Within host selection for faster replicating bacterial symbionts

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Abstract

Wolbachia is a widespread, intracellular symbiont of arthropods, able to induce reproductive distortions and antiviral protection in insects. *Wolbachia* can also be pathogenic, as is the case with *w*MelPop, a virulent variant of the endosymbiont of *Drosophila melanogaster*. An extensive genomic amplification of the 20kb region encompassing eight *Wolbachia* genes, called Octomom, is responsible for *w*MelPop virulence. The Octomom copy number in *w*MelPop can be highly variable between individual *D. melanogaster* flies, even when comparing siblings arising from a single female. Moreover, Octomom copy number can change rapidly between generations. These data suggest an intra-host variability in Octomom copy number between *Wolbachia* cells. Since *w*MelPop *Wolbachia* with different Octomom copy numbers grow at different rates, we hypothesized that selection could act on this intra-host variability. Here we tested if total Octomom copy number changes during the lifespan

of individual *Drosophila* hosts, revealing selection for different *Wolbachia* populations. We performed a time course analysis of Octomom amplification in flies whose mothers were controlled for Octomom copy number. We show that despite the Octomom copy number being relatively stable it increases slightly throughout *D. melanogaster* adult life. This indicates that there is selection acting on the intra-host variation in the Octomom copy number over the lifespan of individual hosts. This within host selection for faster replicating bacterial symbionts may be in conflict with between host selection against highly pathogenic *Wolbachia*.

Introduction

Gene copy number variation is one of the mechanisms allowing rapid evolution across the tree of life [1–3]. In bacteria, growth inhibition by nutrient limitation or antibiotic presence may be overcome by increasing copy number of genes functionally related to these challenges [4]. Moreover, amplified genomic regions allow accumulation of mutations without the risk of loss of the original function. This can lead to the generation of more beneficial variants and subsequent loss of extra copies or repurposing of the new copies for a new function [4]. Thus, genomic amplifications generate extensive and reversible genetic variation, which can either increase the fitness of an individual directly or be a substrate on which adaptive evolution can act.

We have previously found that a genomic amplification affects the biology of the intracellular, maternally transmitted bacterium *Wolbachia* [5]. *Wolbachia* is a widespread endosymbiont of insects, causing an array of phenotypes, including reproductive manipulations [6] and antiviral protection [7,8]. Moreover, some *Wolbachia* strains can strongly reduce the host lifespan. This was first described for

50 *w*MelPop, a laboratory *Wolbachia* variant, in *Drosophila melanogaster* [9]. The
51 Octomom genomic region, which contains eight *Wolbachia* genes, is amplified in
52 *w*MelPop, while it is present as a single copy in closely related non-pathogenic
53 *Wolbachia* variants [10,11]. The number of copies of this region varies greatly
54 between individual *w*MelPop-infected flies from the same population, ranging from
55 two to ten copies [5]. We have previously established *D. melanogaster* lines carrying
56 defined and different Octomom copy numbers and observed that the higher the
57 Octomom copy number, the higher *Wolbachia* levels and the shorter the lifespan of its
58 *D. melanogaster* host [5]. Moreover, a *w*MelPop that reverted to carrying only one
59 Octomom copy proliferates at the same rate as the control *w*MelCS_b variant and is
60 not pathogenic [5]. Thus, we identified Octomom copy number as a pathogenicity
61 determinant of *w*MelPop [5].

62 The high variation in Octomom copy numbers between individual flies can also
63 be observed in the progeny of single *w*MelPop-carrying females [5]. This variation
64 between siblings could be explained by *Wolbachia* variation within a female and
65 differential symbiont assortment to the progeny. The fact that the variability
66 decreased under selection argues for initial high variation within single flies, which is
67 pruned over a few generations of selection for either the highest or the lowest
68 Octomom copy number. However, even under constant selection some variation was
69 either maintained or continuously generated, since reversing the direction of the
70 selection or relaxing it could rapidly change the Octomom copy numbers in these
71 lines [5].

