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1 **Sharing cells with *Wolbachia*: the transovarian vertical**
2 **transmission of *Culex pipiens* densovirus**

3

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17 **Running title: Vertical transmission of *Culex pipiens* densovirus**

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32 **Originality-Significance Statement**

33 This contribution provides a window into the study of a densovirus called CpDV infecting *Culex pipiens*
34 mosquitoes. The originality of the study lies in the fact that CpDV, a DNA virus, was to date almost
35 unstudied, its interaction with its hosts and its transmission mode were unknown. Considering that all
36 *Cx. pipiens* are infected with *Wolbachia*, we studied the influence of this bacterial endosymbiont on
37 CpDV density and transmission. Although *Wolbachia*-RNA virus interactions, especially in non-native
38 *Wolbachia*-host associations have been widely studied, *Wolbachia*-DNA virus interactions were rarely
39 investigated.

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41 In this natural tripartite *Cx. pipiens*-*Wolbachia*-CpDV system:

- 42 1. We revealed the wide prevalence of CpDV, mostly at low densities, in *Cx. pipiens* laboratory lines.
- 43 2. We monitored CpDV presence in several cell types in ovaries including the oocytes where they co-
44 exist with the bacterial endosymbiont *Wolbachia*.
- 45 3. We demonstrated that CpDV density in ovaries was influenced by *Wolbachia* presence and strain
46 variations
- 47 4. We studied CpDV vertical transmission depending on *Wolbachia* presence and strain variations

48

49 We report for the first time the occurrence of widely distributed covert CpDV infections in *Cx. pipiens*
50 laboratory colonies. CpDV is maintained by both vertical and horizontal transmission and under the
51 influence of the vertically transmitted bacterial endosymbiont *Wolbachia*. Taken together our results
52 suggest that CpDV could be very prevalent in nature putatively influencing natural *Cx. pipiens*
53 populations' dynamics.

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60 **SUMMARY**

61 ***Culex pipiens* densovirus (CpDV), a single stranded DNA virus, has been isolated from *Culex***
62 ***pipiens* mosquitoes but differs from other mosquito densoviruses in terms of genome structure**
63 **and sequence identity. Its transmission from host to host, the nature of its interactions with both**
64 **its host and host's endosymbiotic bacteria *Wolbachia* are not known. Here we report the presence**
65 **of CpDV in the ovaries and eggs of *Cx. pipiens* mosquitoes in close encounters with *Wolbachia*. In**
66 **the ovaries, CpDV amount significantly differed between mosquito lines harboring different**
67 **strains of *Wolbachia* and these differences were not linked to variations in *Wolbachia* densities.**
68 **CpDV was vertically transmitted in all laboratory lines to 17%-20% of the offspring. For some**
69 **females, however, the vertical transmission reached 90%. Antibiotic treatment that cured the host**
70 **from *Wolbachia* significantly decreased both CpDV quantity and vertical transmission suggesting**
71 **an impact of host microbiota, including *Wolbachia*, on CpDV transmission. Overall our results**
72 **show that CpDV is transmitted vertically *via* transovarian path along with *Wolbachia* with which**
73 **it shares the same cells. Our results are primordial to understand the dynamics of densovirus**
74 **infection, their persistence and spread in populations considering their potential use in the**
75 **regulation of mosquito vector populations.**

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88 **Introduction**

89 Densoviruses are non-enveloped, single stranded DNA viruses. They constitute a diverse subfamily
90 within the *Parvoviridae* family that includes both vertebrate (*Parvovirinae*) and invertebrate
91 (*Densovirinae*) viruses (Cotmore *et al.*, 2014; Tijssen *et al.*, 2016). Isolated from many insects (e.g.
92 butterflies, moths, mosquitoes, crickets, grasshoppers and cockroaches) and crustaceans (e.g. prawns
93 and crayfish), they are thought to be widely distributed among arthropods (Kerr *et al.*, 2005; Tijssen *et*
94 *al.*, 2016). Recent studies, investigating publicly available transcriptomic and genomic databases, have
95 shown their presence in even broader range of non-vertebrate hosts (e.g., molluscs, annelids, nematodes,
96 cnidarians and sea stars)(François *et al.*, 2016; Kang *et al.*, 2017). In arthropods, most of the
97 densoviruses known so far exhibit a tissue polytropism replicating in many tissues -such as the fat body,
98 hypodermis, epidermis, central nervous system, muscular and tracheal cells, gut, hemocytes, ovaries-
99 and are considered to be lethal for their natural hosts (Tijssen *et al.*, 2016). Due to their observed
100 pathogenicity, narrow host range and sustainability out of their host-cells in nature, they attracted
101 scientific attention as biological control tools especially against mosquitoes (Carlson *et al.*, 2006; Mutuel
102 *et al.*, 2010; Gosselin Grenet *et al.*, 2015; Suzuki *et al.*, 2015; Johnson and Rasgon, 2018).

103

104 Mosquito densoviruses (MDVs) have been described in natural populations (Afanasiev *et al.*, 1991;
105 Boublik *et al.*, 1994; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000; Ng *et al.*, 2011), laboratory
106 colonies (Jousset *et al.*, 2000) and established mosquito cell lines (Jousset *et al.*, 1993; O'Neill *et al.*,
107 1995). The first described mosquito densoviruses were *Aedes aegypti* densovirus (AeDV) (Lebedeva *et*
108 *al.*, 1972) and *Aedes albopictus* densovirus (AalDV) (Jousset *et al.*, 1993). Thereafter many other MDV
109 strains, namely *Toxorhynchites amboinensis* densovirus (TaDV), *Haemagogus equinus* densovirus
110 (HeDV), *Aedes aegypti* Thai strain densovirus (AThDV), *Aedes Peruvian* densovirus (APeDV) have
111 been identified (O'Neill *et al.*, 1995; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000; Ledermann *et*
112 *al.*, 2004). Studies on MDV pathogenicity and transmission modes have generally shown a dose and age
113 dependent infectivity and virulence (Barreau *et al.*, 1996; Kittayapong *et al.*, 1999; Ledermann *et al.*,
114 2004; Rwegoshora and Kittayapong, 2004; Becnel and White, 2007). Vertical transmission of these
115 viruses has only been observed when using sub-lethal doses that reduced the adult mosquitoes' longevity

116 and fertility but did not kill them (O'Neill *et al.*, 1995; Barreau *et al.*, 1996, 1997; Kittayapong *et al.*,
117 1999; Rwegoshora and Kittayapong, 2004; Becnel and White, 2007). In contrast, *Anopheles gambiae*
118 densovirus (AgDV), a recently discovered MDV, which exhibits a negligible pathogenic effect on its
119 host *A. gambiae* mosquitoes (Ren *et al.*, 2008, 2014) has been shown to replicate in its host and to be
120 vertically transmitted to the offspring highlighting its potential as transducing agents for mosquito
121 control (Ren *et al.*, 2008; Suzuki *et al.*, 2014).

122

123 All of the abovementioned MDVs described so far are closely related (82-99% sequence homology),
124 and are considered as strains from two distinct species (dipteran brevidensovirus 1 and 2) belonging to
125 the genus *Brevidensovirus* (Cotmore *et al.*, 2014). To date, the only known densovirus to infect
126 mosquitoes, but does not belong to *Brevidensovirus* genus, is *Culex pipiens* densovirus (CpDV). CpDV
127 is a dipteran ambidensovirus (Jousset *et al.*, 2000; Baquerizo-Audiot *et al.*, 2009) which differs
128 significantly from dipteran brevidensoviruses by the lack of sequence homology (<30% in Non-
129 structural (NS1/Rep) gene) and antigenic cross-reactivity (Jousset *et al.*, 2000; Baquerizo-Audiot *et al.*,
130 2009). It belongs to *Ambidensovirus* genus along with lepidopteran densoviruses, such as *Junonia coenia*
131 densovirus (JcDV) and *Galleria melonella* densovirus (GmDV). Ambidensoviruses have not yet been
132 described in other dipteran species suggesting that they could have been acquired in *Cx. pipiens* by
133 horizontal transfer. CpDV indeed shares the characteristics of other ambidensoviruses having a larger
134 genome size than brevidensovirus (~6kb compared to ~4kb), an ambisense genome organization and
135 significant sequence homology at both capsid and NS gene levels (Jousset *et al.*, 2000; Baquerizo-
136 Audiot *et al.*, 2009).

