The disproportionate effect of drift on a hypervariable master regulator of density

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Abstract

Symbiosis is a continuum of long-term interactions ranging from mutualism to parasitism, according to the balance between costs and benefits for the protagonists. The density of endosymbiont density is in both cases a key factor that determines both the transmission of symbiont and the host extended phenotype and is thus tightly regulated within hosts. However, the evolutionary and molecular mechanisms underlying bacterial density regulation are currently poorly understood. In this context, the symbiosis between the fruit fly and its intracellular bacteria *Wolbachia* (*w*MelPop strain) is particularly interesting to study. Although vertically-transmitted, the symbiont is pathogenic, and a positive correlation between virulence and *w*MelPop density is observed. In addition, the number of repeats of a bacterial genomic region -Octomom- varies between individuals, but most likely also within them, and is positively correlated to the *Wolbachia* density. Such genetic heterogeneity within the host could promote conflicts between partners by increasing within-host competition between symbiont genotypes through a process analogous to the tragedy of the common. To characterize the determinisms at play in the regulation of bacterial density, we first introgressed *w*MelPop in different genetic backgrounds of *D. melanogaster*. We found different density levels and Octomom copy numbers in each host lineage, suggesting a host influence on density regulation through Octomom copy number selection. To confirm this hypothesis, we performed new replicated introgressions on the two *Drosophila* populations that exhibited the most extreme density levels. However, we found no evidence of host influence on density regulation. Instead, we found instability in infection patterns across generations, which rather suggests an influence of drift. Moreover, using reciprocal crosses with the two extreme lineages, we confirmed the absence of host regulation on density levels and Octomom copy number, and a strong influence of drift. We then discuss how drift, both on the symbiont population during transmission and on the host population, could limit the efficiency of selection in such a symbiotic system, and the consequences of drift on the regulation of density and composition of bacterial populations.

Keywords: Symbiosis, *Wolbachia*, *Drosophila*, density regulation, drift

Introduction

A majority of organisms live in symbiosis, a close relationship between two organisms belonging to different species that ranges along the continuum between parasitism and mutualism (De Bary, 1879; Tipton, Darcy and Hynson, 2019). The regulation of the symbiont population, and particularly their abundance within host tissues, are important characteristics that shape the tight relationship between partners and influence the position of the symbiosis along the mutualism-parasitism continuum (Tiivel, 1991; Douglas, 1994). Research on disease evolution has further shown that the evolution of virulence is balanced by the transmission of symbionts to new hosts, and that both virulence and transmission rely on the regulation of the symbiotic density (Anderson and May, 1982). On the one side, an increased virulence can benefit symbionts by increasing their instantaneous transmission, as they exploit more host resources and thus increase their replication within the host. On the other side, the more abundant the symbionts are in host tissues, the more they cost to the host, which shortens the host life span and thereby the window of transmission of the symbiont. As a result, the virulence/transmission trade-off leads to a symbiotic density optimum that optimizes symbiont transmission over the entire life of the host.

Symbiont density is thus under strong regulation (O'Neill, Hoffmann and Werren, 1997; Alizon *et al.*, 2009), and many factors can contribute to its control (López-Madrigal and Duarte, 2019). In insects for instance, host factors can play a major role in regulating the symbiont population (Poinsot *et al.*, 1998; Douglas, 2014) through the activation of immune pathways, such as DUOX or Toll (Douglas, Bouvaine and Russell, 2011; You, Lee and Lee, 2014). Symbionts can also be involved in their own regulation according to particular genetic factors (Ijichi *et al.*, 2002; Chrostek *et al.*, 2013). This is for example the case in symbioses between wasps and vertically-transmitted bacteria, where densities of *Wolbachia* are strain-specific in co-infection (Mouton *et al.*, 2003, 2004). Still, some mechanisms involved in bacterial regulation are poorly understood in insects. For instance, the target of bacterial regulation remains to be clarified: does the host control the overall symbiont population by decreasing symbiont abundance regardless the symbiont genetic specificity or does it target specific variants? Also, control mechanisms that are independent of classical immune pathways are worth exploring. For instance, are cases where hosts sanction symbiont through differential allocation of metabolites frequent and widespread in symbiotic associations (Douglas, 2008)?

Many evidence suggest that selection should lead to symbiotic population regulation systems (Douglas, 2014), but two evolutionary mechanisms could limit the effectiveness of the selection on density regulation and should also be considered: conflicts between different levels of selection and drift. Regarding levels of selection, between-host selection predicts that any excessive replication would be detrimental to the fitness of the host, and therefore would select for symbiotic variants that are the least harmful while being well transmitted (Szathmáry and Smith, 1995). On the contrary, competition that occurs within host tissues should favor symbiont variants that are the most efficient to colonize faster the host, so those with the most proliferative abilities regardless of the cost payed by the host (Alizon, de Roode and Michalakis, 2013). Hence, do within- and between-host selections create an evolutionary conflict regarding the control of symbiont density by favoring symbiont strains with opposite replication profiles (O'Neill, Hoffmann and Werren, 1997; Monnin *et al.*, 2020)? Finally, what is the importance of

drift in vertically-transmitted symbioses? Indeed, bottlenecks during transmission reduce the genetic diversity to the following host generation and may limit the effectiveness of selection upon symbiotic population regulation (Mathé-Hubert *et al.*, 2019). Such molecular and evolutionary mechanisms remain poorly studied, especially in vertically-transmitted symbioses, although they can play an important role in the epidemiological and evolutionary dynamics of symbiotic interactions. A first limitation is conceptual, as populations of vertically-transmitted endosymbionts tend to be considered with little or no heterogeneity, thus limiting the potential for within-host selection. However, while recurrent bottlenecks during transmission tend to reduce diversity, heterogeneity can still be observed in certain systems (Banks and Birky, 1985; Birky, Fuerst and Maruyama, 1989; Abbot and Moran, 2002; Asnicar *et al.*, 2017). A second -more practical- limitation is that if heterogeneity does exist in symbiont populations, it is difficult to trace it experimentally, because of the absence of genetic markers.

A good study model to address questions related to density control is the maternally-transmitted bacterium *Wolbachia* in association with *Drosophila* hosts. In particular, the virulent *w*MelPop strain (Min and Benzer, 1997), which can exhibit heterogeneous density levels between individuals, has differential virulence profiles. Virulence is notably correlated to a tandem amplification of the genomic region "Octomom" (Chrostek *et al.*, 2013). Indeed, flies harboring more copies of Octomom exhibit high density levels in their tissues and a reduced lifespan, while those with less copies exhibit low density levels and survive longer (Chrostek and Teixeira, 2015). This model system is therefore advantageous because hosts and symbionts can exhibit genetic variability, and because the number of Octomom copies can be used as a marker to track the evolution of the symbiotic population. Moreover, Monnin et al. (2000) showed in a previous study that within-host selection can occur in the *w*MelPop in *Drosophila melanogaster*.