72 We hypothesized that variation in Octomom copy number between *Wolbachia*
73 cells within an individual host could lead to a differential growth of these cells. If
74 *Wolbachia* cells with higher Octomom copy number proliferate more, their frequency

in the pool of *Wolbachia* within a host will increase over time, and the average Octomom copy number of the within-host population increases over the host lifespan. We tested this hypothesis through a time course analysis of Octomom copy number in individual *w*MelPop flies originating from mothers with controlled Octomom copy number.

Results

We examined the stability of Octomom copy number over the adult life (from eclosion to the onset of high mortality) in single *w*MelPop-carrying *D. melanogaster* (Fig 1A). The flies were the offspring of parents carrying *w*MelPop with low Octomom copy number (median of 4.5 Octomom copies, range from 4 to 5.5) or high Octomom copy number (median of 9.5, range from 8.5 to 11.5). The low Octomom cohort had, on average, 5.45 (standard deviation, SD = 0.82) Octomom copies per genome and the high Octomom cohort had an average of 10.29 (SD = 1.90) Octomom copies per genome. The fitted generalized additive model (GAM) clearly shows an increase in Octomom copy number in the first few days (six to eight days), which subsequently levels off at the later timepoints (Fig 1A and B). The trend is highly significant (the smooth term for time, $p < 0.001$). This shows that Octomom copy number in the *w*MelPop population increases during most of the adult host lifespan.