137

138 Two decades after its first isolation from *Cx. pipiens* laboratory colonies, due to an unusually high larval
139 mortality, we detected the presence and persistence of CpDV in all life stages of seemingly healthy *Cx.*
140 *pipiens* lines by a PCR diagnostic test (data not shown). This long-term persistence of CpDV in
141 laboratory colonies through generations certainly relies on an efficient transmission. Transmission
142 modes are generally categorized as i) horizontal, transmission from host to host via the environment, ii)
143 vertical transmission from the host parent to offspring, iii) mixed-mode, both horizontal and vertical

144 transmission (Ebert, 2013). Transmission modes have long been investigated in many symbiotic systems
145 especially to understand their impact on virulence evolution (Ewald, 1983, 1987; Alizon *et al.*, 2009;
146 Cressler *et al.*, 2016; Le Clec'h *et al.*, 2017). Empirical studies mostly showed that increased horizontal
147 transmission led to increased virulence, while dominance of vertical transmission mode mostly reduced
148 the virulence [reviewed in (Cressler *et al.*, 2016)]. Therefore transmission modes can have a major
149 impact on the dynamics of virus infection, their persistence and spread in populations.

150

151 To understand CpDV infection dynamics, especially its maintenance through generations, we
152 investigated the ability of *Cx. pipiens* mosquitoes to transmit CpDV vertically. *Cx. pipiens* are all
153 naturally infected with *Wolbachia* strains belonging to one of the five genetically distinct phylogenetic
154 group wPipI to wPipIV (Atyame *et al.* 2011). These wPip strains induce a wide diversity of cytoplasmic
155 incompatibility phenotypes in their mosquito host (Atyame *et al.* 2014). In addition to their ability to
156 manipulate host reproduction to benefit their own vertical transmission (Stouthamer *et al.*, 1999; Werren
157 *et al.*, 2008; Sicard *et al.*, 2014), *Wolbachia* are also known to interact with several of their hosts' viruses
158 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Zug and Hammerstein, 2014). While putative difference in
159 protective ability between the wPip groups identified in *Cx. pipiens* has not yet been investigated, a
160 general protection by wPip has been shown against West Nile Virus infection (Micieli and Glaser, 2014).
161 To date, no *Wolbachia*-mediated "protection" against DNA viruses have been shown in any insects.
162 Contrarily, one study demonstrate that *Wolbachia* makes their lepidopteran host more susceptible
163 against a baculovirus (Graham *et al.*, 2012).

164

165 We thus examined the influence of *Wolbachia* on CpDV density and vertical transmission. To do that,
166 we monitored CpDV density and location in the ovaries and eggs, and then quantified its vertical
167 transmission in *Cx. pipiens* mosquitoes naturally infected with both *Wolbachia* and CpDV. Given their
168 importance in *Cx. pipiens*' phenotype both in natural populations and laboratory colonies, we took
169 *Wolbachia* into account in each step of the study and further investigated the possible effects of their
170 absence on CpDV presence and vertical transmission.

171

172 **Results**

173

174 ***CpDV presence in ovaries and eggs***

175 CpDV was always present, along with *Wolbachia*, in the ovaries of females from ten laboratory lines
176 tested by qPCR and its amount varied between different lines (glm, df=13, dev=82.448, p<0.001, Fig.
177 1). Only two pools of ovaries -one from Ich13 and another from Ich09 line- over 50 pools tested were
178 CpDV negative. We did not detect any statistically significant effect of *Wolbachia* amount (glm, df=1,
179 dev=0.007, p= 0.967, Fig. 1A) on the amount of CpDV in ovaries. To create backcrossed lines for further
180 experimentation, we used “Harash” mosquito line with the highest amount of CpDV in ovaries
181 (120 ± 114 CpDV/host genome, Supplementary Table 1, Fig. 1B) and “Tunis” line with less CpDV in
182 their ovaries (0.005 ± 0.002 CpDV/host genome, Tunis, Supplementary Table 1, Fig. 1B).

183

184 To confirm the presence of virus particles -and not only of the viral genome (*i.e.* as detected by qPCR)-
185 and to investigate their localization in the ovaries, we dissected ovaries from 6 days old Harash females
186 and performed immunolabelling of CpDV using an anti-capsid antibody for confocal observation (Fig.
187 2 and Supplementary Fig. 1). The labelling of CpDV was mainly observed in follicular cells surrounding
188 the follicles (Fig. 2A-B-C) and occasionally in some muscle cells of the sheath enclosing the ovariole
189 (Fig. 2D). In both cell types, the labelling was mainly nuclear with some dots present in the cytoplasm
190 of follicular cells (Fig. 2B to E). Within the follicles, the labelling of CpDV in the nurse cells was not
191 as apparent and rather similar to the background immunofluorescence (Fig. 2D-E). Although we cannot
192 entirely exclude a lack of permeability and accessibility of the nurse cells to the antibodies, a clear
193 labelling was nevertheless observed in a well-defined area of the nucleus of the oocyte (Fig. 2E). As all
194 the *Cx. pipiens* lines were infected, the specificity of the CpDV labeling was tested on ovaries isolated
195 from PCR tested CpDV-free *Ae. albopictus* mosquitoes and no labelling was detected (Supplementary
196 Fig. 2).

197 To examine the CpDV replication, which takes place in the nucleus, we prepared ultrathin sections of
198 ovaries and analyzed the nuclear ultrastructure with transmission electron microscopy (TEM) (Fig. 3).
199 Although no unequivocal densonucleosis was observed, follicular and nurse cells showed hypertrophied

200 nuclei (Fig. 3A and G). Intranuclear structures that could be interpreted as viral factories were observed
201 in several follicular cells (Fig. 3B), nurse cells (Fig. 3E) and oocytes (Fig. 3H). Particles with the
202 expected diameter of CpDV (about 25 nm) were also present in the nucleoplasm (Fig. 3C and F). To
203 confirm the presence of viral particles in ovaries, we prepared filtered homogenate of the full organ for
204 observation using TEM. Micrographs revealed the presence of both icosahedral empty capsids and
205 CpDV virions with the expected diameter (Fig. 3I).

206

207 Same experiments were performed on freshly laid dechorionated eggs from Harash females (Fig. 4).
208 Immunolocalization of CpDV and DAPI staining of *Wolbachia* cells at early stage of the embryo
209 development showed a *Wolbachia* localization especially in the poles, and a CpDV localization both
210 inside and in the periphery of the eggs suggesting no co-localization of these two (Fig. 4A, Top Panel).
211 At the syncytial blastoderm stage, CpDV were co-localized with the nuclei, presumably starting viral
212 replication (Fig. 4A, Bottom Panel). *Wolbachia* were easily detected in the cytoplasm in the ultrathin
213 sections of fertilized eggs even at an early stage of development (Fig. 4C). However, at the early stage
214 of development nuclei were rare, meaning CpDV could only be observed in the cytoplasm where the
215 ribosomes are also present (Fig. 4C). As ribosomes and CpDV have the same diameter, it is not possible
216 to firmly distinguish them (Fig. 4C).