In this study, we take advantage from this *Drosophila*-*w*MelPop symbiosis to shed light on the evolutionary determinisms that act on the regulation of vertically-transmitted symbiont in insects. We investigate whether the host genetic background can directly influence the density of the symbionts, or whether the symbionts self-regulate their density *via* Octomom. Using different host genetic backgrounds and a combination of introgressions and crossing experiments, we analyze the respective role of host and symbiont backgrounds, but also drift, in the evolution of density and genetic composition of the symbiotic population.

Materials and Methods

Model system

Drosophila melanogaster flies have been trapped in different locations (Arabia, Bolivia, China (Canton), France (Sainte-Foy-lès-Lyon), Republic of the Congo - RC (Brazzaville), and USA (Seattle)). These populations have been maintained in the laboratory by regular sib mating for at least 10 years and are considered as genetically homogeneous. In the following experiments, we used these 6 inbred lines (*Wolbachia*-free) plus the *w*1118 line, infected either by the *Wolbachia* strain *w*MelPop *(*provided by Scott O'Neil (Monash University, Australia)) or by the strain *w*MelCS (provided by J. Martinez/F. Jiggins, Cambridge University, UK).

Rearing and collection

Flies were maintained under 12-hour day/night cycles at constant temperature and hygrometry (25°C and 60% relative humidity), and reared on rich medium (for 1 L of medium: 73.3 g of Gaude flour, 76.7 g of inactive brewer's yeast, 8.89 g of agar-agar powder, 4 g of Tegosept - Nipagine, 0.4 L of distilled water and 55.5 mL of 95% ethanol). For the sampling, we controlled the larval competition by depositing 100 *Drosophila* eggs on a rich medium pellet (1 mL) placed in a tube of agarose medium. After hatching, flies were transferred on an agarose medium supplemented with sugar (10 %) and were collected after 7 days to be frozen and stored at -20°C.

Wolbachia introgression within various host genetic backgrounds

As *Wolbachia* is a maternally-transmitted bacterial symbiont, introgression of the infection in a previously uninfected line relies on crosses of uninfected males from this line with females of a *Wolbachia*-infected line. The symbiotic introgression method allows to transmit symbionts from a donor line to a recipient line while conserving most of the genetic background of the recipient line. This method consists in making a first cross between *Wolbachia*-infected females (here, n = 20) from the donor line with *Wolbachia*-uninfected males (here, n= 10) from the recipient line. Then, the F1 progeny of this previous cross carries the *Wolbachia* symbionts from the donor line and shares half of its genetic background between the donor and the recipient lines. Two additional backcrosses between females from the F1 (and then F2) progeny and males of the recipient lines are necessary to restore at the F3 generation 87.5 % of the genetic background of the recipient line (Figure 1).

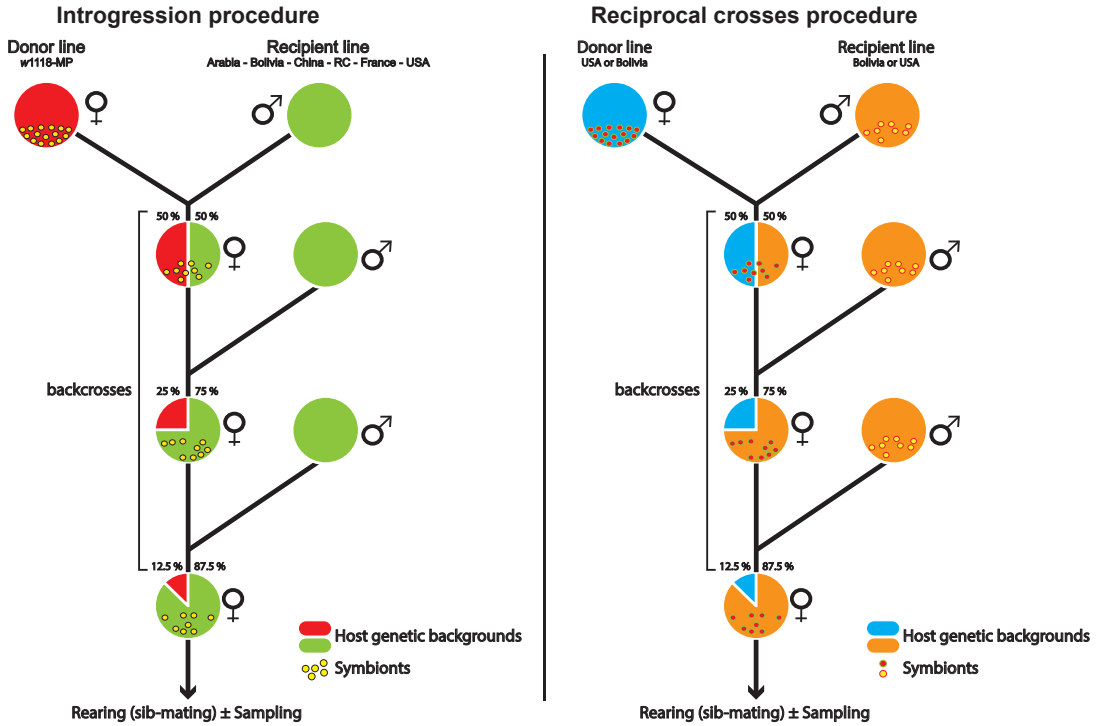

Figure 1: Introgression and reciprocal crosses procedures.
Transmission of symbionts from females of the donor line (red or blue) to a recipient line (green or orange). Serial backcrosses were performed to restore the recipient host genetic background by mating daughters from the previous cross with males from the recipient line. This method was applied to infect the 6 natural *Drosophila melanogaster* populations (experiment #1), to perform new introgressions from Bolivia or USA on 3 replicates (experiment #2) and to conduct reciprocal crosses (experiment #3).

