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 endosymbionts is, in both cases, a key factor that determines both the transmission of symbionts 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- is positively correlated to *Wolbachia* density, underlying a potential genetic mechanism that controls bacterial density. Interestingly, the number of repeats varies between host individuals, but most likely also within them. Such genetic heterogeneity within the host could promote conflicts between bacteria themselves and with the host, notably by increasing within-host competition between symbiont genotypes through a process analogous to the tragedy of the commons. To characterize the determinisms at play in the regulation of bacterial density, we first introgressed *w*MelPop in different genetic backgrounds of *D. melanogaster*, and found different density levels and Octomom copy numbers in each host lineage. To determine whether such variations reflect a host genetic determinism on density regulation through Octomom copy number selection, we replicated the introgressions and performed reciprocal crosses on the two *Drosophila* populations with the most extreme density levels. In both experiments, we detected an absence of directionality in the patterns of infection, associated with a strong instability of these patterns across generations. Given that bacterial density was highly correlated with Octomom copy numbers in all experiments, these results rather suggest a strong influence of drift and a random increase in the frequency of certain bacterial variants. 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). In the case of microorganisms, the regulation of the symbiont population within the host, 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 reproduction rate optimum that optimizes symbiont transmission over the entire life of the host. More specifically in vertically transmitted symbioses, the optimum symbiotic density optimizes both the production of offspring and their colonization by symbionts.

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)?

There is much evidence to suggest that selection should lead to symbiotic population control systems (Douglas, 2014), but two evolutionary mechanisms could limit the effectiveness of selection on density regulation and should also be taken into consideration: conflicts between different levels of selection and drift. In terms of selection levels, between-host selection predicts that any excessive replication would be detrimental to host fitness, thus selecting for symbiotic variants that are the least harmful while being well transmitted (Szathmáry and Smith, 1995). On the contrary, the competition that occurs within the host tissues should favor symbiont variants that are the most efficient to rapidly colonize the host, thus those that have the most proliferative abilities regardless of the cost paid by the host (Alizon, de Roode and Michalakis, 2013). This raises the question of whether within- and between-host

70 selections create an evolutionary conflict regarding the control of symbiont density, by favoring symbiont strains
71 with opposite replication profiles (O'Neill, Hoffmann and Werren, 1997; Monnin *et al.*, 2020). Finally, the
72 importance of drift in vertically transmitted symbioses could be more considered. Indeed, bottlenecks during
73 transmission reduce the genetic diversity to the following host generation and may limit the effectiveness of
74 selection upon symbiotic population regulation (Mathé-Hubert *et al.*, 2019). Such molecular and evolutionary
75 mechanisms remain poorly studied, especially in vertically transmitted symbioses, although they can play an
76 important role in the epidemiological and evolutionary dynamics of symbiotic interactions. A first limitation is
77 conceptual, as populations of vertically transmitted endosymbionts tend to be considered with little or no
78 heterogeneity, thus limiting the potential for within-host selection. However, while recurrent bottlenecks during
79 transmission tend to reduce diversity, heterogeneity can still be observed in certain systems (Banks and Birky, 1985;
80 Birky, Fuerst and Maruyama, 1989; Abbot and Moran, 2002; Asnicar *et al.*, 2017). A second -more practical-
81 limitation is that if heterogeneity does exist in symbiont populations, it is difficult to trace it experimentally, because
82 of the absence of genetic markers.

83 A good study model to address questions related to density control is the maternally transmitted bacterium
84 *Wolbachia* in association with *Drosophila melanogaster* hosts. In particular, the virulent *w*MelPop strain (Min and
85 Benzer, 1997), which can exhibit heterogeneous density levels between individuals, has differential virulence
86 profiles. Virulence is notably correlated to a tandem amplification of the genomic region "Octomom" (Chrostek *et*
87 *al.*, 2013). Indeed, flies harboring *Wolbachia* with more copies of Octomom exhibit high density levels in their
88 tissues and a reduced lifespan, while those harboring *Wolbachia* with less copies exhibit low density levels and
89 survive longer (Chrostek and Teixeira, 2015). This model system is therefore advantageous because hosts and
90 symbionts can exhibit genetic variability, and because the number of Octomom copies can be used as a marker to
91 track the evolution of the symbiotic population. Moreover, previous studies showed that within-host selection can
92 occur in the *w*MelPop in *D. melanogaster* (Chrostek & Teixeira, 2018; Monnin et al. 2020).

93 In this study, we take advantage from this *Drosophila*-*w*MelPop symbiosis to shed light on the evolutionary
94 determinisms that act on the regulation of vertically transmitted symbionts in insects. We investigate whether the
95 host genetic background can directly influence the density of the symbionts, or whether the symbionts self-regulate
96 their density *via* Octomom. Using different host genetic backgrounds and a combination of introgressions and
97 crossing experiments, we analyze the respective role of host and symbiont backgrounds, but also drift, in the
98 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), Republic of the Congo - RC (Brazzaville), USA (Seattle) (Viera et al., 1999) and France (Sainte-Foy-lès-Lyon)). 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'Neill (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). With the exception of introgression experiments (see below), each new generation was established by tube transfer of approximately 80 randomly selected 4/5-day old individuals, to ensure full fertility of the flies. To control larval competition prior to sampling for infection patterns, we pooled about 80 flies in egg-laying cages and transferred 100 eggs laid by 4/5-day old females onto 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

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. As *Wolbachia* is a maternally transmitted bacterial symbiont, this method consists here 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 (n = 20) from the F1 (and then F2) progeny and males (n = 10) from the recipient lines are necessary to restore at the F3 generation 87.5 % of the genetic background of the recipient line (Figure 1).