217

218 ***Effect of infection with distinct Wolbachia strains on CpDV amount in ovaries***

219 To assess the effect of *Wolbachia* (strains and density) on the amount of CpDV in the ovaries, we have
220 established mosquito lines, with help of a backcrossing protocol, with the same host nuclear genetic
221 background but different *Wolbachia* strains (BcTunis, Slab genetic background harbouring *wPip I* from
222 Tunis line; BcHarash, Slab genetic background harbouring *wPip IV* from Harash line, Table 1). We then
223 quantified the amount of *Wolbachia* and CpDV in these lines as well as in Harash, Tunis and Slab lines
224 (Fig. 5). *Wolbachia* amount in the ovaries was not significantly different between the *Cx. pipiens* lines
225 no matter what the *Wolbachia* strain and host genetic background were (*Wolbachia* strain: glm, df=2,
226 dev=17.703, p=0.875; host genetic background: df=1 dev=0.034, p=0.981, Fig. 5A). Indeed, there was
227 no significant effect of the *Wolbachia* amount in the ovaries on CpDV density (lme, dev=0.001, df=1,

228 p=0.9). However, *Wolbachia* strain (*i.e.* the genotype) infecting the mosquito lines explained a
229 significant part of the variance in the CpDV amount in the ovaries (lme, dev=12.236, df=1, p<0.001,
230 Fig. 5B) while the host genetic background had no significant effect on CpDV amount in the ovaries
231 (lme, dev=1.602, df=2, p= 0.449; Fig. 5B).

232

233 ***Effect of infection with distinct Wolbachia strains on CpDV vertical transmission***

234 Using the same mosquito lines, we tested the vertical transmission of CpDV. For this, we let 10 females
235 per line to lay eggs in isolation. To estimate a vertical transmission rate per female mosquito, both
236 females and 10 L1 stage larvae per female were collected and tested for CpDV presence. All the mothers
237 were CpDV positive. CpDV were transmitted to an average of 17% of the offspring (n=49, $0.172 \pm$
238 0.0317 , Fig5C). However, the vertical transmission rate highly diverged between individuals ranging
239 from 0% (n=19 out of total 49 checked) to 90% (n=1, out of total 49 checked). This inter-individual
240 variability was not significantly explained neither by the host genetic background (df=2, dev=0.278, p=
241 0.870, Fig5C), *Wolbachia* strain (df=1, dev=0.152, p= 0.696, Fig3C), *Wolbachia* amount (df=1, dev= -
242 0.135, p=0.713, Fig5C) nor by CpDV amount in the whole body of the parental females (df=1, dev=-
243 0.180, p=0.672, Fig5C).

244

245 ***Effect of Wolbachia curing by tetracycline on the amount of CpDV in ovaries***

246 As all the *Cx. pipiens* isofemale lines were stably infected with *wPip* (Wo+), to test the effect of the
247 *Wolbachia* absence on CpDV presence and vertical transmission, we treated our Slab, BcHarash, and
248 BcTunis lines, all having the same genetic background, with tetracycline (TC) for three generations to
249 cure *Wolbachia*. We quantified *Wolbachia* and CpDV in dissected ovaries and carcasses of Wo+ (before
250 TC treatment) and Wo- females (after TC treatment), as well as females during the 1st and 3rd generation
251 of TC treatment (Fig. 6). There was no significant change between 1st and 3rd generations in terms of
252 CpDV amount (t = -0.643, df = 25.918, p-value = 0.525, Fig. 6B). We thus grouped them together in a
253 “TC treatment” group for further statistical analyses and figures.

254

255 Prior to tetracycline treatment *Wolbachia* were found in higher density in the ovaries compared to the
256 carcasses (Wilcoxon rank sum test, $W= 225$, $p<0.001$; Fig. 6A). By the end of the third generation of
257 tetracycline treatment, as expected, *Wolbachia* totally disappeared both from the ovaries and the
258 carcasses (Fig. 6A).

259

260 In non-treated W_{o+} females, higher amount of CpDV were measured in ovaries compared to carcasses
261 (Wilcoxon rank sum test, $W = 168$, $p = 0.021$, Fig. 6B). In the carcasses, TC treatment did not cause
262 any significant effects on CpDV amount (Fig. 6B, Wilcoxon rank sum test with Bonferroni correction;
263 W_{o+} vs. during TC treatment, $W= 132.5$, $p=0.08$; W_{o+} vs. W_{o-} , $W= 100$, $p=0.138$; during TC treatment
264 vs. W_{o-} , $W=308$, $p=1$). On the contrary, in the ovaries CpDV amount significantly decreased during the
265 TC treatment (Dunnnett's test with Bonferroni Correction; W_{o+} vs. during TC treatment, $t=2.302$,
266 $p=0.025$; Fig. 6B). However, in W_{o-} lines (three generations after the end of the tetracycline treatment)
267 CpDV amount increased and reached a level not significantly different from non-treated W_{o+} lines
268 (Dunnnett's test with Bonferroni Correction; W_{o+} vs. W_{o-} , $t= 0.118$, $p=1$).

269

270 ***Effect of Wolbachia curing by tetracycline on CpDV vertical transmission rate***

271 CpDV vertical transmission decreased significantly from an average of 25% to an average of 13% during
272 tetracycline treatment (Wilcoxon rank sum test with Bonferroni correction; W_{o+} vs. TC treatment, $W=$
273 488 , $p= 0.004$, Fig. 7). Three generations after the end of tetracycline treatment, vertical transmission in
274 W_{o-} lines stayed marginally lower than non-treated W_{o+} lines (Wilcoxon rank sum test with Bonferroni
275 correction; W_{o+} vs. W_{o-} , $W= 576.5$, $p= 0.05$, Fig. 7).

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283 Discussion

284

285 CpDV was isolated for the first time from *Cx. pipiens* larvae almost two decades ago (Jousset *et al.*,
286 2000). The features of densonucleosis (hypertropied nuclei and electron-dense virogenic stroma) was
287 then observed in larval tissues (hypodermis, fat body, midgut, imaginal disks and nervous tissue)
288 suggesting CpDV's role on the larval mortality (Jousset *et al.*, 2000). Here, we showed that CpDV was
289 also present in apparently healthy adults especially in their ovaries along with endosymbiotic bacteria
290 *Wolbachia*. Even if the densonucleosis was not unequivocal, intranuclear structures similar to viral
291 factories were observed and viral particles were isolated from ovaries. Furthermore immunolabelling
292 revealed virus particles in the follicular cells and the oocytes. Only couple of studies reported the
293 presence of densovirus in the ovaries. *Helicoverpa armigera* densovirus (*Ambidensovirus*) and
294 *Anopheles gambiae* densovirus (*Brevidensovirus*) have been shown to reach high densities in ovaries
295 of their hosts while they were also able to infect other host tissues (Ren *et al.*, 2008; Suzuki *et al.*, 2014;
296 Xu *et al.*, 2014).

297

298 The presence of CpDV capsids in the ovaries and eggs highlighted the possibility of vertical transovarial
299 transmission. Transmission mechanisms of densovirus are generally thought to be horizontal causing
300 epizootics (Tijssen *et al.*, 2016). Nevertheless, vertical transmission has been shown both for some
301 ambidensoviruses and brevidensoviruses. Interestingly, ambidensoviruses with different vertical
302 transmission rates had different types of symbiotic interactions: while the 100% vertically transmitted
303 ambidensovirus of *H. armigera* is mutualistic (Xu *et al.*, 2014), the *Myzus persicae* densovirus which is
304 transmitted to 30-40% of the offspring has been shown to affect its hosts adversely (Van Munster *et al.*,
305 2003). In mosquitoes experimentally infected with brevidensoviruses, the vertical transmission ranged
306 between 28 to 62%: i) for *Aedes albopictus* densovirus, the vertical transmission rate ranged between
307 28-55% depending on the virus titer in parental females (Barreau *et al.*, 1997); ii) for *Aedes aegypti*
308 densovirus vertical transmission ranged from 42% to 62% depending on the timing of the horizontal
309 infection of the larvae (the larvae that were infected during L3 stage transmitted the virus less, as they
310 became adults, than the larvae infected during L1 stage) (Suchman *et al.*, 2006), and iii) for *Aedes*

311 gambiae densovirus vertical transmission was detected in 28% of larval offspring (Ren *et al.*, 2008). We
312 showed that CpDV was transmitted to an average of 17% of the larval offspring in naturally infected
313 *Cx. pipiens* lines. Even if the vertical transmission rate varied between individuals, this rate was quite
314 stable (15%-21%) between the *Cx. pipiens-Wolbachia* lines tested through the different replicates. The
315 importance of inter-individual variability in vertical transmission rate of CpDV revealed the complexity
316 of the factors affecting vertical transmission. Individual differences in vertical transmission, for instance,
317 could be due to when females have been infected by horizontal transmission (Suchman *et al.*, 2006), or
318 whether they have been infected vertically or horizontally. Due to the lack of CpDV free lines, it was
319 not possible to quantify the horizontal transmission or venereal transmission by experimental infections.
320 Nevertheless, the infection rate changed from 17% at the L1 stage to 100% in tested mothers suggesting
321 a high level of horizontal transmission.