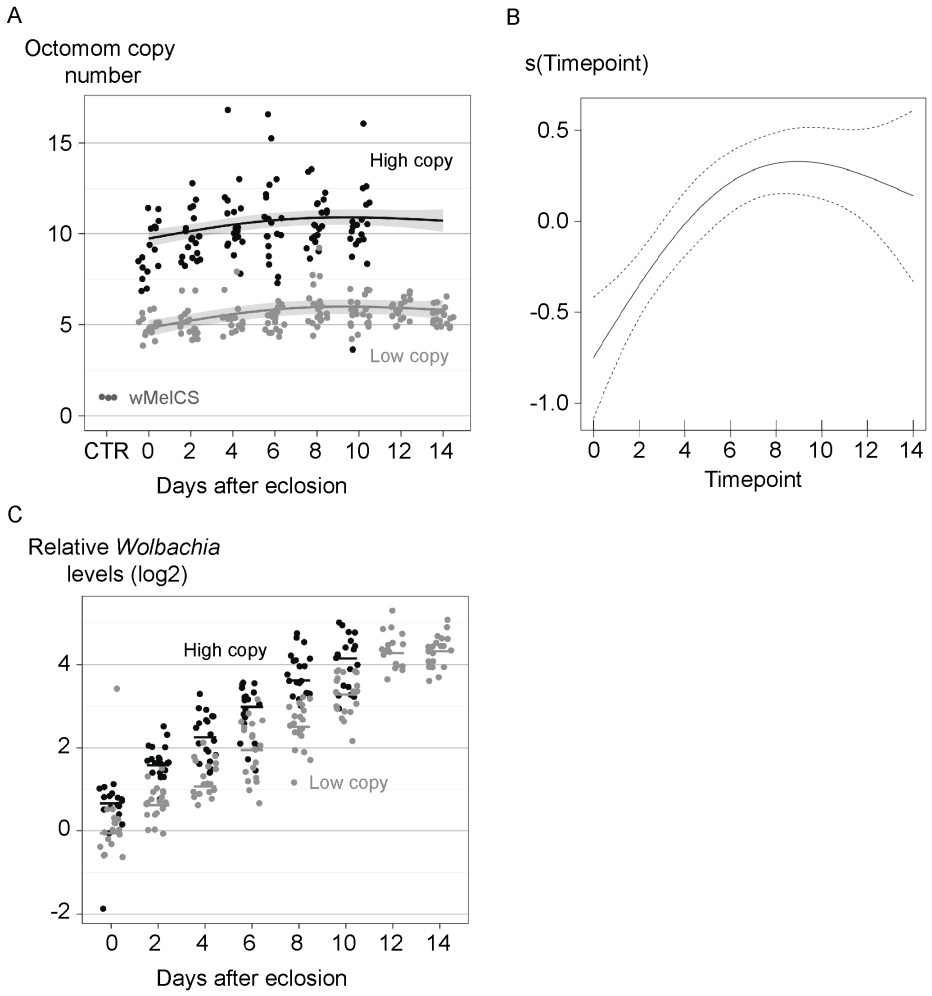

Figure 1. Octomom copy number and *Wolbachia* levels during adult *D. melanogaster* life. (A) Each dot represents *WD0513* genomic levels in a single fly, as we have previously shown that this gene can be used to estimate Octomom region copy number [5]. The values were obtained using the Pfaffl method with *wsp* as a reference gene and calibrated using the median of three samples of control *w*MelCS_b flies (CTR). The lines represent the fit of the generalized additive model (GAM) and shaded area - the 95% confidence interval. (B) Fitted GAM smooth of Octomom copy number in response to the age of adults. The 95% confidence interval is indicated by the dashed lines. Note the Y-axis is standardized so that average is zero. (C) Each dot represents *wsp* levels in a single fly. Lines are medians of the replicates. *wsp* levels were obtained using Pfaffl method with *Rpl32* as a reference gene and calibrated using the median of the low copy number samples at time zero. (A) and (C) represent data from single females derived from the 3rd generation of selection for Octomom copy number in the DrosDel isogenic w^{1118} genetic background [5]. Females were raised and kept at 25ºC (survival of their siblings is shown in Fig. S5A of [5]). Supporting data can be found in S1 Data.

In parallel, we tested *Wolbachia* titer in the same flies. *Wolbachia* levels increase with the age of flies (log-linear mixed-effect model (lme), $p < 0.001$) and flies carrying *w*MelPop with higher Octomom copy numbers have 1.85 times higher *Wolbachia* levels compared to flies carrying *w*MelPop with lower Octomom copy numbers (lme , $p < 0.001$), confirming previous results (Fig 1C). However, we do not see a significant interaction between cohort and growth rate (lme, $p = 0.102$). We also tested if Octomom copy number could be an explanatory variable for *Wolbachia* levels within each cohort. This variable was not significant (lme, $p = 0.48$).

Discussion

By analyzing cohorts of individuals from mothers carrying *Wolbachia* with controlled Octomom copy number we observed that Octomom copy number is relatively stable over the life of the host. However, we detected a small, but clear and statistically significant, increase in Octomom copy number over time in the first six to eight days of the adult *D. melanogaster* life. This could be explained by selection acting on the heterogeneity of *w*MelPop copy number between *Wolbachia* cells within a single host. Since bacteria with higher copy numbers grow faster over time, they contribute more to the total pool of *w*MelPop in an older fly and therefore increase total Octomom copy number. In our dataset, the Octomom copy number stops increasing at the last days of the host life. This is surprising and could be explained by the initial heterogeneity in the flies being reduced in the course of selection. If *Wolbachia* cells with the maximal Octomom copy number within each fly reach a very high frequency or are fixed, there is no genetic variation for selection to act on,

132 and the Octomom copy number does not continue to increase. Alternatively, the
133 differential fitness between *Wolbachia* harboring different Octomom copy numbers
134 may decrease with the age of the fly, weakening the strength of the selection and
135 preventing continuous Octomom copy number growth.

136 Importantly, our data indicate that the selective pressures acting on *w*MelPop
137 within and between hosts are different. Within a host, there may be a selection for
138 *Wolbachia* that grow fast. On the other hand, competition between flies could select
139 for *Wolbachia* that grow slower and have a lower cost for the host [5]. These
140 opposing selective pressures may play a role in shaping the evolution of *Wolbachia* in
141 natural populations.

142 Although the results presented here are compatible with our prediction of within
143 host heterogeneity and selection, other forces may contribute to the changes in
144 Octomom copy number during host lifespan. Amplification and deletion of Octomom
145 copies may also occur throughout *w*MelPop host's adult life. The rates of these two
146 opposing mutations will determine an overall tendency of Octomom copy number to
147 increase or decrease over time. Therefore, the dynamics of Octomom copy number
148 changes may be the combination of selection and mutation. A mutation bias for
149 deletion of Octomom copies could temper the effect of selection for higher Octomom
150 copy numbers. On the other hand, a bias for amplification could explain the observed
151 increase in Octomom copy number, even in the absence of selection. Nonetheless, this
152 would also result in an increased proportion of more pathogenic *w*MelPop with host
153 age. The influence of mutation and selection on Octomom copy number change
154 during host lifespan may vary with intrinsic and extrinsic factors. For instance,
155 mutation bias towards amplification or deletion could change with Octomom copy

156 number itself. On the other hand, since temperature has a strong effect on *w*MelPop
157 pathogenicity [9,12], it may also affect the differential growth of *Wolbachia* with
158 different Octomom copy numbers and, therefore, within host selection on this trait. A
159 mechanistic understanding of how Octomom copy number changes and influences
160 *Wolbachia* phenotype and which factors modulate it will help disentangling the
161 relative contribution of selection and mutation to Octomom copy number changes
162 during *D. melanogaster* life.