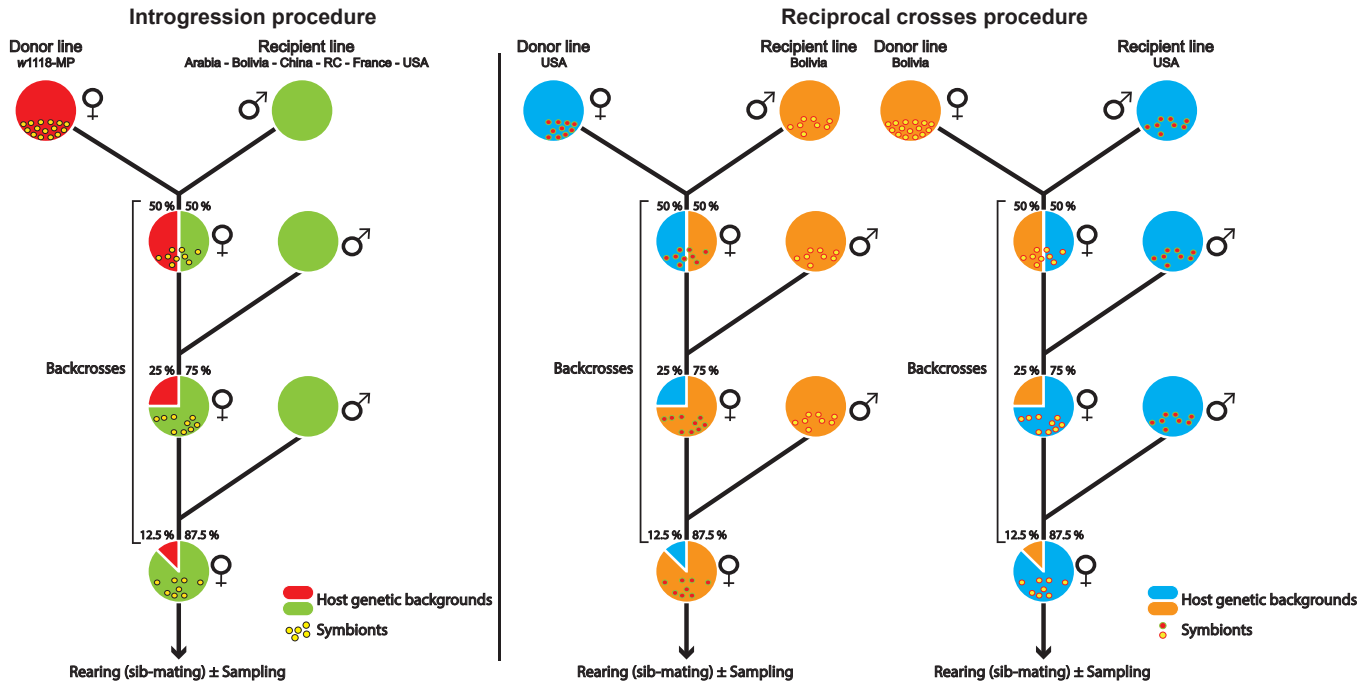


Figure 1: Introgression and reciprocal crosses procedures.
Transmission of symbionts from females of the donor line (red or blue) to a recipient line (green or orange). Serial backcrosses were performed to restore the recipient host genetic background by mating daughters from the previous cross with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

We first applied this method to infect the 6 natural *Drosophila melanogaster* population lines by the *w*MelPop strain (experiment #1, MP1 lines). For that purpose, we used an iso-female *w*1118 line infected by *w*MelPop (IsoA3) as the donor line and the other populations as recipient lines (1 introgression / line). After two generations of regular sib-mating, flies were placed in egg-laying cages for sampling (see 'rearing and collection' protocol above), and infection patterns (*i.e.*, *w*MelPop relative density and the average Octomom copy number per bacteria in flies) were checked by qPCR in these lines.

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 8 generations later (experiment #2) 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 first 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 (except the generation preceding each sampling, for which the larval density was controlled as described above).

In parallel to experiment #2, we independently performed reciprocal crosses between the Bolivia-MP1 and USA-MP1 lines (*i.e.*, lines infected by *w*MelPop during the first introgression experiment) to test the respective influence of 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 (experiment #3; 3 independent

155 replicates). After 3 backcrosses, flies were maintained under regular sib-mating (except the generation preceding
156 each sampling, in which the larval density was controlled).
157

158 **Quantification of *w*MelPop density and Octomom copy number**

159 *Wolbachia* density and Octomom copy number were measured on 7-day old females (n = 10 flies / line (experiment
160 #1) and n = 5 flies / line / timepoint (experiments #2 and #3)), whose DNA was extracted using the EZ-10 96-well
161 Plate Animal Genomic DNA® kit (Bio Basic). In brief, flies were individually crushed in 400 µL of lysis buffer by a
162 sterile 5-mm stainless bead shacked by a TissueLyser® (Qiagen) for 30 s at 25 Hz. DNA was extracted following the
163 instructions from the manufacturer, eluted in 100 µL of elution buffer and stored at -20°C.

