Within host selection for faster replicating bacterial symbionts

Ewa Chrostek[1,2] and Luis Teixeira[1,3]*

1 Instituto Gulbenkian de Ciência, Oeiras, Portugal

2 Current Address: Max Planck Institute for Infection Biology, Berlin, Germany

3 Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal

* Corresponding author:

E-mail: lteixeira@igc.gulbenkian.pt

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 bacteria 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 increases 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.

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, $p = 0.48$).

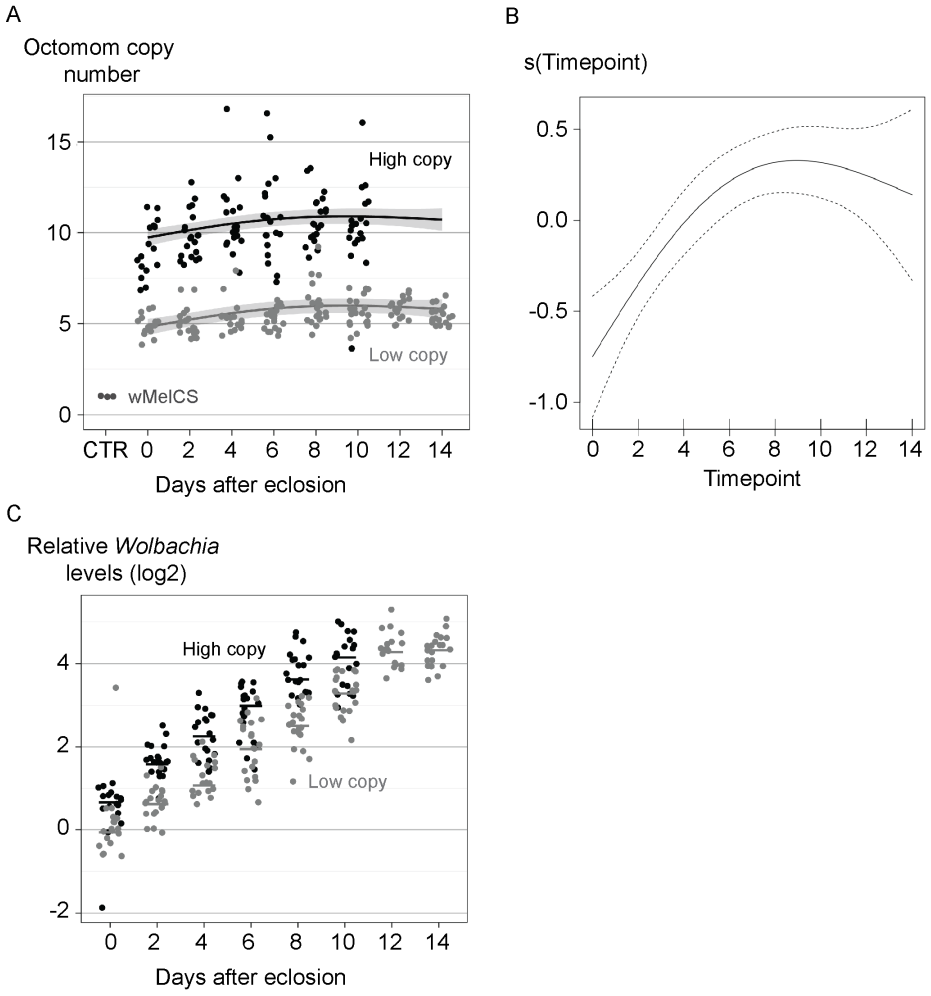


Figure 1. Octomom copy number and *Wolbachia* levels during adult *D. melanogaster* life. (A) Each dot represents *WD0513* genomic levels in a single fly, as we have previously shown that this gene can be used to estimate Octomom region copy number [5]. The values were obtained using the Pfaffl method 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.

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

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

Wolbachia with higher Octomom copy number proliferate more and are more pathogenic to their hosts [5]. The within host selection for bacteria that proliferate more and have a higher potential of being deleterious to the host may be a common phenomenon. For instance, *Staphylococcus aureus* variants that cause blood or deep tissue infection are, in the majority, the result of within-host selection from non-pathogenic nose colonizing variants [12]. The adaptations conferring high virulence identified in this study do not favor *S. aureus* dissemination and onward transmission (discussed in [12]). Thus, the selective pressure acting on the bacteria within a single host may be in conflict with the selective pressure acting on the entire bacterial population. This implies that although the more pathogenic bacterial variants arise throughout the life of the hosts, they are constantly purged from the overall bacterial population by selection either on the fitness of the host (vertically transmitted symbionts) or on the bacterial transmission capacity (horizontally transmitted symbionts).