163 *Wolbachia* with higher Octomom copy number proliferate more and are more
164 pathogenic to their hosts [5]. The within host selection for bacteria that proliferate
165 more and have a higher potential of being deleterious to the host may be a common
166 phenomenon. For instance, *Staphylococcus aureus* variants that cause blood or deep
167 tissue infection are, in the majority, the result of within-host selection from non-
168 pathogenic nose colonizing variants [13]. The adaptations conferring high virulence
169 identified in this study do not favor *S. aureus* dissemination and onward transmission
170 (discussed in [13]). Thus, the selective pressure acting on the bacteria within a single
171 host may be in conflict with the selective pressure acting on the entire bacterial
172 population. This implies that although the more pathogenic bacterial variants arise
173 throughout the life of the hosts, they are constantly purged from the overall bacterial
174 population by selection either on the fitness of the host (vertically transmitted
175 symbionts) or on the bacterial transmission capacity (horizontally transmitted
176 symbionts).

177 A recent report suggested that *w*MelPop Octomom copy numbers change
178 drastically during *D. melanogaster* lifespan, increasing more than two-fold in the first
179 ten days of adult life and then decreasing more than four-fold over the next thirty days

180 [14]. These results differ from the ones we present here and may be explained by the
181 lack of experimental control for the Octomom copy number in these flies and
182 determination of the Octomom copy numbers after the onset of mortality. Using data
183 from Chrostek and Teixeira 2015 [5] we constructed a model showing that in a mixed
184 population of flies the differential growth of *Wolbachia* with different Octomom copy
185 numbers, combined with differential death of flies carrying *Wolbachia* with different
186 Octomom copy numbers, leads to initial increase in Octomom copy number, followed
187 by a decrease due to death of the flies carrying *Wolbachia* with higher Octomom copy
188 number at the host population level [15].

189 Here we confirmed that *w*MelPop with higher Octomom copy number has higher
190 *Wolbachia* titers, supporting our conclusion that this amplification controls *w*MelPop
191 levels. However, the difference in growth rate between these lines was not statistically
192 significant. This may be due to the high variability in the data and the differential
193 growth between these lines being potentially small. We have previously shown
194 different growth rates between *w*MelPop carrying one and two copies of Octomom
195 and between these and *w*MelPop carrying 12 or 15 copies [5]. However, the growth
196 rate of *w*MelPop carrying 12 and 15 copies was not significantly different [5]. This
197 indicates that the relationship between growth rate and Octomom copy number is not
198 linear and that differences in *w*MelPop carrying higher copy numbers may have a
199 smaller impact on growth. Therefore, the difference in growth between *w*MelPop
200 carrying five and ten copies may indeed be small. The difference in *Wolbachia* titers
201 despite the lack of measurable difference in the growth rate might be the result of the
202 cumulative effect of small growth rate differences throughout the fly development
203 from egg to adult, the result of a differential growth rate at different development

204 stages, or even the accumulation of small differences in growth for more than one
205 generation.

206 The labile nature of the Octomom amplification and the resulting phenotypes of
207 this amplification make *w*MelPop an interesting case study to understand genome
208 dynamics and selective forces acting on endosymbionts. This system may be further
209 used in the future to reveal general principles in host-bacteria symbiosis.

210

211 Material and methods

212 Fly strains

213 *D. melanogaster* DrosDel isogenic background (*iso*) flies with *w*MelCS_b and
214 *w*MelPop were described before [5,10]. Selection on *D. melanogaster* lines carrying
215 *w*MelPop with different Octomom copy number was described in [5].