We first applied this method to infect the 6 natural *Drosophila melanogaster* population lines with the *w*MelPop strain (experiment #1, MP1 lines). For that purpose, we used an iso-female *w*1118 line infected with *w*MelPop (IsoA3) as the donor line and the other populations as recipient lines (1 introgression / line). Eight generations after the introgression procedure (under the same rearing conditions as explain above), each new generation was established by tube transfer of 80 randomly selected individuals. Infection patterns in these lines were checked by qPCR to measure *w*MelPop relative density and the average Octomom copy number per bacteria per fly. As the introgression of *w*MelPop in different recipient lines (experiment #1) resulted in different infection patterns (*i.e.*, density and number of Octomom copies), we tested in a second experiment the replicability of the infection pattern after a new introgression procedure. For that purpose, we selected two recipient lines (USA and Bolivia) that exhibited extreme infection patterns after introgression (*i.e.*, USA-MP1 exhibited a high *w*MelPop density whereas Bolivia-MP1 exhibited a low *w*MelPop density, see results), and performed anew 3 independent symbiotic introgressions, using the same iso-female line (IsoA3, 12 generations after the introgression procedure) as the donor line and these two populations (USA and Bolivia) as recipient lines. After 3 generations of backcrosses, Bolivia-MP2 and USA-MP2 flies were maintained under regular sib-mating.

We also independently performed reciprocal crosses between the Bolivia-MP1 and USA-MP1 lines (*i.e.*, lines infected with *w*MelPop during the first introgression experiment) to test the respective influence of the host and symbiotic genetic backgrounds on the *w*MelPop proliferation within flies. For this purpose, we reciprocally backcrossed Bolivia-MP1 and USA-MP1 individuals for 3 generations (Figure 1 right; 3 independent replicates). After 3 backcrosses, flies were maintained under regular sib-mating.

Quantification of *w*MelPop density and Octomom copy number

Wolbachia density and Octomom copy number were measured on 7-days old flies, whose DNA was extracted using the Biobasic EZ-10 96-well Plate Animal Genomic DNA kit. In brief, flies were individually crushed in 400 µL of lysis buffer by a sterile 5-mm stainless bead shacked by a TissueLyser (Qiagen). DNA was extracted following the instructions from the manufacturer, eluted in 100 µL of elution buffer and stored at -20°C.

Relative *Wolbachia* density and Octomom copy number were measured in the same extract by quantitative real-time PCR using SYBR® green and following the MIQE guideline applied to DNA samples (Bustin *et al.*, 2009). To quantify the average amount of *w*MelPop per fly, we used primers targeting a monocopy reference gene in the host (*RP49* in *Drosophila melanogaster*) and primers targeting a monocopy gene outside of the Octomom region in *Wolbachia* (*WD0505* in *w*MelPop). Then, we normalized the number of copies of *WD0505* by the number of copies of the reference gene *RP49* to estimate the relative density of *w*MelPop per fly (Monnin *et al.*, 2020). To quantify the average Octomom copy number of the *w*MelPop population within a fly, we used primers targeting the same gene located outside the Octomom copy number in the *w*MelPop genome (*WD0505*) and primers targeting a gene inside the Octomom region (*WD0513*). Then, we normalized the number of copies of *WD0513* by the number of copies of *WD0505* to estimate the mean Octomom copy number of the *w*MelPop population per fly (Chrostek *et al.*, 2013). The sequences of the primers used (synthesis by Eurogentec) are available in the Table s1.

The PCR amplifications were performed on a CFX96 instrument (Bio-Rad), independently for each target gene. Four µL of a diluted DNA sample (1/25), 0.5 µL of each forward and reverse primer (100 µM) and 5 µL of SsoADV Universal SYBR® Green Supermix (BioRad) were used, for a total volume of 10 µL. The reaction conditions for amplification were 95 °C for 3 min of preincubation, followed by 40 cycles of {95 °C for 10 s for denaturation, 60 °C for 10 s for hybridization and 68 °C for 15 s for elongation}. The mean primer efficiencies were calculated using 6 points (in duplicate) from a 10-fold dilution series (10^3 to 10^8 copies) of previously purified PCR products (*Table s1*). The cycle quantification (Cq) values were estimated by the regression method, and the mean Cq value between technical duplicates was used for the determination of individual DNA quantities (deviation between duplicates below 0.5 cycles).

Statistical analyses

We used the R software (version 3.6.3) for all analyses (R Core Team, 2020). Density and Octomom copy number ratios were estimated and normalized from the Cq values using the EasyqpcR package (Le Pape, 2012), based on the qBase algorithms published by Hellemans *et al*. (2007), taking into account the efficiency of primers. We first used a control sample from an aliquoted DNA extract (w1118 line infected by the *Wolbachia w*MelCS strain) as a calibrator, to check for inter-plate variability. This variability was found to be small enough to be disregarded. Using the EasyqpcR package, we determined the quantity of *WD0505* relative to *RP49* and of *WD0513* relative to *WD0505*. In addition, as the *w*MelCS genome contains only one copy of Octomom, we confirmed that the Octomom copy number measured was close to one and set its values to exactly 1. We used this transformation of the calibrator value as a standardization for all the samples.

The relative density data were analyzed by general linear models. Normality and homoscedasticity were checked graphically. The Octomom copy number data were analyzed with general linear models with gamma distribution, as the distribution of this factor did not fit to a normal distribution. We confirmed graphically that the gamma distribution used in the model fitted to the Octomom copy number data with the package fitdistrplus (Delignette-Muller and Dutang, 2015). The significance of the factors in these models were checked graphically with confidence intervals and considering p-values.

In the first experiment, we focused on the overall effect of the host genetic background on the relative density and Octomom copy number. The host genetic background of the lineages was thus set as the explanatory variable. We used the method of contrasts with *p*-values adjusted by Tukey method to obtain the pairwise differences between the lineages for both the relative density and the Octomom copy number, and created statistical groups with these estimated pairwise differences. The correlation between the relative density and the Octomom copy number was performed by a linear model with the Octomom copy number set as an explanatory variable.

In the second and third experiments, we focused on the differences between replicates. Then, the replicate label was set as the explanatory factor. The statistical analyses were performed independently for the Bolivia and USA host genetic backgrounds. We also used the method of contrasts with p-values adjusted by Tukey method to obtain the pairwise differences between the replicates for both the relative density and the Octomom copy number.

Results

To characterize the determinisms at play in the regulation of bacterial density, we first investigated if host genetic background can have an active influence on density levels. As the Octomom region is also implied in density regulation, we additionally tested the influence of its amplification on density levels and the potential interaction between host and bacterial genotypes on density levels.