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

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

184 **Statistical analyses**

185 We used the R software (version 4.0.3) for all analyses (R Core Team, 2020). Density and Octomom copy number
186 ratios were estimated and normalized from the Cq values using the EasyqpcR package (Le Pape, 2012), based on
187 the qBase algorithms published by Hellemans et al. (2007), taking into account the efficiency of primers. We first
188 used a control sample from an aliquoted DNA extract (w1118 line infected by the *Wolbachia w*MelCS strain) as a
189 calibrator, to estimate the inter-plate variability. We took this variability into account to normalize data between

190 plates using the EasyqpcR package and determined the quantity of *WD0505* relative to *RP49* and of *WD0513*
191 relative to *WD0505*. In addition, as the *w*MelCS genome contains only one copy of Octomom, we confirmed that
192 the Octomom copy number measured was close to one and set its values to exactly 1. We used this transformation
193 of the calibrator value as a standardization for all the samples.

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

200 In the first experiment, we focused on the overall effect of the host genetic background on the relative density and
201 Octomom copy number. The host genetic background of the lineages was thus set as the explanatory variable. We
202 used the method of contrasts with *p*-values adjusted by Tukey method to obtain the pairwise differences between
203 the lineages for both the relative density and the Octomom copy number. To determine the potential role of
204 Octomom in the control of the bacterial population, we estimated the correlation between the relative density and
205 the Octomom copy number (log-transformed data) by a linear model with the average Octomom copy number set
206 as an explanatory variable.

207 In the second and third experiments, we focused on the differences between replicates. Then, the replicate label
208 was set as the explanatory factor. The statistical analyses were performed independently for the Bolivia and USA
209 host genetic backgrounds. We also used the method of contrasts with p-values adjusted by Tukey method to obtain
210 the pairwise differences between the replicates for both the relative density and the Octomom copy number. We
211 finally estimated the correlation between the relative density and the Octomom copy number (log-transformed
212 data) by a linear model with the average Octomom copy number set as an explanatory variable. We performed
213 these correlation analyses: 1) for each genetic background, separately for each timepoint, and 2) for each replicate
214 line, with all timepoint grouped.

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 involved 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) 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 per *Wolbachia*) varied significantly among introgressed lines (Linear regression model; *w*1118 – MP; Population effect on relative density: $P = 2.33 \times 10^{-09}$; Population effect on Octomom copy number: $P = 2.19 \times 10^{-08}$; see statistical details in Table s2). Introgressed lines differed from each other (see pairwise comparisons in Table s3), with a maximum difference in bacterial density and mean Octomom copy number per *Wolbachia* of respectively 8.3 and 5.8-fold between Bolivia and USA lines (Table s2).

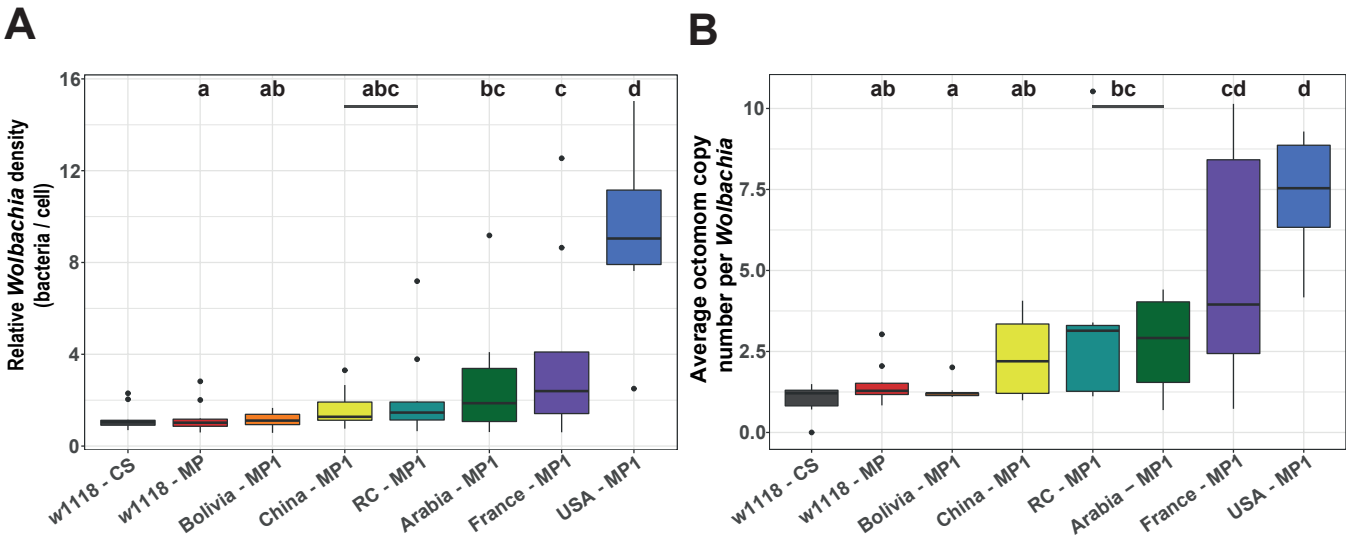
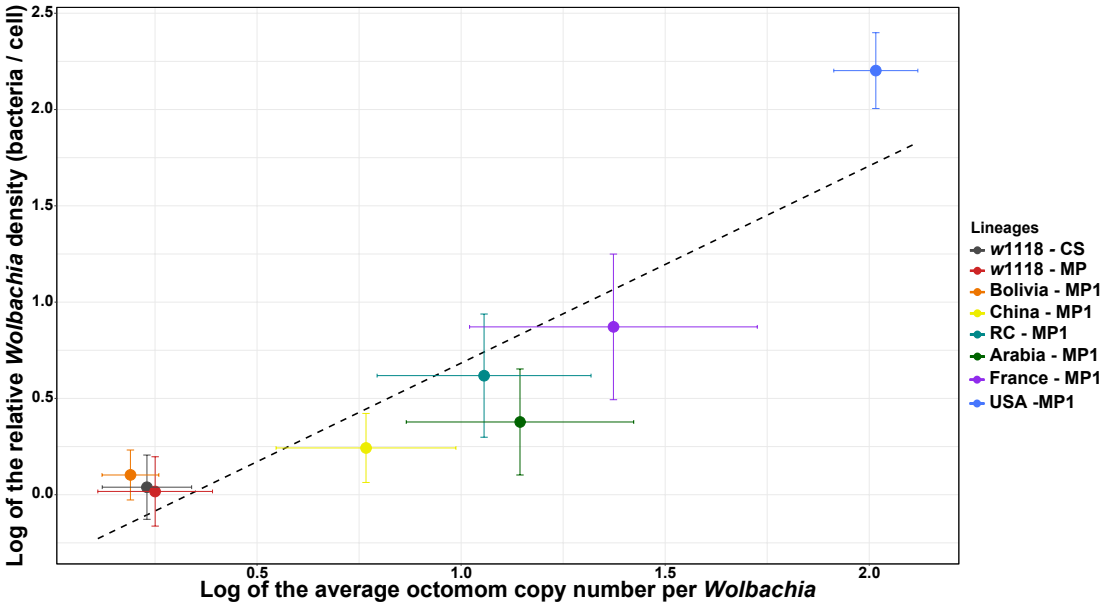