322

323 The co-existence of endosymbiotic bacteria *Wolbachia* and CpDV within the host ovary cells suggested
324 that they might be interacting. Indeed, symbiotic bacteria and viruses have been shown to interact in
325 several ways. Firstly, symbiotic bacteria can “protect” their hosts against pathogens by either modulating
326 the host immunity or tolerance, or by competing for host resources. A famous example of such
327 interaction is the *Wolbachia* mediated protection of insect hosts against RNA viruses (Hedges *et al.*,
328 2008; Teixeira *et al.*, 2008). In *Drosophila* this “protection” strongly changed according to *Wolbachia*
329 strains differing in their titers (Martinez *et al.*, 2017). Similar “protection” has been reported in
330 mosquitoes, especially in *Ae. aegypti* transinfected with non-native *Wolbachia* strains from *Drosophila*,
331 against several arboviruses such as Dengue, Chikungunya, West Nile and Yellow fever viruses (Moreira
332 *et al.*, 2009; Mousson *et al.*, 2010; Hussain *et al.*, 2012). On the other hand, native *Wolbachia* in *Ae.*
333 *albopictus* that exhibit low densities, even in the ovaries, do not influence virus replication (Mousson *et*
334 *al.*, 2010; Lu *et al.*, 2012). A global pattern both in mosquitoes and *Drosophila* is that *Wolbachia*-
335 mediated antiviral protection seems to depend on *Wolbachia* density (Lu *et al.*, 2012; Osborne *et al.*,
336 2012; Bian *et al.*, 2013). Our results showed, for the first time, a significant effect of *Wolbachia* strain
337 on the quantity of a DNA virus, the CpDV, in the ovaries suggesting an interaction between these two
338 symbionts sharing the same cells. Indeed by using a backcrossing protocol in mosquitoes naturally

339 infected both with their respective wPip strain and CpDV, we have demonstrated that CpDV density in
340 the ovaries was not controlled by host nuclear genetic background but most likely by maternally
341 transmitted cytoplasmic factors such as *Wolbachia*. However, *Wolbachia* density was really stable
342 between naturally infected *Cx. pipiens* lines for different *Wolbachia* strains tested suggesting variations
343 of symbiont titer was not responsible for variations in CpDV titers. Nevertheless further research
344 including the establishment of CpDV free lines from putative uninfected natural populations followed
345 by experimental infections would be useful to delineate the complex tripartite interactions in this new
346 mosquito-virus-*Wolbachia* natural system.

347

348 To further investigate whether CpDV and *Wolbachia* had reciprocal interactions and whether CpDV
349 could still be vertically transmitted in the absence of *Wolbachia*, we treated our lines with antibiotics.
350 This treatment decreased both the amount of CpDV in the ovaries and the vertical transmission alike.
351 However, following the end of the treatment CpDV amount in the ovaries increased in the *Wolbachia*-
352 free lines. This suggested that this sudden decrease could be caused by either the direct influence of
353 antibiotics on the host metabolism, or by the rest of the microbiota that putatively recovered in the
354 generations following the end of the antibiotic treatment. Nevertheless, even though CpDV was able to
355 be transmitted vertically in the absence of *Wolbachia*, the vertical transmission was lower in *Wolbachia*-
356 free lines, suggesting their effect on CpDV transmission. Several endosymbiotic bacteria have been
357 reported to modulate virus transmission. For instance Tomato yellow leaf curl virus' (a single stranded
358 DNA plant virus) transmission is facilitated by GroEL protein secreted by its vector's endosymbiotic
359 bacteria *Hamiltonella* (Gottlieb *et al.*, 2010). More recently, Jia *et al.* (2017) showed that Rice Dwarf
360 virus (a plant double stranded RNA virus) binds to the envelopes of endosymbiotic bacteria *Sulcia*
361 improving its vertical transmission in leafhoppers. Similarly, vertical transmission of *Wolbachia* could
362 be hitchhiked by CpDV in *Cx. pipiens*. CpDV could potentially achieve this through the interactions of
363 its capsids with bacterial surface glycans. Dengovirus virions, as of many non-enveloped viruses, are
364 constituted by a single major capsid protein with supersecondary structures which can act as interaction
365 motifs (*e.g.* Single Jelly Roll) (Neu *et al.*, 2011; Huang *et al.*, 2014). These motifs can attach to bacteria's
366 surface glycans which can serve as initial attachment factors for many viruses (Neu *et al.*, 2011; Huang

367 *et al.*, 2014). Thus CpDV capsids could potentially interact with a wide array of glycans, including
368 *Wolbachia*'s surface glycans and such interactions could influence the infection outcome and lead to
369 their co-vertical transmission (Kuss *et al.*, 2011; Berger and Mainou, 2018).

370

371 Overall we observed a low amount of CpDV in our samples highlighting a covert infection in laboratory
372 *Cx. pipiens* mosquitoes. Viruses exhibiting covert infections can differ in their replication strategy and
373 virulence (Kane and Golovkina, 2010; Williams *et al.*, 2017). They can either cause persistent infections
374 with low levels of virus replication, producing virions but only causing sub-lethal disease or causing
375 latent infections, without any production of virions and limited number of viral gene expression (Kane
376 and Golovkina, 2010; Williams *et al.*, 2017). In the case of CpDV, we were able to observe CpDV
377 virions but not a particularly high mortality in the mosquito lines even in the heavily infected Harash
378 line. This type of covert infection combined with the mixed mode transmission can allow the persistence
379 of CpDV in laboratory as well as natural populations of *Cx. pipiens* mosquitoes. In *Cx. pipiens*
380 mosquitoes even low rate vertical transmission can have a large effect on the epidemiology of CpDV
381 by allowing the transmission of the virus even after acute reduction of host density, e.g. during
382 overwintering diapause (Ebert, 2013; Williams *et al.*, 2017). While still not well studied this type of
383 covert persistent infections by insect specific viruses can largely affect the ecology of the insect
384 populations and vector competence.

385

386 **Experimental procedures**

387 **Mosquito Lines**

388 All the mosquito lines used in this study (Supplementary Table 1) have been maintained in insectary
389 conditions (at 25 ± 2 °C and $75 \pm 2\%$ relative humidity and a 12:12 h photoperiod). During larval stage,
390 they have been fed with a mixture of shrimp powder and rabbit pellets. Adults have been kept in 65 dm³
391 cages and have been fed with a honey solution. Females were fed weekly with turkey blood using a
392 Hemotek membrane feeding system (Discovery Workshops, United Kingdom).