Wolbachia introgression within various *Drosophila melanogaster* lines is associated with contrasted infection patterns

During a preliminary experiment, we checked the infection status of six *D. melanogaster* populations with contrasted genotypes, introgressed with the heterogeneous strain *w*MelPop originating from the same isoA3 line. When we quantified the relative *Wolbachia* density (Figure 2A) and the average copy number of the genomic region Octomom (Figure 2B) eight generations after the introgression protocol, we found contrasted infection patterns in the different *D. melanogaster* lines tested.

Both *Wolbachia* density and composition (*i.e.*, measured as the mean number of Octomom copies) are significantly influenced by the host genotype (Linear regression model; *w*1118 – MP; Population effect on relative density: $P = 2.33 \times 10^{-09}$; Population effect on Octomom copy number: $P = 2.62 \times 10^{-15}$; see statistical details in Table s2). Introgressed lines thus differed between each other (see pairwise comparisons in Table s3)., with a maximum difference in bacterial density and mean Octomom copy number of respectively 8.3 and 5.8-fold between Bolivia and USA lines (Table s2).

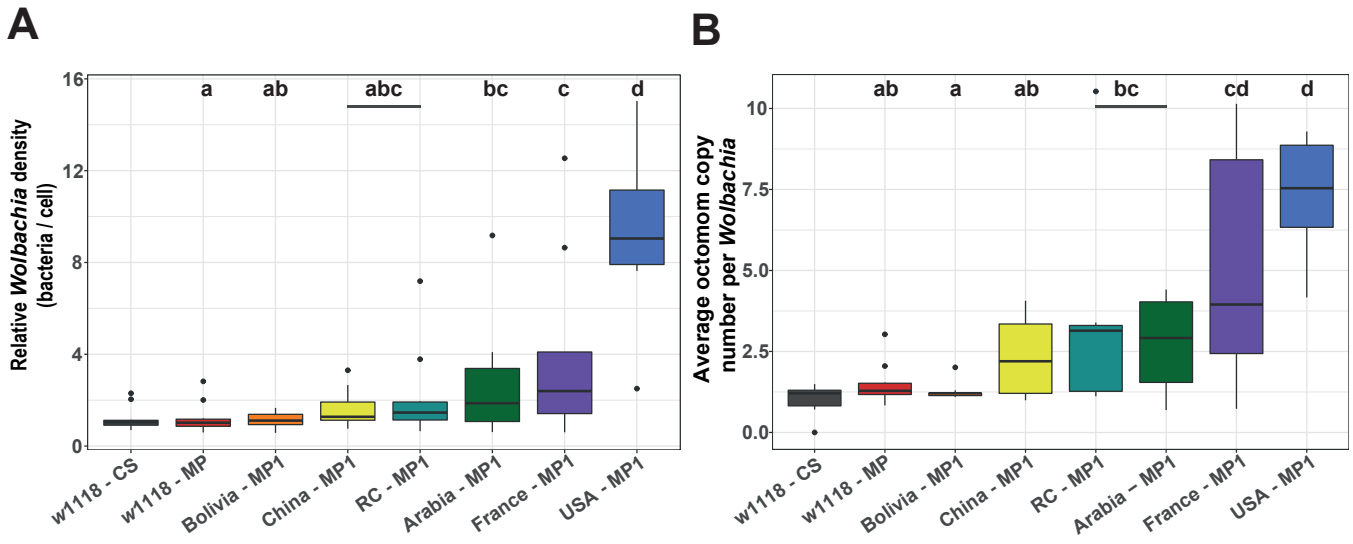

Figure 2: Effect of the host genetic background on infection patterns.
1A: relative *Wolbachia* density per cell, **1B:** average Octomom copy number per *Wolbachia*). Each color represents a host genetic background (n = 10 flies / background). Letters above boxplots show the significative groups performed by pairwise comparisons. The w1118-CS line is an experimental control infected with *w*MelCS and is not integrated in the statistical analyses. The *w*1118-MP line, infected with *w*MelPop, is the line initially used as 'donor' for the introgression procedure. All the other lines were infected with *w*MelPop by introgression (MP1).

We also observed a positive relationship between the relative density in *w*MelPop and the mean Octomom copy number per line (*Intercept* = -0.83, *SE(intercept)* = 0.60, *slope* = 1.23, *SE(slope)* = 0.16, r^2 = 0.89, Linear regression model on median of each host genetic background : *P* = 0.0003) (Figure 3). This strong correlation suggests that the Octomom region is involved in the control of the bacterial density and confirms previous results highlighted by Chrostek et al. (2013) (Chrostek *et al.*, 2013). The number of repeats could thus provide a way to monitor the evolution of bacterial populations across generations and to better characterize selective pressures associated with the control of bacterial populations.

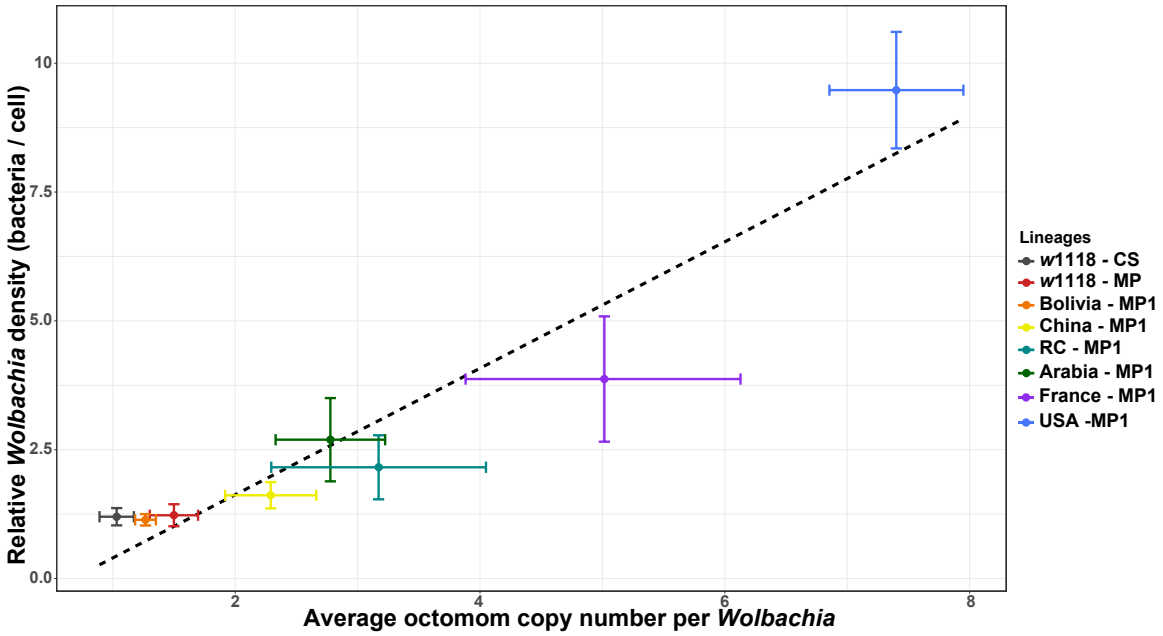

Figure 3: Relationship between the relative *w*MelPop density and the average Octomom copy number per *Wolbachia* cell. Median ± SE. Each color represents a host genetic background (n = 10 flies / background), and the dashed line represents the linear regression.