Figure 2: Infection patterns after introgression of *Wolbachia* in different host genetic backgrounds.
2A: relative *Wolbachia* density per cell, **2B:** average Octomom copy number per *Wolbachia*. Each color represents a host genetic background (n = 10 flies / background). Box plots indicate 'minimum', 1st quartile, median, 3rd quartile, and 'maximum' ± outliers (dots). Different letters above boxplots indicate a significant difference between lines after pairwise comparisons (Table s3). The *w*1118-CS line is an experimental control infected by *w*MelCS and is not integrated in the statistical analyses. The *w*1118-MP line, infected by *w*MelPop, is the line initially used as 'donor' for the introgression procedure. All the other lines were infected by *w*MelPop by introgression (MP1).

244 We observed a positive relationship between the relative density per line and the associated mean Octomom copy
245 number per *Wolbachia* (*Intercept* = -0.40, *SE(intercept)* = 0.25, *slope* = 1.07, *SE(slope)* = 0.22, r^2 = 0.83, Linear
246 regression model on the median of each host genetic background : *P* = 0.005; Figure 3). This strong correlation
247 suggests that variation in the number of Octomom copies is a genetic mechanism involved in the control of bacterial
248 density and confirms previous results highlighted by Chrostek *et al.* (2013). The number of Octomom repeats could
249 thus provide a way to monitor the evolution of bacterial populations across generations and to better characterize
250 selective pressures associated with the control of bacterial populations.



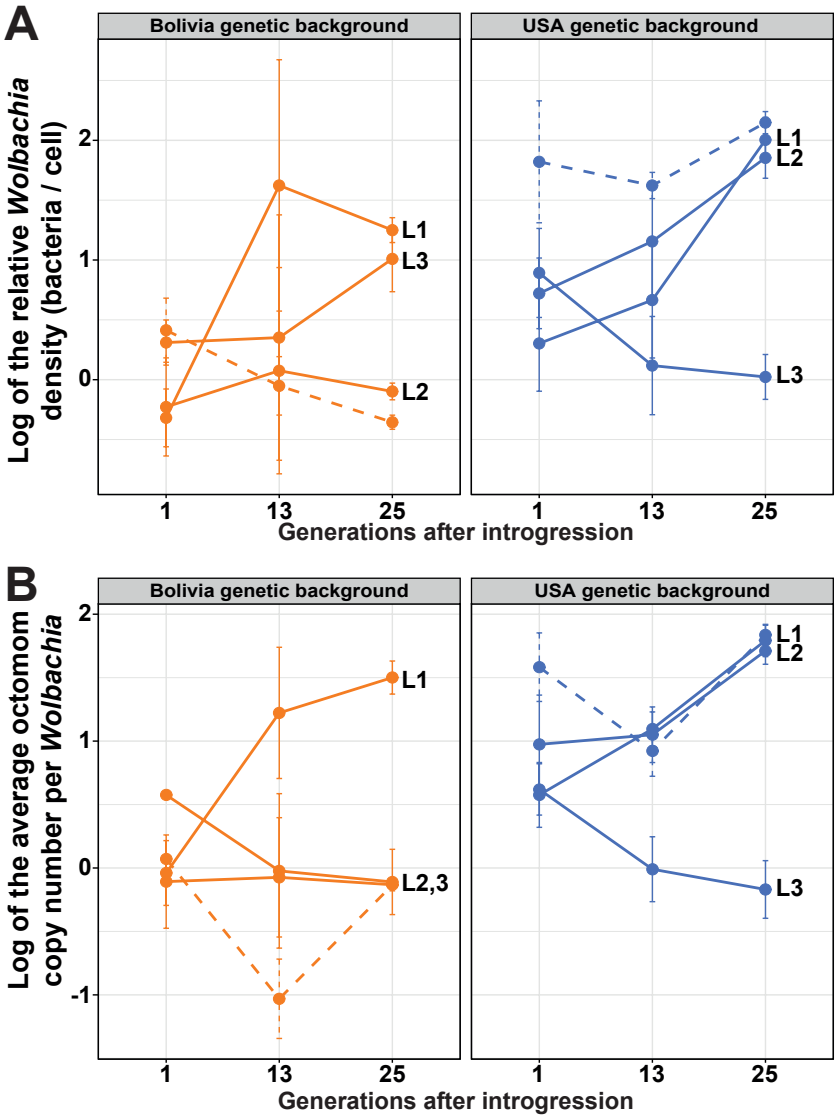
251
252
253 **Figure 3: Relationship between the relative *w*MelPop density (log) and the average Octomom copy number per *Wolbachia***
254 **cell (log).** Median ± SE. Each color represents a host genetic background (n = 10 flies / background), and the dashed line
255 represents the linear regression.
256

