequency to decrease. Under these conditions, the currently most frequent infection may be maintained, even if coinfected individuals are present. This effect was stronger when considering the variation in infection proportions in the uni–bidirectional CI model.

3.3.3 Coinfection Decrease Rates

We tested different values for coinfection decrease. As a result, after incorporating male infection decrease rate, we observe chaotic behaviour in the prevalence of infection and a high dependence of initial proportions. We simulate initial frequencies of $Pt = (0.25, 0.25, 0.25, 0.25)^t$ and include H, μ and F values following experimental data. Previous models suggest that coinfection proportions should increase until fixation (Turelli 1994; Frank 1998). However, under unidirectional CI, it is not possible to predict the final outcome. It changes according to reduction parameters (Fig. 5a). In other way, under uni–bidirectional CI, we can only predict the final outcome if the reduction rate is extremely low. In this scenario, coinfection prevails (Fig. 5b).

In addition, we simulate the asymptotic state for an initial datum in the diagonal for different July's parameter (a_0). We obtain a very oscillating behaviour (blue line). We also observe that if we refine the parameter's mesh, we obtain a different result (red line). This implies that the basins of attraction are very sensitive to the parameters for some initial data in the diagonal (Fig. 5c).

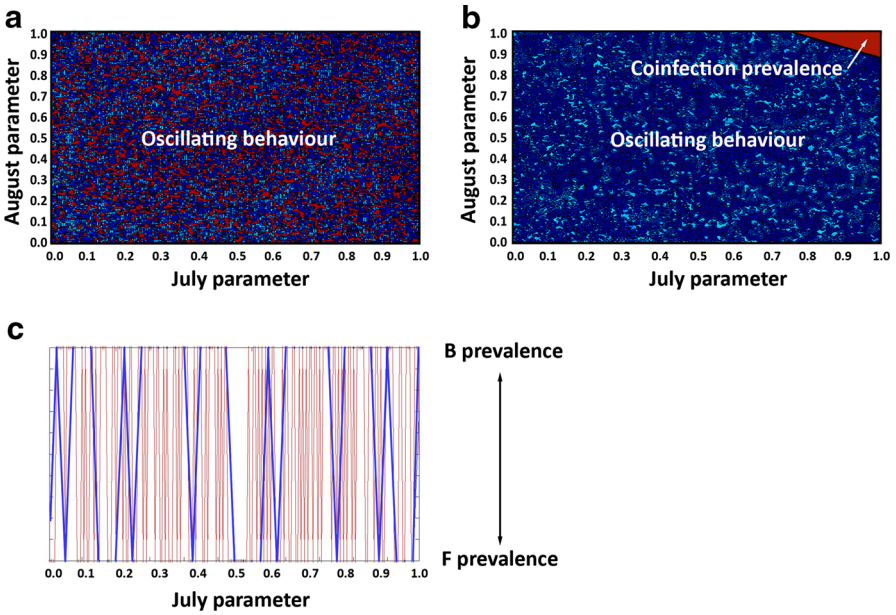

Fig. 5 Progression of infection proportions considering monthly infection proportions fluctuations. Simulations based on (*A*) unidirectional and (*B*) bidirectional incompatibility models. The final outcome is summarised in (*A*) and (*B*), indicating the main infection at equilibrium: *Dark* and *light blue* mean *F* and *B* infection, respectively, while *red* indicates coinfection. (*C*) Illustrates chaotic behaviour in the prevalence of infection. Both horizontal axes indicate the prevalence of *B* and *F* strains. The *red line* indicates a redefined parameter mesh with respect to the *thick blue line* (Color figure online)

3.4 Biological Hypotheses

The high coinfection frequencies in SAL have been explained by strong positive selection for coinfected individuals (Zabal-Aguirre et al. 2010) according to theoretical models (Frank 1998; Turelli 1994). However, if the same factors drive the evolution of infection proportions in infected populations, how can we explain the current differences in proportions between populations such as SAL and NAV (Tables S-1, S-2 and S-3)? Many factors influence bacterial spread and the evolution of the infection proportions. We suggest that any factors acting against coinfected males explain stable, low-level-coinfected populations in NAV and other Iberian Peninsula populations in contrast to SAL.

Several factors have been proposed to explain *Wolbachia* infection variation during development. For instance, male ageing and mating frequency may reduce infection density in several species (Kittayapong et al. 2002; Noda et al. 2001). We suggest that any factors acting against coinfected males explain stable, low-level-coinfected populations in NAV and other Iberian Peninsula populations in contrast to SAL. In our model, the influence of ageing should be similar in both populations, so we have ruled out this possibility. However, we have no data about mating frequency, since our study is based on natural populations. Other genetic and environmental factors should be considered.

As suggested by Koehncke et al. (2009), selection could act differently in *Wolbachia*-infected males and females that experience CI. Coinfected females are at a selective advantage because of their capacity to rescue all sperm modifications. So, with independent coinfection proportions, selection would be expected to increase the transmission of both strains of *Wolbachia* and, indirectly, the frequency of host genes favouring *Wolbachia* transmission. By contrast, with low coinfection proportions (as we observed in NAV), the reduction of the fertility of males involved in bidirectional CI crosses (expressed as embryo deaths) could be a target for selection, thereby favouring genes that eliminate or reduce infection in males (sex-specific modifiers).

Previous studies have shown that *Wolbachia* coinfection spread could be affected by environmental conditions (Mouton et al. 2006, 2007). So, we cannot discount the possibility of a direct or indirect positive relationship between temperature and the strength of the male response to bacterial infection. Note that our results show different bacterial dynamics in the distinct infected populations. Their environmental conditions differ with respect to temperature, and *Wolbachia* is known to be a temperature-sensitive bacterium. Indeed, insect heat shocks, between 30 and 37 °C, are used to eliminate it (Clancy and Hoffmann 1998; Van Opijnen and Breeuwer 1999; Wiwatanaratanabutr and Kittayapong 2006). NAV temperatures frequently exceed 30 °C during July and August, occasionally rising above 35 °C (see Supplemental Material).

This hypothesis could also explain the current distribution of infection in the European populations of *C. parallelus.* It is worth pointing out that levels of B infection are markedly lower in the Iberian Peninsula than in European populations. Note that mean temperatures in the locations of the Iberian populations are higher than those for other European populations. In addition, the *Chorthippus* immune system is activated at high temperatures (Ouedraogo et al. 2003; Springate and Thomas 2005). This coincidence suggests a relationship between geographic patterns of infection and environmental factors that could modulate the infection in some way.

4 Conclusions

Intra-generational variation in infection proportions of the populations can affect the medium to long term spread of *Wolbachia* infection. We found this effect to be strongest under the uni–bidirectional CI model proposed here, in which partial rescue between strains of *Wolbachia* is possible. In this model, the previously predicted coinfected stable equilibrium could change to a single infection at stable equilibrium for a wide range of values of the parameters H and μ. This could explain, independently of any biological reasons, the infection pattern observed in most Iberian populations, in which the F strain prevails over the expected coinfection equilibrium.

Acknowledgments We thank Dr Phil Mason (Glasgow University) for his useful advice and our anonymous referees for their helpful comments. We also thank Prof Juan Orellana for providing us with the mill for the DNA extraction. We are grateful to the Comunidad de Madrid and the Gobierno de Aragón for permission to collect grasshoppers. This work has been supported by Spanish Grants CGL2009-08380/BOS and CGL2012-35007 and the collaboration of Chromacell S.L. Rafael Granero-Belinchón is supported by Grants MTM2011-26696 from the Ministerio de Ciencia e Innovación (MICINN), Spain.

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