In this preliminary experiment, we thus observed diversity in term of bacterial density and composition between the 6 lines of *Drosophila melanogaster* introgressed with *w*MelPop. These results suggest that different host genetic backgrounds selected specific variants of the symbiotic community. However, other factors, like genetic drift through a founder effect during the vertical transmission of symbionts from the donor line and / or from one host generation to the other, could also explain this pattern.

The infection pattern can change rapidly over generations, regardless of the host genetic background

To disentangle the effects of host genotype and genetic drift, we performed a similar experiment of introgression of the *w*MelPop *Wolbachia* strain in different genetic backgrounds, but established three independent replicate lines for each host genetic background. While the three replicates should exhibit the same response under host control, variation among the three replicates is expected under the drift hypothesis. This experiment was performed using the lines that exhibited the most extreme patterns of infection in the preliminary experiment (*i.e.*, Bolivia-MP1 and USA-MP1). We then evaluated the stability of the infection pattern over generations, by tracking the relative density (Figure 4A) and the Octomom copy number (Figure 4B) immediately after the first introgression event, after 13 generations, and after 25 generations (see Tables S4 & S5 for details).

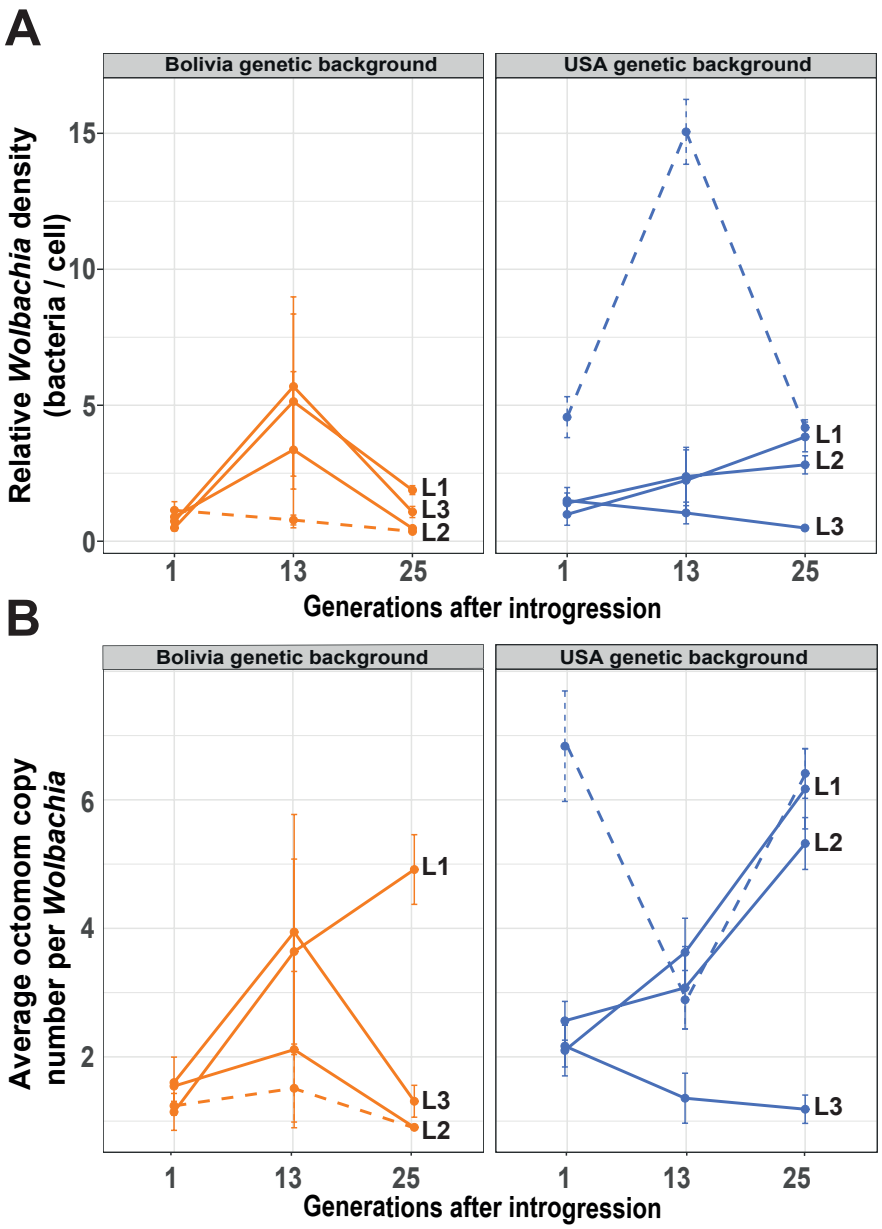

Figure 4: Replicability of the infection patterns over generations, after a new introgression procedure.
4A: relative *Wolbachia* density, **4B:** average Octomom copy number per *Wolbachia*, n = 10 / condition / timepoint, median ± SE). Each color represents a host genetic background. Plain lines represent the replicate lineages from the new introgression procedure (MP2), dashed lines represent the lineages from the initial introgression procedure (MP1), which was set as reference in the statistical analyses. L1, L2 and L3 represent the replicate lines.