A recent report suggested that *w*MelPop Octomom copy numbers change drastically during *D. melanogaster* lifespan, increasing more than two-fold in the first ten days of adult life and then decreasing more than four-fold over the next thirty days [13]. These results differ from the ones we present here and may be explained by the

159 lack of experimental control for the Octomom copy number in these flies. Using data
160 from Chrostek and Teixeira 2015 [5] we constructed a model showing that in a mixed
161 population of flies the differential growth of *Wolbachia* with different Octomom copy
162 numbers, combined with differential death of flies carrying *Wolbachia* with different
163 Octomom copy numbers, leads to initial increase in Octomom copy number, followed
164 by a decrease due to death of the flies carrying *Wolbachia* with higher Octomom copy
165 number, at the host population level [14].

166 Here we confirmed that *w*MelPop with higher Octomom copy number has higher
167 *Wolbachia* titers, supporting our conclusion that this amplification controls *w*MelPop
168 levels. However, the difference in growth rate between these lines was not statistically
169 significant. This may be due to the high variability in the data and the differential
170 growth between these lines being potentially small. We have previously shown
171 different growth rates between *w*MelPop carrying one and two copies of Octomom
172 and between these and *w*MelPop carrying 12 or 15 copies [5]. However, the growth
173 rate of *w*MelPop carrying 12 and 15 copies was not significantly different [5]. This
174 indicates that the relationship between growth rate and Octomom copy number is not
175 linear and that differences in *w*MelPop carrying higher copy numbers may have a
176 smaller impact on growth. Therefore, the difference in growth between *w*MelPop
177 carrying five and ten copies may indeed be small. The difference in *Wolbachia* titers
178 despite the lack of measurable difference in the growth rate might be the result of the
179 cumulative effect of small growth rate differences throughout the fly development
180 from egg to adult, the result of a differential growth rate at different development
181 stages, or even the accumulation of small differences in growth for more than one
182 generation.

Although the results present here are compatible with our prediction based on within host heterogeneity and selection, alternative explanations exist. For example, the increase in Octomom copy number with time could reflect an intrinsic property of these duplications independent of selection. Nonetheless, this would also result in an increase in more pathogenic *w*MelPop with host age and an outcome at odds with the selective pressure at the inter-host level. Future approaches may try to relate the rate of change of Octomom copy number within host in different conditions that affect the amplification-induced phenotype. For example, at lower temperatures the phenotype of *w*MelPop is reduced [9,15] and the selection for *w*MelPop with higher Octomom copy number may be absent.

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

Material and methods

Fly strains

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

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

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

DNA extractions

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

Real-time quantitative PCR

The real-time qPCR reactions were performed using CFX384 Real-Time PCR Detection System (Bio-Rad) as described before [5,10]. Each reaction contained 6 µl of iQ SYBR Green Supermix (Bio-Rad), 0.5 µl of each primer (3.6 mM), and 5 µl of

diluted DNA. We performed two technical replicates for each sample for each set of primers. Primer sequences were described before [5]. For all three genes assayed: *Wolbachia WD0513* and *wsp,* and *Drosophila Rpl32* the following thermal cycling protocol was applied: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C, 1 min at 59°C, and 30 s at 72°C. Melting curves were examined to confirm the specificity of amplified products. Ct values were obtained using Bio-Rad CFX Manager software with default threshold settings. Ct values were subjected to a quality check - samples with standard deviation between technical replicates exceeding 0.5 for one of the genes were discarded. The experiment spanned six qPCR plates and three samples of ten wMelCS_b flies (extracted and aliquoted beforehand and assayed on every qPCR plate) were used to normalize between plates. Relative amounts of genes were calculated by the Pfaffl method [16]. To apply the method, the efficiency of each primer set was predetermined in a separate experiment. For relative Octomom copy number quantification, *WD0513* was the target gene and the single-copy *wsp* gene was used as a reference. The medians of three samples of pools of ten wMelCS_b flies were used as control values for the Pfaffl method. *w*MelCS_b has one copy of the Octomom region in the genome, determined by the coverage analysis of sequencing data [10]. This sample, with known Octomom copy number, is required to estimate Octomom copy number of the remaining samples [17]. For *Wolbachia* quantification, *wsp* was the target gene and *Drosophila Rpl32* gene was used as a reference. The levels of *wsp* are relative to the median of the samples of the low Octomom cohort at time zero.