257 To summarize, we observed in this preliminary experiment a large variation of *Wolbachia* densities between the 6
258 lines of *Drosophila melanogaster* introgressed with the same *w*MelPop line – densities that were strongly correlated
259 to the average Octomom copy number per *Wolbachia*. These results are consistent with the selection of specific
260 variants by different host genetic backgrounds. However, other factors, like genetic drift by founder effect during
261 the vertical transmission of symbionts from the donor line and / or from one host generation to another, could
262 explain this pattern. To disentangle these hypotheses, we thus performed two sets of experiments using the two
263 lines that exhibited the most extreme patterns of infection in the preliminary experiment (*i.e.*, Bolivia and USA).

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

265 In the first set of experiments, we performed a similar introgression of the *w*MelPop *Wolbachia* strain in the Bolivia
266 and USA genetic backgrounds, but established three independent replicate lines for each host background (Bolivia-
267 MP2 and USA-MP2 lines). While the three replicates should exhibit the same response under host control, variation
268 among the three replicates is expected under the drift hypothesis. We additionally evaluated the stability of the
269 infection pattern over generations, by tracking the relative *Wolbachia* density (Figure 4A) and the average

Octomom copy number per *Wolbachia* (Figure 4B) immediately after the first introgression event, after 13 generations, and after 25 generations (see Tables s4 & s5 for details).



<u>Figure 4:</u> Replicability of the infection patterns after a new introgression procedure, and their evolution over generations. **4A:** relative *Wolbachia* density (log; median ± SE), **4B:** average Octomom copy number per *Wolbachia* (log; median ± SE), n = 5 / line / timepoint. Each color represents a host genetic background. Plain lines represent the replicate lineages from the new introgression procedure (MP2), L1, L2 and L3 indicating the replicates. Dashed lines represent the lineages from the initial introgression procedure (MP1), which were set as references in the statistical analyses.

Just after the introgression event (t = 1), the relative *Wolbachia* density and the average Octomom copy number per *Wolbachia* did not differ significantly between the Bolivia-MP2 replicate 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 in *Wolbachia* from the Bolivia-MP2 replicate lines did not differ significantly between replicates (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 *Wolbachia* from the USA-MP2 replicate lines tend to differ from the USA-MP1 line from the first experiment used here as the reference (Linear

288 regression model; experiment group effect on relative density: $P = 0.08$; experiment group effect on Octomom
289 copy number: $P = 0.025$; see statistical details in Table s4). The relative density and the average Octomom copy
290 number in *Wolbachia* from the USA-MP2 replicates lines did not differ between replicates (see pairwise
291 comparisons in Table s5) and the number of octomom copies per *Wolbachia* did not show significant difference
292 with the donor line (w1118-MP) (Linear regression model; maternal transmission effect on Octomom copy number:
293 $P = 0.35$; Table s4). These results are more consistent with a maternal transmission effect from the donor line to
294 the recipient ones, with infection patterns mirroring the bacterial composition of the donor line.

295 We then examined the stability of the infection pattern over generations, for each replicate line (Figure 4). After 25
296 generations post introgression, the relative density and Octomom copy number per *Wolbachia* in Bolivia-MP2 and
297 USA-MP2 replicate lines differed significantly from the quantities measured immediately after introgression (Linear
298 regression model; generational effect on relative density: $P_{Bolivia} = 6.22\text{x } 10^{-06}$, $P_{USA} = 6.23\text{x } 10^{-05}$; generational effect
299 on Octomom copy number: $P_{Bolivia} = 7.25\text{x } 10^{-04}$, $P_{USA} = 0.005$; see statistical details in Table s4). Moreover, the
300 infection patterns between the Bolivia-MP2 or between the USA-MP2 replicate lines significantly differed (see
301 pairwise comparisons in Table s5).

302 Finally, we noticed that the correlation between the bacterial density and the average number of Octomom copies
303 per *Wolbachia* remained high over generations ($R^2 > 71\%$, Figure s2) and within lines, when variation in *Wolbachia*
304 density was observed (Figure s3 and associated statistics).

305 All together, these results show an absence of host control on the density and composition of the bacterial
306 population, and an influence of the number of Octomom copies on the control of *Wolbachia* densities. The
307 variations observed between replicate lines and over time may thus reflect drift.

308 **<u>The bacterial composition initially transmitted strongly influences the patterns of infection observed</u>**
309 **<u>over generations</u>**

310 In the second set of experiments, we performed reciprocal crosses to test the respective influence of the host
311 genetic background, the bacterial population, the maternal effect of transmission, and drift on the *w*MelPop
312 proliferation within flies (experiment #3). In order to jumble the host-*Wolbachia* associations, we made reciprocal
313 crosses in 3 independent replicates using the Bolivia-MP1 and USA-MP1 lines from the first experiment. Then, to
314 evaluate the stability of the infection pattern over generations, we measured the relative density of *w*MelPop
315 (Figures 5A & 5B) and the average Octomom copy number per *Wolbachia* per fly (Figures 5C & 5D) one generation
316 after the final homogenizing cross, 13 generations and 25 generations post introgression (see Tables s6 & s7 for
317 details).