Just after the introgression event (t = 1), the relative density and the average Octomom copy number per *Wolbachia* did not differ significantly between the Bolivia-MP2 replicates lines and the Bolivia-MP1 line from the first experiment used here as the reference (Linear regression model; experiment group effect on relative density : $P > 0.1$; experiment group effect on Octomom copy number: $P > 0.1$; see statistical details in Table s4). The relative density and Octomom copy number of the Bolivia-MP2 replicates lines did not differ significantly between them (see pairwise comparisons in Table s5), which does not contradict a host determinism for density regulation trough Octomom copy number selection in this genetic background. However, at the same timepoint (t = 1), the relative density and the average Octomom copy number in the USA-MP2 replicates lines differed significantly from the USA-MP1 line from the first experiment used here as the reference (Linear regression model; experiment group effect on relative density : $P = 0.012$; experiment group effect on Octomom copy number: $P = 7.6 \times 10^{-04}$; see statistical

details in Table s4). Nevertheless, the relative density and the average Octomom copy number of the USA-MP2 replicates lines did not differ between them (see pairwise comparisons in Table s5), and did not show significant difference with the donor line (w1118-MP)(Linear regression model; maternal transmission effect on relative density: $P = 0.38$; maternal transmission effect on Octomom copy number: $P = 0.35$; Table s4). These results could suggest a maternal transmission effect from the donor line to the recipient ones, with infection patterns mirroring the bacterial composition of the donor line.

We then examined the stability of the infection pattern over generations, for each replicate line (Figure 4). After 25 generations post introgression, the relative density and Octomom copy number per *Wolbachia* in Bolivia-MP2 and USA-MP2 replicate lines differed significantly from the quantities measured immediately after introgression (Linear regression model; generational effect on relative density: $P_{Bolivia} = 0.011$, $P_{USA} = 0.022$; generational effect on Octomom copy number: $P_{Bolivia} = 0.044$, $P_{USA} = 0.009$; see statistical details in Table s4). Moreover, the infection patterns between the Bolivia-MP2 or between the USA-MP2 replicate lines significantly differed (see pairwise comparisons in Table s5). All together, these results show an absence of host control on the density and composition of the bacterial population. The variations observed between replicate lines and over time may thus reflect drift.

The bacterial composition initially transmitted strongly influences the patterns of infection observed over generations

In parallel with the second experiment, we performed reciprocal crosses to test the respective influence of the host genetic background, the bacterial population, the maternal effect of transmission, and drift on the *w*MelPop proliferation within flies (experiment #3). In order to jumble the host-*Wolbachia* associations, we made reciprocal crosses in 3 independent replicates with the Bolivia-MP1 line and USA-MP1 line from the first experiment. Then, to evaluate the stability of the infection pattern over generations, we measured the relative density of *w*MelPop (Figure **5A** & **5B**) and the average Octomom copy number per fly (Figure **5C** & **5D**) one generation after the final homogenizing cross, 13 generations and 25 generations post introgression (see Tables S6 & S7 for details).

Just after the introgression event (t = 1), the relative density and the average Octomom copy number per *Wolbachia* differed significantly between the Bolivia(USA-MP1) replicate lines and the Bolivia-MP1 line (Linear regression model, experiment group effect on relative density: $P = 0.016$; experiment group effect on Octomom copy number: $P = 4.19 \times 10^{-07}$, see statistical details in Table s6), but not from the USA-MP1 line (Linear regression model, experiment group effect on relative density: $P = 0.550$; experiment group effect on Octomom copy number: $P = 0.864$, see statistical details in Table s6). In addition, the infection patterns of the Bolivia (USA-MP1) replicate lines did not differ significantly between them (see pairwise comparisons in Table s7). Similarly at the same timepoint, the relative density and the average Octomom copy number in the USA(Bolivia-MP1) replicate lines differed significantly from the USA-MP1 line (Linear regression model, experiment group effect on relative density $P = 8.53 \times 10^{-05}$; experiment group effect on Octomom copy number $P = 2.20 \times 10^{-16}$, see statistical details in Table s6) , but not from the Bolivia-MP1 line (Linear regression model, experiment group effect on relative density: $P = 0.352$; experiment group effect on Octomom copy number: $P = 0.789$, see statistical details in Table s6). In addition, the

infection patterns of the USA(Bolivia-MP1) replicate lines did not differ significantly between them (see pairwise comparisons in Table s7). These results confirm an absence of control from the host on the establishment of the infection pattern and rather suggest a homogeneous symbiont transmission from the donor line.