393

394

395 *Backcrosses*

396 To test the effect of the *Wolbachia* genetic differences on CpDV amount and their vertical transmission
397 we used three isofemale lines Tunis, Harash and Slab (Supplementary Table 1) that are infected with
398 *wPip* I, *wPip* IV and *wPip* III respectively. *wPip* groups of these lines have been assigned based on a
399 PCR/RFLP test on pool of larvae using *pk1* primers as previously described (Dumas *et al.* 2013, Altinli
400 *et al.*, 2018) and were regularly verified during the experiments. We introduced the cytoplasm of Tunis
401 and Harash lines, along with their respective *wPip* strains, into the Slab nuclear background as described
402 by Duron *et al.* (Duron *et al.*, 2012). With this backcrossing protocol we have established BcTunis and
403 BcHarash lines with the same genetic background (over 97% of genome replacement of the original
404 lines by Slab nuclear genome is estimated) and different *Wolbachia* types (*wPip*I and *wPip*IV
405 respectively, Table 1). *wPip* types were verified with the PCR/RFLP test on pool of larvae upon the
406 establishment of the lines and regularly verified for the duration of the experiments .

407

408 *Wolbachia free lines*

409 BcTunis, Slab and BcHarash lines, all stably infected with *wPip* (*Wo*+), were treated with tetracycline
410 (50 mL/L for larval treatment using a 0.4g/L solution) for 3 generations to create *Wolbachia* free lines
411 (*Wo*-: BcTunisTC, SlabTC, BcHarashTC, Table1). Experiments were conducted using the females
412 from: 1) the first generation right after we started the antibiotic treatment, 2) the third generation during
413 the antibiotic treatment to be able to follow the effect of the treatment and 3) from at least three
414 generations after the end of the antibiotic treatment, to exclude the direct effect of TC treatment on host
415 metabolism, and consequently on CpDV quantity and transmission (*Wo*-).

416

417 **Immunolabelling**

418 Immunolabeling were performed on ovaries from 6 days old females and dechorionated eggs. The egg
419 rafts were collected in 1-4 hours following egg laying. The egg- rafts were bleached (active ingredient,
420 9.6% of sodium hypochlorite) to separate the individual eggs and rinsed with distilled water. They were
421 then fixed with 3.2% para-formaldehyde diluted in PBS-T (PBS 1X with Tween 0,02%) for 2 hours,

422 and rinsed with PBS 1X. The chorion of each individual egg was removed manually with a needle under
423 an optical microscope (Leica MZ 8).

424

425 All the samples were fixed for 1 h in 4% paraformaldehyde (PFA) and washed 3 times with PBS, then
426 treated with 50 mM of NH₄Cl for 30 min to minimize autofluorescence. Ovaries and eggs were
427 permeabilized with PBS containing 0.5% Triton and blocking was performed with 1% bovine serum
428 albumin (BSA) in PBS for 1 h. Immunolabelling of CpDV were performed using a mouse primary JcDV
429 anti-capsid antibody that co-reacts with CpDV capsids (Jousset et al., 2000), diluted at 1:300 in PBS
430 with 0.2% Tween and incubated overnight at 4°C. Secondary antibodies (Alexa Fluor® 488 and 568;
431 1:500; Invitrogen) were used at 1:500 dilutions and incubated for 1 h (ovaries) or 6 h (eggs). Samples
432 were incubated 30 min with Phalloidin-FITC (1:300; Sigma) to label actin cytoskeleton, and 5 min with
433 DAPI (1 µg/ml; Invitrogen) to label nuclei and *Wolbachia*. Finally, labeled tissues and eggs were
434 mounted in Dako (Sigma) on coverslips for observation with a Leica SPE confocal microscope equipped
435 with suitable lasers and bandpass for the dyes (excitation 405 and emission 420-480 for DAPI; excitation
436 488 and emission 505-550 for Alexa Fluor® 488; emission 532 and emission 580-650 for TexasRed),
437 using a 20X/0.55 HCX APO L W and a 40X/1.15 ACS APO oil objectives. We used the LAS AF (Leica)
438 software to operate the microscope and all images were processed with ImageJ software.

439

440 **Electron microscopy**

441 To observe CpDV particles, a pool of ovaries from 6 days old females were dissected, crushed in PBS
442 and filtered with a 0.22 µm pore size filter. The filtered homogenate was subjected to a negative staining
443 using 2% phosphotungstic acid (PTA, Electron Microscopy Sciences) for observation using
444 transmission electron microscopy (TEM, see above).

445

446 Ultrathin sections were prepared from ovaries of 6 days old females and dechorionated eggs. Dissected
447 ovaries and eggs were first fixed in 2.5% glutaraldehyde in PBS for 2 h at 4°C, and post fixed in 2%
448 osmium tetroxide in the same buffer for 1 h at room temperature. Samples were then thoroughly rinsed
449 in distilled water, stained *en bloc* in 2% aqueous uranyl acetate solution for 2 h at room temperature,

450 dehydrated in increasing-concentration ethanol baths, embedded overnight in Embed 812 resin (Electron
451 Microscopy Sciences), and further polymerized for 2 days at 60°C. Then, ultrathin sections of
452 approximately 100 nm, performed using an LKB Ultratome III on copper grids, were counterstained
453 with uranylless (Delta Instruments) and lead citrate and examined using a JEOL 1200 EX II transmission
454 electron microscope operating at 100 kV and equipped with an Olympus camera (Quemesa model, 4008
455 x 2664 pixels) (MEA Platform, University of Montpellier, France).

456

457 **Vertical transmission assays**

458 Adult mosquitoes were collected from population cages with a mouth aspirator after 5 days following
459 blood meal. CO₂ was used to immobilize the mosquitoes. Ten females from each line (to test the effect
460 of distinct *Wolbachia* strains: Slab, Harash, Tunis, BcTunis, BcHarash; effect of curing *Wolbachia* with
461 tetracycline: SlabTC, BcTunisTC, BcHarashTC, Table 1) were isolated in individual autoclaved glass
462 containers covered with a net. Once females were awake, one third of the glass containers were filled
463 with bottled water that has been tested negative for CpDV prior to use and females were left to lay eggs.
464 Egg-rafts were then transferred to a new sterile glass container and water to hatch, to prevent any
465 possible contamination from females to the water. Five egg-rafts were randomly chosen per line
466 whenever possible and 10 L1 stage larvae from each females have been collected right after they
467 hatched. This was repeated twice adding up to about 10 females and 100 L1 stage larvae per line with a
468 total of 500 L1 tested.

469

470 Each L1 stage larva was individually collected to a 1.5 µl Eppendorf tube and crushed in 10 µl of STE
471 buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to obtain a crude DNA extraction. Two
472 PCR reactions were conducted using these crude extractions, 1) to check the presence of CpDV using
473 CpDVquantif and CpDVquantir primers and 2) to check the amplifiability of DNA using ace-2 gene
474 primers, acequantidir and acequantirev (see below for the sequences of the primers). The larvae crude
475 extraction that did not allow any amplification in the latter were excluded from further analysis. PCR
476 amplification conditions were as following: i) for CpDVquantif and CpDVquantir primers initial
477 denaturation for 5 min at 94 °C, followed by 33 cycles of denaturation, annealing and elongation

478 respectively at 94 °C for 30 s, 56 °C for 30s, and 72 °C for 90 s, and a final elongation at 72 °C for 5
479 min ii) for ace2quantif primers initial denaturation for 5 min at 94 °C, followed by 35 cycles of
480 denaturation, annealing and elongation respectively at 94 °C for 30 s, 52 °C for 30s, and 72 °C for 90 s,
481 and a final elongation at 72 °C for 5 min.

482 The proportion of CpDV-positive over CpDV-negative L1 larvae per female is calculated as vertical
483 transmission rate.

484

485 **Quantification of CpDV and *Wolbachia***

486 Ovaries were dissected from 6 days old female mosquitoes. Three ovaries were pooled together and 5
487 replicates were done per line. Qiagen extraction kit was used to extract DNA of ovary pools according
488 to the manufacturer's instructions. DNA was eluted in 55 µl PCR gradient water. Negative controls were
489 made to confirm that there was no contamination from the extraction kit and were always negative.
490 Additionally to check the effect of *Wolbachia* and CpDV amount in the females on vertical transmission
491 rate, DNA from the females that laid eggs have been extracted using CTAB technique (Rogers and
492 Bendich, 1989).