Statistical analysis

The statistical analysis was performed in R [18]. The script of the analysis is provided in S1 Text. Graphs were generated using the package ggplot2 [19].

Since the temporal trend over time of the number of Octomom copies was not linear we analyse it by fitting a Generalized Additive Model (GAM, package mgcv in R [20]). We included time and line as independent variables and PCR plate as a random effect. The smooth terms for the interaction between time and lines were non-significant ($p > 0.114$) and were removed form the final model.

Analysis of *wsp* levels over time was performed with log-linear mixed-effect model fits (package lme4 in R [21]). The effect of interaction between factors was determined by an ANOVA comparing models fit to the data with and without the interaction.

Acknowledgments

We would like to thank Tiago Marques for advice on the statistical analysis.

References

1. Ohno S. Evolution by Gene Duplication. Berlin, Heidelberg: Springer Berlin Heidelberg; 1970. doi:10.1007/978-3-642-86659-3
2. Bergthorsson U, Andersson DI, Roth JR. Ohno's dilemma: evolution of new genes under continuous selection. Proc Natl Acad Sci U S A. 2007;104: 17004–9. doi:10.1073/pnas.0707158104
3. Romero D, Palacios R. Gene Amplification and Genomic plasticity in

Prokaryotes. Annu Rev Genet. 1997;31: 91–111. doi:10.1146/annurev.genet.31.1.91

4. Andersson DI, Hughes D. Gene amplification and adaptive evolution in bacteria. Annu Rev Genet. 2009;43: 167–95. doi:10.1146/annurev-genet-102108-134805

5. Chrostek E, Teixeira L. Mutualism breakdown by amplification of Wolbachia genes. PLoS Biol. 2015;13: e1002065. doi:10.1371/journal.pbio.1002065

6. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6: 741–751. doi:10.1038/nrmicro1969

7. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. Wolbachia and virus protection in insects. Science. 2008;322: 702. doi:10.1126/science.1162418

8. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. 2008;6: e2. doi:10.1371/journal.pbio.1000002

9. Min KT, Benzer S. Wolbachia, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. Proc Natl Acad Sci U S A. 1997;94: 10792–6.

10. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, et al. Wolbachia variants induce differential protection to viruses in Drosophila melanogaster: a phenotypic and phylogenomic analysis. PLoS Genet. 2013;9: e1003896. doi:10.1371/journal.pgen.1003896

11. Woolfit M, Iturbe-Ormaetxe I, Brownlie JC, Walker T, Riegler M, Seleznev A, et al. Genomic Evolution of the Pathogenic Wolbachia Strain, wMelPop. Genome Biol Evol. 2013;5: 2189–204. doi:10.1093/gbe/evt169

12. Young BC, Wu C-H, Gordon NC, Cole K, Price JR, Liu E, et al. Severe infections emerge from the microbiome by adaptive evolution. bioRxiv. 2017; doi: 10.1101/116681

13. Rohrscheib CE, Frentiu FD, Horn E, Ritchie FK, Swinderen B Van, Ii MWW, et al. Intensity of Mutualism Breakdown Is Determined by Temperature Not Amplification of Wolbachia Genes. PLoS Pathog. 2016; 1–15. doi:10.1371/journal.ppat.1005888

14. Chrostek E, Teixeira L. Comment on Rohrscheib et al . 2016 " Intensity of mutualism breakdown is determined by temperature not amplification of Wolbachia genes ." PLoS Pathog. 2017; 2–7. doi:10.1371/journal.ppat.1006540

15. Reynolds KT, Thomson LJ, Hoffmann AA. The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent Wolbachia strain popcorn in Drosophila melanogaster. Genetics. 2003;164: 1027–34.

16. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29: e45.

17. D 'haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. Methods. 2010;50: 262–270. doi:10.1016/j.ymeth.2009.12.007