318 Just after the introgression event (t = 1), the relative density and the average Octomom copy number per *Wolbachia*
319 differed significantly between the Bolivia(USA-MP1) replicate lines and the Bolivia-MP1 line (Linear regression
320 model, experiment group effect on relative density: $P = 0.047$; experiment group effect on Octomom copy number:
321 $P = 4.88 \text{ x } 10^{-09}$, see statistical details in Table s6), but not from the USA-MP1 line (Linear regression model,
322 experiment group effect on relative density: $P = 0.798$; experiment group effect on Octomom copy number: $P =$

0.586, 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 per *Wolbachia* from the USA(Bolivia-MP1) replicate lines differed significantly from those from the USA-MP1 line (Linear regression model, experiment group effect on relative density $P = 0.018$; experiment group effect on Octomom copy number $P = 6.31 \times 10^{-08}$, see statistical details in Table s6), but not from the Bolivia-MP1 line (Linear regression model, experiment group effect on relative density: $P = 0.416$; experiment group effect on Octomom copy number: $P = 0.559$, 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.

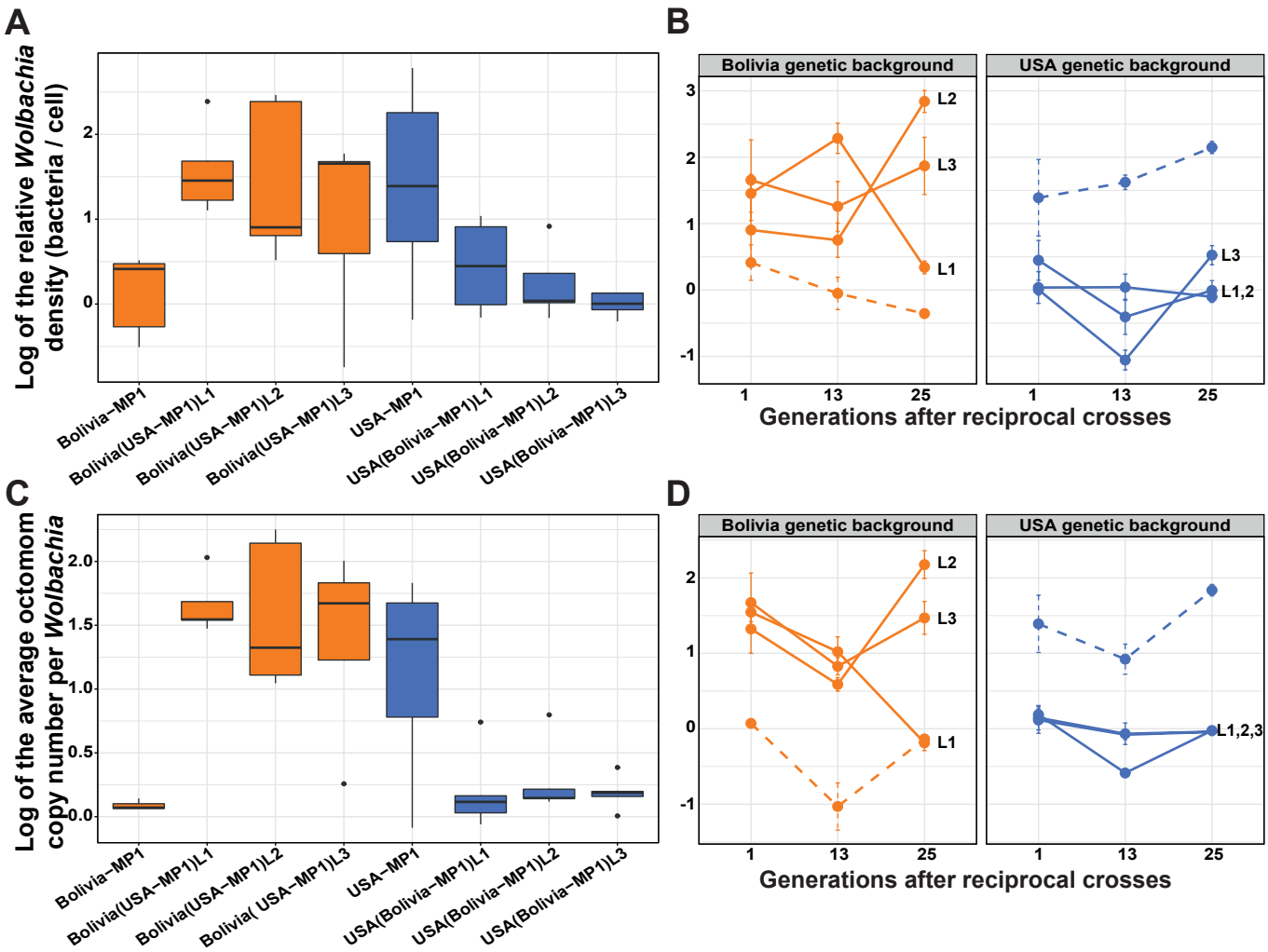


Figure 5: Evolution of infection patterns after reciprocal crosses.
5A: Relative density (log) one generation post introgression (box plot with 'minimum', 1st quartile, median, 3rd quartile, and 'maximum' ± outliers (dots)). **5B:** Evolution of the relative density (log) over generations (median ± SE). **5C**: Average Octomom copy number per *Wolbachia* (log) one generation post introgression. **5D:** Evolution of the average Octomom copy number per *Wolbachia* (log) over generations (median ± SE). N = 5 flies / line / timepoint. 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, L1, L2 and L3 indicating the replicates. Dashed lines represent the lineages from the initial introgression procedure (MP1), which were used as references in the statistical analyses.