216

217 Experimental setup for time-course analysis of *WD0513* 218 and *Wolbachia* levels

219 Female progeny of females from the 3rd generation of selection for Octomom
220 copy number in the *D. melanogaster* DrosDel isogenic background (*iso*) [5] was
221 collected at eclosion (ten females per tube), allowed to mate with brothers for 24 h
222 (five males per tube), separated from males, and 20 females were sacrificed every
223 second day for *WD0513* and *Wolbachia* density quantification. Females were
224 maintained at 25°C on a standard cornmeal diet without live yeast and were passed to
225 fresh vials every 3 days. We sampled only until the onset of high mortality in the
226 different lines in order to avoid sampling bias for surviving, low Octomom copy
227 number bearing flies.

228

229 ## DNA extractions

230 DNA was extracted from single flies (*w*MelPop) or pools of ten flies (*w*MelCS_b
231 controls). Each fly or pool of flies was squashed in 250 µl of 0.1 M Tris HCl, 0.1 M
232 EDTA, and 1% SDS (pH 9.0) and incubated 30 min at 70°C. Next, 35 µl of 8 M
233 CH_3CO_2K was added, and samples were mixed by shaking and incubated on ice for
234 30 min. Subsequently, samples were centrifuged for 15 min at 13,000 rpm at 4°C, and
235 the supernatant was diluted 100× for qPCR.

236

237 ## Real-time quantitative PCR

238 The real-time qPCR reactions were performed using CFX384 Real-Time PCR
239 Detection System (Bio-Rad) as described before [5,10]. Each reaction contained 6 µl
240 of iQ SYBR Green Supermix (Bio-Rad), 0.5 µl of each primer (3.6 mM), and 5 µl of
241 diluted DNA. We performed two technical replicates for each sample for each set of
242 primers. Primer sequences were described before [5]. For all three genes assayed:
243 *Wolbachia WD0513* and *wsp,* and *Drosophila Rpl32* the following thermal cycling
244 protocol was applied: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C, 1
245 min at 59°C, and 30 s at 72°C. Melting curves were examined to confirm the
246 specificity of amplified products. Ct values were obtained using Bio-Rad CFX
247 Manager software with default threshold settings. Ct values were subjected to a
248 quality check - samples with standard deviation between technical replicates
249 exceeding 0.5 for one of the genes were discarded. The experiment spanned six qPCR
250 plates and three samples of ten wMelCS_b flies (extracted and aliquoted beforehand
251 and assayed on every qPCR plate) were used to normalize between plates. Relative
252 amounts of genes were calculated by the Pfaffl method [16]. To apply the method, the

253 efficiency of each primer set was predetermined in a separate experiment. For relative
254 Octomom copy number quantification, *WD0513* was the target gene and the single-
255 copy *wsp* gene was used as a reference. The medians of three samples of pools of ten
256 wMelCS_b flies were used as control values for the Pfaffl method. *w*MelCS_b has
257 one copy of the Octomom region in the genome, determined by the coverage analysis
258 of sequencing data [10]. This sample, with known Octomom copy number, is required
259 to estimate Octomom copy number of the remaining samples [17]. For *Wolbachia*
260 quantification, *wsp* was the target gene and *Drosophila Rpl32* gene was used as a
261 reference. The levels of *wsp* are relative to the median of the samples of the low
262 Octomom cohort at time zero.
263

264 ## Statistical analysis

265 The statistical analysis was performed in R [18]. The script of the analysis is
266 provided in S1 Text. Graphs were generated using the package ggplot2 [19].
267 Since the temporal trend over time of the number of Octomom copies was not
268 linear we analyse it by fitting a Generalized Additive Model (GAM, package mgcv in
269 R [20]). We included time and line as independent variables and PCR plate as a
270 random effect. The smooth terms for the interaction between time and lines were non-
271 significant ($p > 0.114$) and were removed form the final model.
272 Analysis of *wsp* levels over time was performed with log-linear mixed-effect
273 model fits (package lme4 in R [21]). The effect of interaction between factors was
274 determined by an ANOVA comparing models fit to the data with and without the
275 interaction.
276

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Supporting information

S1 Data – Relative levels of *WD0513* and *wsp* in single females carrying wMelPop.

S1 Text – R script with the statistical analysis of the data.