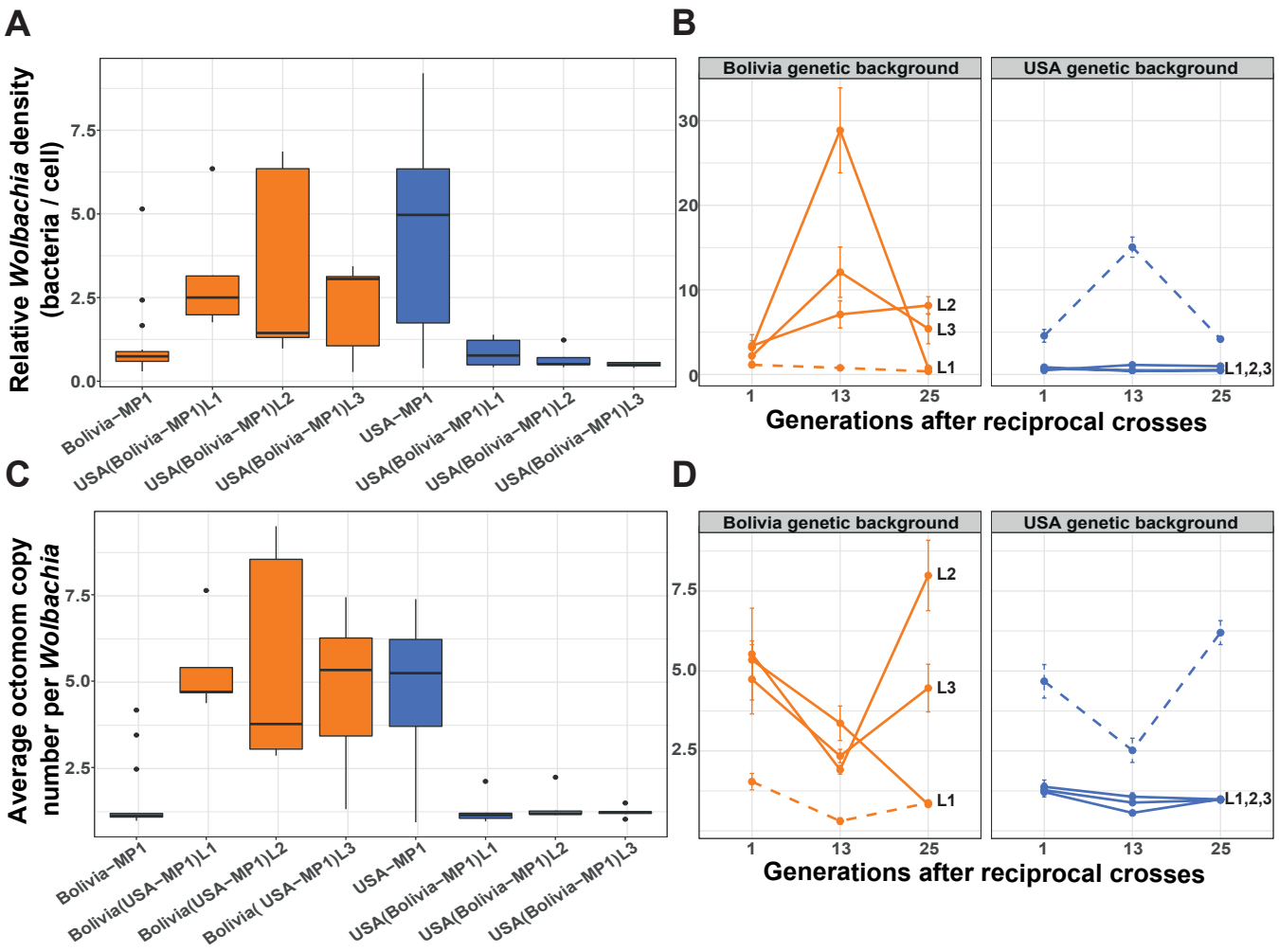

Figure 5: Infection patterns in reciprocal crosses over generations.
Relative density: **(5A)** one generation post introgression (box plot with 'minimum', 1st quartile, median, 3rd quartile, and 'maximum' ± outliers (dots)) and **(5B)** over generations (median ± SE). Average Octomom copy number per *Wolbachia*: **(5C)** one generation post introgression and **(5D)** over generations (median ± SE)). Each color represents a host genetic background and the information in brackets represents the bacterial genetic background. Plain lines represent the replicate lineages from the reciprocal crosses, and dashed lines from the initial introgression procedure (MP1) that was used as reference in the statistical analyses. L1, L2 and L3 represent the replicate lines.

Then, we examined whether the infection patterns were stable within each of the replicate lines over generations by measuring the relative density and average Octomom copy number per *Wolbachia* 13 and 25 generations after the last backcross. After 25 generations post introgression, the relative density in Bolivia(USA-MP1) replicate lines differed significantly from their reference at t = 1 (Linear regression model, generational effect on the relative density: $P = 0.006$; generational effect on the Octomom copy number: $P = 7.79 \times 10^{-05}$, see statistical details in Table s6). On the contrary, the USA(Bolivia-MP1) replicate lines did not differ from their reference at t = 1 (Linear regression model, generational effect on the relative density: $P = 0.060$; generational effect on the Octomom copy number: $P = 0.060$, see statistical details in Table s6). Moreover, the infection pattern of the Bolivia (USA-MP1) replicate lines differed significantly between them, as well as the USA(Bolivia-MP1) did (see pairwise comparisons

in Table s7). All together, these results confirm an absence of host control on the density and composition of the bacterial population. Moreover, the variability observed between the replicates indicates a random transmission of the symbiont over generations.

In conclusion, we failed to reveal any influence of the host genotype on the control of the *w*MelPop proliferation through the selection of bacteria containing high or low Octomom copy number. Instead, we found a strong maternal effect of transmission and an instability of the infection patterns over generations. These experiments lead us to consider that drift could be an important evolutionary force responsible for the diversification of infection patterns observed.

Discussion

In this study we sought to identify the determinisms involved in the regulation of endosymbiotic populations and used the *Drosophila melanogaster* – *w*MelPop symbiotic system to track the influence of host and symbiont genotypes on the density regulation, as well as the evolutionary forces at play. Indeed, this symbiotic model is particularly relevant because it exhibits genetic variability among the vertically transmitted symbiont population, whose evolution can be tracked by a genomic amplification (Octomom). While we first found large differences when comparing infection patterns (*i.e.*, bacterial density and Octomom copy number) in different host genetic backgrounds, such host control on bacterial proliferation/selection was not confirmed after new experiments of introgression on more replicate lines and crossing experiments between lines exhibiting the most extreme infection patterns. Instead, we showed that the infection patterns were initially set up by the bacterial genotype and became very unstable over generations. These results suggest that, in this symbiotic system, drift strongly influences the evolution of the symbiont density -and thus its stability over generations-, contrarily to what is generally described in the literature of insect endosymbioses (*e.g.*, Mouton et al., 2003; Hosokawa *et al.*, 2006).

Numerous examples in insects support an active regulation of symbiotic populations by the host, with stable density over generations when the environment remains constant (Ikeda, Ishikawa and Sasaki, 2003; Mouton *et al.*, 2004, 2007; Funkhouser-Jones *et al.*, 2018). The orchestrated modulation of the symbiont proliferation rate throughout insect development also suggests a fine-tuned host control of the bacterial density (Rio *et al.*, 2006; Login *et al.*, 2011; Vigneron *et al.*, 2012; Duarte *et al.*, 2020). On the opposite, bacterial factors alone can also explain variation in bacterial densities within some hosts. For example, different strains of *Wolbachia* are known to exhibit different, but stable, density levels in the same host lines based on their genetic particularities (Mouton *et al.*, 2003; Chrostek *et al.*, 2013). Proliferation of symbionts within the host is under strong selection as it is a key factor influencing the trade-off between symbiont transmission (*i.e.*, the highest is the symbiont density, the highest is the probability of transmission) and virulence (*i.e.*, the highest is the symbiont density, the highest are the cost on host survival and fecundity) (Anderson and May, 1982; Ewald, 1983). This transmission/virulence trade-off often leads to an optimal density, which can be controlled by host or bacterial determinants. In insect hosts, the main molecular mechanisms that determine the abundance and composition of symbionts are associated with immune response (Lemaitre and Hoffmann, 2007; Zug and Hammerstein, 2015) or resource allocation (Kiers *et al*., 2003), and microbial

365 communities can in turn select resistance mechanisms against host effectors or trigger antagonist regulators of the
366 host immunity (Vallet-Gely *et al.*, 2008; Lindsey, 2020).