346
347 Next, we examined whether infection patterns were stable within each of the replicate lines over generations by
348 measuring the relative density and average Octomom copy number per *Wolbachia* 13 and 25 generations after the
349 last backcross. After 25 generations post introgression, the relative density in Bolivia(USA-MP1) replicate lines
350 differed significantly from their reference at t = 1 (Linear regression model, generational effect on the relative
351 density: $P = 0.015$; generational effect on the Octomom copy number: $P = 7.79 \times 10^{-05}$, see statistical details in Table
352 s6). On the contrary, the USA(Bolivia-MP1) replicate lines did not differ from their reference at t = 1 (Linear
353 regression model, generational effect on the relative density: $P = 0.052$; generational effect on the Octomom copy
354 number: $P = 0.059$, see statistical details in Table s6). Moreover, the infection patterns of the Bolivia(USA-MP1)
355 replicate lines differed significantly from each other, as did the infection patterns of the USA(Bolivia-MP1) replicate
356 lines (see pairwise comparisons in Table s7). All together, these results confirm an absence of host control on the
357 density and composition of the bacterial population. Moreover, the variability observed between the replicates
358 indicates a random transmission of the symbiont over generations.

359 Finally, we confirmed that the correlation between the bacterial density and the average number of Octomom
360 copies per *Wolbachia* remained high over generations ($R^2 > 83\%$, Figure s4) and within the lines, when variation in
361 *Wolbachia* density was observed (Figure s5 and associated statistics).

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

367

368 # Discussion

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

382 Numerous examples in insects support an active regulation of symbiotic populations by the host, with stable density
383 over generations when the environment remains constant (Ikeda, Ishikawa and Sasaki, 2003; Mouton *et al.*, 2004,
384 2007; Funkhouser-Jones *et al.*, 2018). The orchestrated modulation of the symbiont proliferation rate throughout
385 insect development also suggests a fine-tuned host control of the bacterial density (Rio *et al.*, 2006; Login *et al.*,
386 2011; Vigneron *et al.*, 2014). On the opposite, bacterial factors alone can also explain variation in bacterial densities
387 within some hosts. For example, different strains of *Wolbachia* are known to exhibit different, but stable, density
388 levels in the same host lines based on their genetic particularities (Mouton *et al.*, 2003; Chrostek *et al.*, 2013).
389 Proliferation of symbionts within the host is under strong selection as it is a key factor influencing the trade-off
390 between symbiont transmission (*i.e.*, the higher the symbiont density, the higher the probability of transmission)
391 and virulence (*i.e.*, the higher the symbiont density, the higher the cost on host survival and fecundity) (Anderson
392 and May, 1982; Ewald, 1983). This transmission/virulence trade-off often leads to an optimal density, which can be
393 controlled by host or bacterial determinants. In insect hosts, the main molecular mechanisms that determine the
394 abundance and composition of symbionts are associated with immune response (Lemaitre and Hoffmann, 2007;
395 Zug and Hammerstein, 2015) or resource allocation (Kiers *et al*., 2003). Microbial communities can in turn select
396 resistance mechanisms against host effectors or trigger antagonist regulators of the host immunity (Vallet-Gely *et*
397 *al.*, 2008; Lindsey, 2020).

398 In the *D. melanogaster*-*w*MelPop system, however, we observed a strong instability of infection patterns. Because
399 the introgression was limited to 87.5%, a few alleles from the donor background – different in each replicate line –
400 could marginally influence the *Wolbachia* load. However, incomplete introgression should not affect the results of
401 the experiment where lines with the two most extreme phenotypes were crossed. Alternatively, the high instability
402 could be due either to an instability of the optimum, or to a large influence of drift that limits the ability of the
403 system to reach the optimum. Our results, and notably the variations observed between replicate lines in controlled

404 rearing conditions, rather suggest a strong influence of drift on the regulation of bacterial density. Bacterial factors,
405 such as the number of Octomom copies, could fluctuate through time and be at the origin of variation in density
406 levels. However, the genomic region 'Octomom' has recently been questioned regarding its involvement in the
407 establishment of density levels. Initially, it has been shown that proliferation rate and virulence of *w*MelPop are
408 correlated with genomic amplification of the Octomom region (Chrostek *et al.*, 2013; Chrostek and Teixeira, 2015,
409 2018), but this relationship has been challenged by Rohrscheib and her collaborators (Rohrscheib *et al.*, 2016,
410 2017), who support that the virulence of *w*MelPop rather depends on an increase in the extrinsic rearing
411 temperature. However, to exclude any influence of the Octomom copy number on *Wolbachia* growth and
412 pathogenicity, these variables should be tested independently of the temperature (Chrostek and Teixeira, 2017).
413 At constant temperature, our results show a clear link between *Wolbachia* density in adults and the average
414 number of Octomom copies per *Wolbachia*, and are in accordance with the current literature (Chrostek *et al.*, 2013;
415 Chrostek and Teixeira, 2015, 2018; Monnin *et al.*, 2020). While we cannot exclude that another gene or set of
416 *Wolbachia* genes different from Octomom could determine symbiont density, the strong correlation between the
417 density observed and the number of Octomom repeats in all the experiments (generally >80% when the density
418 was variable) suggests that Octomom is the main determinant of bacterial density in this biological system and in
419 our controlled conditions. Consequently, this genomic amplification can be used as a marker of bacterial diversity
420 and evolution of our experimental system.