18. Team R. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2012.

19. Wickham H. ggplot2 Elegant Graphics for Data Analysis. Media. 2009. doi:10.1007/978-0-387-98141-3

20. Wood SN. Generalized additive models : an introduction with R. Texts Stat

Sci. 2006; xvii, 392 . doi:10.1111/j.1541-0420.2007.00905_3.x

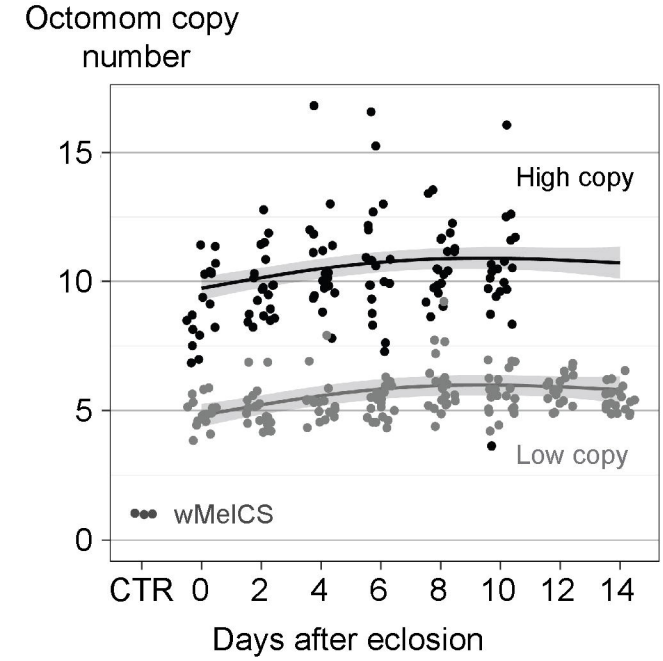
21. Bates D, Machler, M, Bolker BM, Walker, SC. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software. 2015;67. doi:10.18637/jss.v067.i01

Supporting information

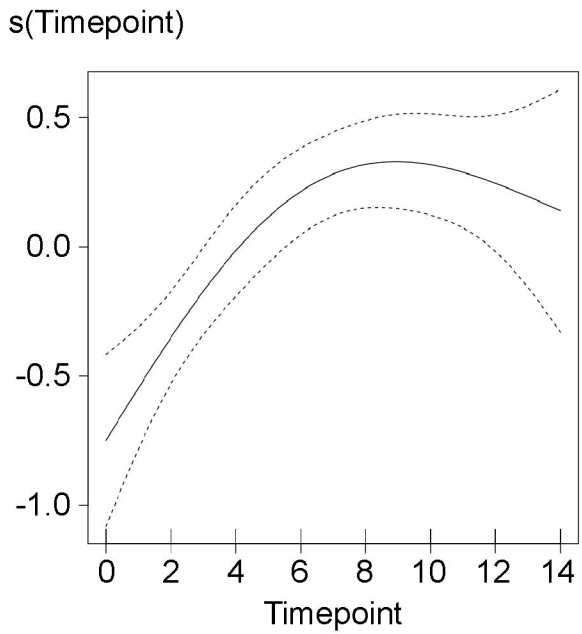
S1 Data – Relative levels of *WD0513* and *wsp* in single females carrying wMelPop.

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

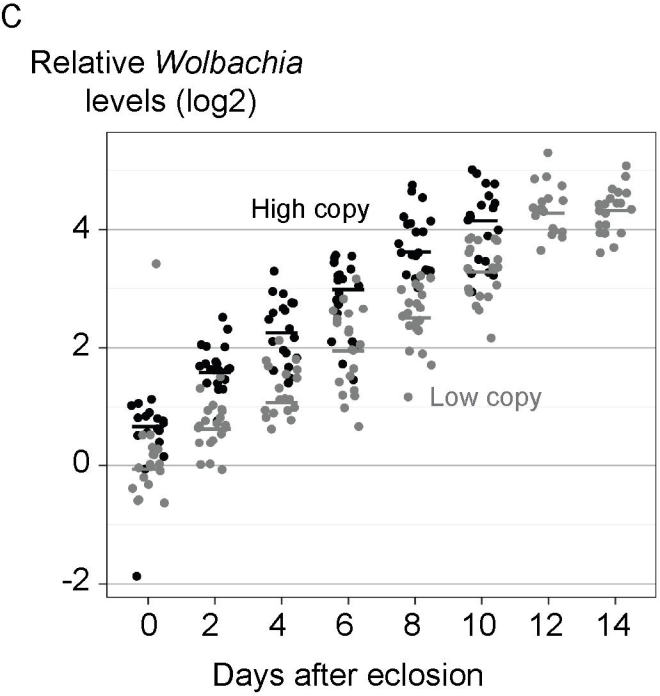
A


Octomom copy number
15
10
5
0
High copy
Low copy
wMelCS
CTR 0 2 4 6 8 10 12 14
Days after eclosion

B


s(Timepoint)
0.5
0.0
-0.5
-1.0
0 2 4 6 8 10 12 14
Timepoint

C


Relative Wolbachia levels (log2)
4
2
0
-2
High copy
Low copy
0 2 4 6 8 10 12 14
Days after eclosion