493

494 Real-time quantitative PCR (qPCR) was used to estimate the amount of *Wolbachia* and CpDV in each
495 sample. qPCR were performed using SYBR Green I Master (Roche) and analysed on a LightCycler 480
496 Instrument (Roche) using the LightCycler 480 software (version 1.5.1.62). On each DNA sample three
497 PCR reactions were performed in triplicates using i) NS2 protein coding region specific primers to
498 quantify CpDV (CpDVquantif: 5'-CATTGGAGGAGAAAGTTGGAA-3' , CpDVquantir: 5'-
499 TCCCATTATCTTGCTGTCG-3'), ii) wsp specific primers to quantify *Wolbachia* (wolpipdir 5'-
500 AGAATTGACGGCATTGAATA-3' and wolpiprev 5'-CGTCGTTTTTGTGTTAGTTGTG-3' (Berticat
501 *et al.*, 2002)) and iii) *Culex* Ace-2 locus specific primers to quantify host genome copy as a reference
502 (Acequantidir: 5'-GCAGCACCAAGTCCAAGG-3', Acequantirev: 5'-
503 CTTACGGCCGTTCAAGTAG-3') (Weill *et al.*, 2000). Standard curves were created using dilutions
504 of a pCR®4-TOPO- Vector (Invitrogen) containing a single copy of each *ace-2*, *wsp* and *ns2* gene
505 fragments. Plates were designed to have all the compared groups of a biological replicate and prepared

506 using Echo 525 Liquid Handler, Labcyte Inc. The amount of *Wolbachia* and CpDV were normalized
507 with the amount of Ace-2 copies in order to obtain a number of virus or *Wolbachia* per cell.

508

509 **Data Analysis**

510

511 All data analyses have been performed in R version 3.3.1 (R Core Team, 2016).

512

513 To analyze the effects of *Wolbachia* amount and diversity, on the amount of CpDV in the ovaries, one
514 highly infected outlier from Harash line has been removed from the analysis and normality of the
515 continuous response variable (log₁₀ transformed amount of CpDV genome/ amount of host genome)
516 has been checked with Shapiro–Wilk normality test. A linear mixed effect model is fitted with genetic
517 background (Slab, Harash, Tunis), *Wolbachia* type (*wPip* I, *wPip* III and *wPip* IV) and *Wolbachia*
518 amount as fixed effects and replicates as random effects using lme4 package (Bates, 2010).
519 Homoscedasticity and normality of the residuals were checked using Breusch-Pagan test (ncvTest, in
520 car package (Fox and Weisberg, 2011)) and Shapiro-Wilk test respectively. Likelihood ratio tests of the
521 full model against the model without a given effect were used to obtain deviance and p-values.

522

523 To analyze the effects of *Wolbachia* diversity on vertical transmission of CpDV, a binomial generalized
524 mixed effect model is fitted with genetic background (Slab, Harash, Tunis), *Wolbachia* type (*wPip* I,
525 *wPip* III and *wPip* IV), *Wolbachia* amount and CpDV amount in the full body of the female as fixed
526 effects and replicates as random effects.

527

528 CpDV in females were checked for the first and third generation of tetracycline treatment, they were
529 grouped under ‘during tetracycline treatment group’ given that these two groups did not show any
530 significant difference in terms of CpDV amount in the ovaries and represented the same treatment status.

531 A Dunnett’s test (glhrt function in multcomp package) (Hothorn *et al.*, 2008) has been performed for
532 pairwise comparison of treatment stages to the non-treated control group.

533

534 In this model genetic background (Slab, Harash, Tunis) and treatment status (no treatment, during TC
535 treatment and after TC treatment) were fixed effects and different replicates were added as random
536 effects. This last dataset also have been analysed using pairwise Wilcoxon test with Bonferroni
537 correction to compare the different treatment stages to the non-treated control group.

538

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562 **References**

- 563 Afanasiev, B.N., Galyov, E.E., Buchatsky, L.P., and Kozlov, Y. V (1991) Nucleotide sequence and
564 genomic organization of *Aedes densonucleosis* virus. *Virology* 336: 323–336.
- 565 Alizon, S., Hurford, A., Mideo, N., and Van Baalen, M. (2009) Virulence evolution and the trade-off
566 hypothesis: History, current state of affairs and the future. *J. Evol. Biol.* 22: 245–259.
- 567 Altinli, M., Gunay, F., Alten, B., Weill, M., and Sicard, M. (2018) *Wolbachia* diversity and
568 cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey. *Parasit. Vectors* 11:
569 198.
- 570 Atyame, C.M., Delsuc, F., Pasteur, N., Weill, M., and Duron, O. (2011) Diversification of *Wolbachia*
571 endosymbiont in the *Culex pipiens* mosquito. *Mol. Biol. Evol.* 28: 2761–2772.
- 572 Baquerizo-Audiot, E., Abd-alla, A., Tijssen, P., and Bergoin, M. (2009) Structure and expression
573 strategy of the genome of *Culex pipiens* Densovirus, a mosquito densovirus with an ambisense
574 organization. 83: 6863–6873.
- 575 Barreau, C., Jousset, F.X., and Bergoin, M. (1996) Pathogenicity of the *Aedes albopictus* Parvovirus
576 (AaPV), a denso-like virus, for *Aedes aegypti* mosquitoes. *J. Invertebr. Pathol.* 68: 299–309.
- 577 Barreau, C., Jousset, F.X., and Bergoin, M. (1997) Venereal and vertical transmission of the *Aedes*
578 *albopictus* parvovirus in *Aedes aegypti* mosquitoes. *Am. J. Trop. Med. Hyg.* 57: 126–131.
- 579 Bates, D. (2010) Mixed models in R using the lme4 package Part 5 : Inference based on profiled
580 deviance. 1–7.
- 581 Becnel, J.J. and White, S.E. (2007) Mosquito pathogenic viruses--the last 20 years. *J. Am. Mosq.*
582 *Control Assoc.* 23: 36–49.
- 583 Berticat, C., Rousset, F., Raymond, M., Berthomieu, A., and Weill, M. (2002) High *Wolbachia*
584 density in insecticide-resistant mosquitoes. *Proc. R. Soc. B Biol. Sci.* 269: 1413–1416.
- 585 Bian, G., Zhou, G., Lu, P., and Xi, Z. (2013) Replacing a Native *Wolbachia* with a Novel Strain
586 Results in an Increase in Endosymbiont Load and Resistance to Dengue Virus in a Mosquito
587 Vector. *PLoS Negl. Trop. Dis.* 7: e2250.
- 588 Boublik, Y., Jousset, F.X., and Bergoin, M. (1994) Complete nucleotide sequence and genomic
589 organization of the *Aedes albopictus* Parvovirus (AaPV) pathogenic for *Aedes aegypti* larvae.

590 Virology 200: 752–763.

591 Carlson, J., Suchman, E., and Buchatsky, L. (2006) Densoviruses for control and genetic manipulation
592 of mosquitoes. *Adv. Virus Res.* 68: 361–92.

593 Clements, A.N. (1992) *The biology of mosquitoes*. CABI Publishing, Cambridge

594 Cotmore, S.F., Agbandje-McKenna, M., Chiorini, J.A., Mukha, D. V., Pintel, D.J., Qiu, J., et al.
595 (2014) The family Parvoviridae. *Arch. Virol.* 159: 1239–1247.

596 Cressler, C.E., McLeod, D. V., Rozins, C., Van Den Hoogen, J., and Day, T. (2016) The adaptive
597 evolution of virulence: A review of theoretical predictions and empirical tests. *Parasitology* 143:
598 915–930.