367 In the *D. melanogaster*-*w*MelPop system, however, we observed a strong instability of infection patterns that could
368 be due either to an instability of the optimum, or to a large influence of drift that limits the ability of the system to
369 reach the optimum. Our results, and notably the variations observed between replicate lines in controlled
370 conditions, rather suggest a strong influence of drift on the regulation of bacterial density. Bacterial factors, such
371 as the number of Octomom copy, could fluctuate through time and be at the origin of variation in density levels.
372 However, the Octomom genomic region has recently been questioned regarding its involvement in the
373 establishment of density levels. Historically, *w*MelPop proliferation rate and virulence have been shown to be
374 correlated to the genomic amplification of the Octomom region (Chrostek *et al.*, 2013; Chrostek and Teixeira, 2015,
375 2018), but this relationship has been challenged by Rohrscheib *et al.* (Rohrscheib *et al.*, 2016, 2017), who support
376 that the virulence of *w*MelPop rather depends on an increase in the extrinsic rearing temperature. However, to
377 exclude any influence of the Octomom copy number on *Wolbachia* growth and pathogenicity, these variables
378 should be tested independently of the temperature (Chrostek and Teixeira, 2017). At constant temperature, our
379 results show a clear link between the density in adults and the number of copies of Octomom, and are in accordance
380 with the current literature (Chrostek *et al.*, 2013; Chrostek and Teixeira, 2015, 2018; Monnin *et al.*, 2020).
381 Consequently, this genomic amplification can be used as a marker of bacterial diversity and evolution of our model
382 system.

383 We can thus wonder why we observed such variability and temporal instability within lineages, and why drift
384 overcomes this potential bacterial regulation through Octomom? Indeed, this pattern contrasts with what is
385 observed in already well-established symbioses, where one symbiont genotype is fixed (Werren, Baldo and Clark,
386 2008). In our experiments, we were able to show very similar levels of infection between mothers and daughters
387 just after introgression or reciprocal crosses procedures, suggesting a maternal effect. However, instability
388 observed across generations suggests that this effect is probably non-genetic: when a large number of bacteria is
389 observed in the mother's tissues, a large number of bacteria is transmitted to the oocytes and maintained in the
390 adult stage (Veneti *et al.*, 2004; Hosokawa, Kikuchi and Fukatsu, 2007; Parkinson, Gobin and Hughes, 2016).
391 However, there may still be random variability between mothers regarding the amount of bacteria transmitted to
392 their eggs, and between these eggs (Mira and Moran, 2002). Bottlenecks during transmission can thus eventually
393 lead to a gradual shift of the 'initial' density over time. Bottlenecks can also influence density levels through random
394 differential transmission of bacterial variants from one generation to the next (Funk, Wernegreen and Moran, 2001;
395 Kaltenpoth *et al.*, 2010), especially if these variants exhibit different reproductive rates (as it is the case with variants
396 carrying different numbers of Octomom copies (Duarte *et al.*, 2020)). To summarize, if not counteracted by host or
397 symbiont density control, drift is expected to induce instability over generations by a combination of quantitative
398 (*i.e.*, transmission of a non-equivalent number of bacteria to the eggs) and qualitative/genetic (*i.e.*, random
399 transmission of different variants) bottlenecks. Hence, the high variability and the temporal instability depicted in
400 our study could reflect the random transmission of different *w*MelPop quantities and variants during transmission
401 bottlenecks. Interestingly, we observed a higher temporal instability when the inter-individual variability was higher

in the donor lines; variability which was associated with higher values of density and Octomom copy numbers. Indeed, the higher were the density and number of Octomom copies of the transmitted symbionts in the lines, the more unpredictable was the outcome of infection patterns after several generations in these lines. As observed with microsatellites, the mutation rate of a repeated sequence depends strongly on the number of motifs present in the sequence (Whittaker *et al.*, 2003). Assuming the same rules on the number of Octomom copies present in the population (*i.e.*, a higher propensity for duplication when the number of copies is high), the outcomes of drift could be more unpredictable for individuals harboring the highest average number of Octomom copies. Indeed, in the presence of moderate bottlenecks and a mutation rate increasing with the number of copies, a strong variability of infection patterns is expected over generations. Finally, we cannot exclude any drift at the level of the host population, as we selected around 80 flies to establish the next generation (from ~200 eggs laid). However, previous experiments using a similar number of founding flies demonstrated stability in bacterial densities and Octomom copy numbers (Monnin *et al.*, 2020), suggesting that drift at the population level is negligible compared to transmission drift.

The presence of Octomom may be at the origin of a strong genetic instability of this strain, which, associated with drift, may counteract any fixation of a particular genotype, and generate instability of infection patterns as well as a variation in virulence. Even higher instability is expected to occur with lines carrying a high number of Octomom copies, because of the increased mutation rate. Under these conditions, between-host selection should therefore not be efficient, and would explain why the vertically transmitted *w*MelPop strain exhibits a strong virulence, whereas the overall alignment of interests between the host and vertically transmitted symbionts generally leads to the selection of low virulent symbionts that maximize host survival and indirectly their own transmission (Anderson and May, 1982; O'Neill, Hoffmann and Werren, 1997). Maintenance of this virulence phenotype has also been associated with the fact that virulence is only expressed in conditions rarely observed in nature, so that the between-host selection against highly prolific variants (such as those with high Octomom copy numbers) is weak at 25°C. A recent study however shows that strains with 8-9 Octomom copies are pathogenic from 18°C to 29°C (Duarte *et al.*, 2020). In addition, another selective force, the within-host selection, could explain the virulence of *w*MelPop in certain environmental conditions. Indeed, when fly populations are reared at 28°C, Monnin *et al.* (2020) showed that the population evolved toward a higher virulence, which may be due to the stronger effect of within-host selection compared to between-host selection. Thus, when selective pressures are strong, within and between-host selection could modulate symbiont virulence in the *Drosophila*-*w*MelPop association, whereas drift might not allow any co-evolution between partners and co-adaptation to environmental changes when selective pressures are limited.

To conclude, we showed that the host did not control for bacterial density and composition in the symbiosis between *D. melanogaster* and *w*MelPop, and that the infection patterns were very instable across generations, suggesting a strong influence of drift that could limit the effects of within- and between-host selections. As the transmission of symbionts in vertically-transmitted symbiosis is subject to potential bottlenecks both in terms of quantity and genetic diversity (Mira and Moran, 2002; Galbreath *et al.*, 2009; Kaltenpoth *et al.*, 2010), it seems necessary to further characterize the intensity of bottlenecks in this symbiotic system, in order to better evaluate

the impact of drift on the evolution of bacterial populations in vertically-transmitted symbiosis and its impact on host phenotypes.

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Conflict of interest disclosure

The authors of this preprint declare that they have no financial conflict of interest with the content of this article. NK & FV are recommenders for PCI Evolutionary Biology.

Data accessibility & Supplementary material

Raw data and scripts are available online: https://doi.org/10.5281/zenodo.4288852 and https://doi.org/10.5281/zenodo.4288890, respectively.

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