421 We can thus wonder why we observed such variability and temporal instability within lineages, and why drift
422 overcame this potential bacterial regulation through Octomom? Indeed, this pattern contrasts with what is
423 observed in already well-established symbioses, where one symbiont genotype is fixed (Werren, Baldo and Clark,
424 2008). In our experiments, we were able to show very similar levels of infection between mothers and daughters
425 just after introgression or reciprocal crosses procedures, suggesting a maternal effect. However, instability detected
426 across generations suggests that this maternal effect is probably non-genetic: when a large number of bacteria is
427 quantified in the mother's tissues, a large number of bacteria is transmitted to the oocytes and maintained in the
428 adult stage (Veneti *et al.*, 2004; Hosokawa, Kikuchi and Fukatsu, 2007; Parkinson, Gobin and Hughes, 2016).
429 However, there may still be random variability between mothers regarding the amount of bacteria transmitted to
430 their eggs, and between these eggs (Mira and Moran, 2002). Bottlenecks during transmission can thus eventually
431 lead to a gradual shift of the 'initial' density over time. Bottlenecks can also influence density levels through random
432 differential transmission of bacterial variants from one generation to the next (Funk, Wernegreen and Moran, 2001;
433 Kaltenpoth *et al.*, 2010), especially if these variants exhibit different reproductive rates (as it is the case with variants
434 carrying different numbers of Octomom copies (Duarte *et al.*, 2021)). To summarize, if not counteracted by host or
435 symbiont density control, drift is expected to induce instability over generations by a combination of quantitative
436 (*i.e.*, transmission of a non-equivalent number of bacteria to the eggs) and qualitative/genetic (*i.e.*, random
437 transmission of different variants) bottlenecks. Hence, the high variability and the temporal instability depicted in
438 our study could reflect the random transmission of different *w*MelPop quantities and variants during transmission
439 bottlenecks. In addition, drift at the transmission level alone cannot explain variation of the average density at the
440 population level but should be associated to other factors. Drift at the host population level could play a role by

441 fixing hosts with symbiotic populations with a different average of Octomom copy number. Alternatively, selection,
442 or mutation bias could also play a role. For instance, the mutation rate of a repeated sequence in microsatellites
443 can strongly depend on the number of motifs present in the sequence (Whittaker *et al.*, 2003). Assuming the same
444 rules on the number of Octomom copies present in the population (*i.e.*, a higher propensity for duplication when
445 the number of copies is high), the outcomes of drift could be more unpredictable for individuals harboring the
446 highest average number of Octomom copies. Indeed, in the presence of moderate bottlenecks and a mutation rate
447 increasing with the number of copies, a strong variability of infection patterns is expected over generations.
448 However, we did not observe such higher temporal instability when the values of density and Octomom copy
449 numbers were high in the donor lines. These results suggest that the influence of the mutation rate was negligible
450 compared to the transmission bottleneck. Under conditions of genetic instability linked to transmission
451 bottlenecks, between-host selection should not be efficient, and would explain why the vertically transmitted
452 *w*MelPop strain exhibits a strong virulence, whereas the overall alignment of interests between the host and
453 vertically transmitted symbionts generally leads to the selection of low virulent symbionts that maximize host
454 survival and indirectly their own transmission (Anderson and May, 1982; O'Neill, Hoffmann and Werren, 1997).

455 Different environmental conditions can also modify the optimum density of the symbiont. Optima can be different
456 for the host and the symbionts, and lead to antagonistic interactions between symbiotic partners and to variations
457 in bacterial density (Parker *et al.,* 2021). In the *D. melanogaster* / *w*MelPop system, the maintenance of the
458 virulence phenotype has thus frequently been associated with the fact that virulence is only expressed in conditions
459 rarely observed in nature, so that the between-host selection against highly prolific variants (such as those with
460 high Octomom copy numbers) is weak at 25°C. A recent study however shows that strains with 8-9 Octomom copies
461 are pathogenic from 18°C to 29°C (Duarte *et al.*, 2021). In addition, another selective force, the within-host
462 selection, could explain the virulence of *w*MelPop in certain environmental conditions. Indeed, when fly
463 populations are reared at 28°C, Monnin *et al.* (2020) showed that the population evolved toward a higher virulence,
464 which may be due to the stronger effect of within-host selection compared to between-host selection. Thus, when
465 selective pressures are strong, within and between-host selection could modulate symbiont virulence in the
466 *Drosophila*-*w*MelPop association, whereas drift might not allow any co-evolution between partners and co-
467 adaptation to environmental changes when selective pressures are limited.

468 To conclude, we showed that the host did not control for bacterial density and composition in the symbiosis
469 between *D. melanogaster* and *w*MelPop, and that the infection patterns were very instable across generations,
470 suggesting a strong influence of drift that could limit the effects of within- and between-host selections. As the
471 transmission of symbionts in vertically transmitted symbiosis is subject to potential bottlenecks both in terms of
472 quantity and genetic diversity (Mira and Moran, 2002; Galbreath *et al.*, 2009; Kaltenpoth *et al.*, 2010), it seems
473 necessary to further characterize the intensity of bottlenecks in this symbiotic system, in order to better evaluate
474 the impact of drift on the evolution of bacterial populations in vertically transmitted symbioses and its impact on
475 host phenotypes.
476

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483 # Conflict of interest disclosure

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

486 # Data accessibility & Supplementary material

487 Raw data and scripts are available online: https://doi.org/10.5281/zenodo.4607210 and
488 https://doi.org/10.5281/zenodo.4607223, respectively.
489

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