599 Duron, O., Bernard, J., Atyame, C.M., Dumas, E., and Weill, M. (2012) Rapid evolution of *Wolbachia*
600 incompatibility types. *Proc. R. Soc. B Biol. Sci.* 279: 4473–4480.

601 Ebert, D. (2013) The epidemiology and evolution of symbionts with mixed-mode transmission. *Annu.*
602 *Rev. Ecol. Evol. Syst.* 44: 623–643.

603 Ewald, P.W. (1983) Host-parasite relations, vectors, and the evolution of disease severity. *Annu. Rev.*
604 *Ecol. Syst.* 14: 465–485.

605 Ewald, P.W. (1987) Transmission modes and evolution of the parasitism-mutualism continuum. *Ann.*
606 *N. Y. Acad. Sci.* 503: 295–306.

607 Fox, J. and Weisberg, S. (2011) *An {R} Companion to Applied Regression Second*. Sage, Thousand
608 Oaks {CA}.

609 François, S., Filloux, D., Roumagnac, P., Bigot, D., Gayral, P., Martin, D.P., et al. (2016) Discovery
610 of parvovirus-related sequences in an unexpected broad range of animals. *Sci. Rep.* 6: 1–13.

611 Gosselin Grenet, A.S., Salasc, F., Francois, S., Mutuel, D., Dupressoir, T., Multeau, C., et al. (2015)
612 *Les densovirus: Une «massive attaque» chez les arthropodes*. *Virologie* 19: 19–31.

613 Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Kontsedalov, S., Skaljic, M., Brumin, M., et al.
614 (2010) The transmission efficiency of Tomato Yellow Leaf Curl Virus by the whitefly *Bemisia*
615 *tabaci* Is correlated with the presence of a specific symbiotic bacterium species. *J. Virol.* 84:
616 9310–9317.

617 Graham, R.I., Grzywacz, D., Mushobozi, W.L., and Wilson, K. (2012) *Wolbachia* in a major African

618 crop pest increases susceptibility to viral disease rather than protects. *Ecol. Lett.* 15: 993–1000.

619 Hedges, L.M., Brownlie, J.C., O’Neill, S.L., and Johnson, K.N. (2008) *Wolbachia* and virus protection
620 in insects. *Science* (80). 322: 702.

621 Hothorn, T., Bretz, F., and Westfall, P. (2008) Simultaneous inference in general parametric models.
622 *Biometrical J.* 50: 346–363.

623 Huang, L.Y., Halder, S., and Agbandje-Mckenna, M. (2014) Parvovirus glycan interactions. *Curr.*
624 *Opin. Virol.* 7: 108–118.

625 Hussain, M., Lu, G., Torres, S., Edmonds, J.H., Kay, B.H., Khromykh, A.A., and Asgari, S. (2012)
626 Effect of *Wolbachia* on replication of West Nile Virus in mosquito cell line and adult
627 mosquitoes. *J. Virol.* 87: 851–858.

628 Johnson, R.M. and Rasgon, J.L. (2018) Densonucleosis viruses (‘densoviruses’) for mosquito and
629 pathogen control. *Curr. Opin. Insect Sci.* 28: 90–97.

630 Jousset, F.X., Baquerizo, E., and Bergoin, M. (2000) A new densovirus isolated from the mosquito
631 *Culex pipiens* (Diptera: Culicidae). *Virus Res.* 67: 11–16.

632 Jousset, F.X., Barreau, C., Boublik, Y., and Cornet, M. (1993) A Parvo-like virus persistently
633 infecting a C6/36 clone of *Aedes albopictus* mosquito cell line and pathogenic for *Aedes aegypti*
634 larvae. 29: 99–114.

635 Kane, M. and Golovkina, T. (2010) Common threads in persistent viral infections. *J. Virol.* 84: 4116–
636 4123.

637 Kang, Y.J., Huang, W., Zhao, A. li, Lai, D.D., Shao, L., Shen, Y.Q., et al. (2017) Densoviruses in
638 oyster *Crassostrea ariakensis*. *Arch. Virol.* 162: 2153–2157.

639 Kerr, J., Cotmore, S., and Bloom, M.E. (2005) *Parvoviruses* Taylor & Francis, Boca Raton.

640 Kittayapong, P., Baisley, K.J., and O’Neill, S.L. (1999) A mosquito densovirus infecting *Aedes*
641 *aegypti* and *Aedes albopictus* from Thailand. *Am. J. Trop. Med. Hyg.* 61: 612–617.

642 Lebedeva, O.P., Zelenko, A.P., and Kuznetsova, M. (1972) The detection of viral infection in larvae of
643 *Aedes aegypti*. *Mikrobiol. Zh.* 34: 70–73.

644 Ledermann, J.P., Suchman, E.L., Black IV, W.C., and Carlson, J.O. (2004) Infection and
645 pathogenicity of the mosquito densoviruses AeDENV, HeDENV, and APeDENV in *Aedes aegypti*

646 mosquitoes (Diptera: Culicidae). *J. Econ. Entomol.* 97: 1828–1835.

647 Le Clec'h, W., Dittmer, J., Raimond, M., Bouchon, D., and Sicard, M. (2017) Phenotypic shift in
648 *Wolbachia* virulence towards its native host across serial horizontal passages. *Proc. R. Soc. B*
649 *Biol. Sci.* 284: 20171076.

650 Lu, P., Bian, G., Pan, X., and Xi, Z. (2012) *Wolbachia* induces density-dependent inhibition to
651 Dengue virus in mosquito cells. *PLoS Negl. Trop. Dis.* 6: 1–8.

652 Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J.P., and Jiggins, F.M. (2017) Symbiont
653 strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses
654 across *Drosophila* species. *Mol. Ecol.* 26: 4072–4084.

655 Micieli, V.M. and Glaser, R.L. (2014) Somatic *Wolbachia* (Rickettsiales : Rickettsiaceae) levels in
656 *Culex quinquefasciatus* and *Culex pipiens* (Diptera : Culicidae) and resistance to West Nile Virus
657 infection. *J. Med. Entomol.* 51: 189–199.

658 Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., et al. (2009) A
659 *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and
660 *Plasmodium*. *Cell* 139: 1268–1278.

661 Mousson, L., Martin, E., Zouache, K., Madec, Y., Mavingui, P., and Failloux, A.B. (2010) *Wolbachia*
662 modulates Chikungunya replication in *Aedes albopictus*. *Mol. Ecol.* 19: 1953–1964.

663 Van Munster, M., Dullemans, A.M., Verbeek, M., Van Den Heuvel, J.F.J.M., Reinbold, C., Brault, V.,
664 et al. (2003) Characterization of a new densovirus infecting the green peach aphid *Myzus*
665 *persicae*. *J. Invertebr. Pathol.* 84: 6–14.

666 Mutuel, D., Ravallec, M., Chabi, B., Multeau, C., Salmon, J.M., Fournier, P., and Ogliastro, M. (2010)
667 Pathogenesis of *Junonia coenia* densovirus in *Spodoptera frugiperda*: A route of infection that
668 leads to hypoxia. *Virology* 403: 137–144.

669 Neu, U., Bauer, J., and Stehle, T. (2011) Viruses and sialic acids: Rules of engagement. *Curr. Opin.*
670 *Struct. Biol.* 21: 610–618.

671 Ng, T.F.F., Willner, D.L., Lim, Y.W., Schmieder, R., Chau, B., Nilsson, C., et al. (2011) Broad
672 surveys of DNA viral diversity obtained through viral metagenomics of mosquitoes. *PLoS One*
673 6: e20579.

674 O'Neill, S.L., Kittayapong, P., Braig, H.R., Andreadis, T.G., Gonzalez, J.P., and Tesh, R.B. (1995)
675 Insect densovirus may be widespread in mosquito cell lines. *J. Gen. Virol.* 76: 2067–2074.

676 Osborne, S.E., Iturbe-Ormaetxe, I., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2012) Antiviral
677 protection and the importance of *Wolbachia* density and: Tissue tropism in *Drosophila simulans*.
678 *Appl. Environ. Microbiol.* 78: 6922–6929.

679 R Core Team (2016) R: A Language and Environment for Statistical Computing.

680 Raikhel, A.S. and Lea, A.O. (1983) Previtellogenic development and vitellogenin synthesis in the fat
681 body of a mosquito: An ultrastructural and immunocytochemical study. *Tissue Cell* 15: 281–299.

682 Ren, X., Hoiczky, E., and Rasgon, J.L. (2008) Viral paratransgenesis in the malaria vector *Anopheles*
683 *gambiae*. 4: 4–11.

684 Ren, X., Hughes, G.L., Niu, G., Suzuki, Y., and Rasgon, J.L. (2014) *Anopheles gambiae* densovirus
685 (AgDENV) has negligible effects on adult survival and transcriptome of its mosquito host. *PeerJ*
686 2: e584.

687 Rogers, S.O. and Bendich, A.J. (1989) Extraction of DNA from plant tissues. In, Gelvin, S.B.,
688 Schilperoort, R.A., and Verma, D.P.S. (eds), *Plant Molecular Biology Manual*. Springer
689 Netherlands, Dordrecht.

690 Rwegoshora, R.T., Baisley, K.J., and Kittayapong, P. (2000) Seasonal and spatial variation in natural
691 densovirus infection in *Anopheles minimus* s.l. in Thailand. *Southeast Asian J. Trop. Med.*
692 *Public Health* 31: 3–9.

693 Rwegoshora, R.T. and Kittayapong, P. (2004) Pathogenicity and infectivity of the Thai-strain
694 densovirus (A_{Th}DENV) in *Anopheles minimus* s.l. *Southeast Asian J. Trop. Med. Public Health*
695 35: 630–634.

696 Sicard, M., Dittmer, J., Grève, P., Bouchon, D., and Braquart-Varnier, C. (2014) A host as an
697 ecosystem: *Wolbachia* coping with environmental constraints. *Environ. Microbiol.* 1924: 2922–
698 2936.

699 Stouthamer, R., Breeuwer, J.A.J.A.J., and Hurst, G.D.D. (1999) *Wolbachia pipientis* : microbial
700 manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 53: 71–102.

701 Suchman, E.L., Kononko, A., Plake, E., Doehling, M., Kleker, B., Black, W.C., et al. (2006) Effects of

702 AeDNV infection on *Aedes aegypti* lifespan and reproduction. 39: 465–473.

703 Suzuki, Y., Barik, T.K., Johnson, R.M., and Rasgon, J.L. (2015) In vitro and in vivo host range of
704 *Anopheles gambiae* densovirus (AgDNV). *Sci. Rep.* 5: 5–10.

705 Suzuki, Y., Niu, G., Hughes, G.L., and Rasgon, J.L. (2014) A viral over-expression system for the
706 major malaria mosquito *Anopheles gambiae*. *Sci. Rep.* 4: 32–35.

707 Teixeira, L., Ferreira, Á., and Ashburner, M. (2008) The bacterial symbiont *Wolbachia* induces
708 resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* 6: 2753–2763.

709 Tijssen, P., Péntzes, J.J., Yu, Q., Pham, H.T., and Bergoin, M. (2016) Diversity of small, single-
710 stranded DNA viruses of invertebrates and their chaotic evolutionary past. *J. Invertebr. Pathol.*
711 140: 83–96.

712 Weill, M., Berticat, C., Raymond, M., and Chevillon, C. (2000) Quantitative polymerase chain
713 reaction to estimate the number of amplified esterase genes in insecticide-resistant mosquitoes.
714 *Anal. Biochem.* 285: 267–270.

715 Werren, J.H., Baldo, L., and Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate
716 biology. *Nat. Rev. Microbiol.* 6: 741–751.

717 Williams, T., Virto, C., Murillo, R., and Caballero, P. (2017) Covert infection of insects by
718 baculoviruses. *Front. Microbiol.* 8: 1–13.

719 Xu, P., Liu, Y., Graham, R.I., Wilson, K., and Wu, K. (2014) Densovirus Is a mutualistic symbiont of
720 a global crop pest (*Helicoverpa armigera*) and protects against a baculovirus and Bt biopesticide.
721 *PLoS Pathog.* 10: 2–12.

722 Zug, R. and Hammerstein, P. (2014) Bad guys turned nice? A critical assessment of *Wolbachia*
723 mutualisms in arthropod hosts. *Biol. Rev.* 49: 89–111.

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730 **Figure Legends**

731 **Figure 1. The two symbionts *Wolbachia* and CpDV coexist in *Cx. pipiens* ovaries of ten laboratory**
732 **lines. *Wolbachia* amount (A)** Ovaries from 15 females per line were dissected and grouped in five
733 pools (3 individuals per pool). *wsp* copy number/*ace-2* copy number was quantified for the 5 pools per
734 line by qPCR assays. **CpDV amount (B)** NS2 copy number and *ace-2* copy number have also been
735 quantified in the same pools. Harash line with the highest quantity of CpDV in ovaries and Tunis line
736 with less CpDV in ovaries were selected for further experimentation.

737

738 **Figure 2. Immunolabeling of CpDV in *Cx. pipiens* ovaries.**

739 **Structure of a *Cx. pipiens* ovariole (A).** The mosquito ovaries are paired and composed of functional
740 units called ovarioles, containing primary (F1) and secondary follicles (F2) (Clements, 1992). Each
741 primary follicle contains seven nurse cells and one oocyte, both surrounded by follicular epithelium.
742 The secondary follicle is composed of undifferentiated cells surrounded by follicular epithelium. The
743 secondary follicle will turn into a primary follicle after oviposition of the oocyte contained in the
744 previous primary follicle (Raikhel and Lea, 1983). The sheath that enclosed each ovariole is a thin
745 membrane with an outer muscle layer composed of isolated cell bodies interconnected by muscle fibers.
746 **Confocal images of ovaries isolated from 6 days old *Cx. pipiens* females (B,C,D,E).** Immunolabeling
747 of CpDV (in red) was performed with an anti-capsid antibody, actin was labeled with phalloidin-FITC
748 (green) and nuclei with DAPI (blue). Arrows show CpDV-infected cells and arrowheads point
749 cytoplasmic dots of CpDV labelling. Images of the surface of an ovary and an ovariole (composed of a
750 primary and a secondary follicles) are shown in B and C, respectively. Internal views of primary follicles
751 are shown in D and E. Oo = oocyte.

752

753 **Figure 3. Electron micrographs of *Cx. pipiens* ovaries.**

754 **Ultrathin sections of ovaries isolated from 6 days old females (Harash line) (A to H).** Large views
755 of an ovariole (primary follicle) in A, D and G (bar scale: 5 μ m). Ultrastructure of the hypertrophied
756 nuclei and putative virus factories in a follicular cell (A and B), in an oocyte (D and E) and in a nurse

757 cell (G and H). Zooms in the nucleoplasm of the follicular cell (C) and in the oocyte (F) show particles
758 with the expected size of CpDV (25 nm).

759 **Virus particles observed in a filtered homogenate of ovaries (I).** Arrows show icosahedral virus
760 particles and arrowheads point empty particles with the expected size of CpDV (bar scale : 50 nm).

761

762

763 **Figure 4. Localization of CpDV in *Cx. pipiens* eggs.**

764 **Confocal images of dechorionated eggs from *Cx. pipiens* females (Harash line) (A).** CpDV (in red)
765 were immunolabeled with an anti-capsid antibody; *Wolbachia* and nuclei (in blue) were labeled with
766 DAPI. Top panel shows eggs in the first hour after laying (early developmental stage) and bottom panel
767 eggs 2 hours after laying (late developmental stage). The second part of the top panel is a zoom of one
768 egg pole in the first hour after laying; the second part of the Bottom panel is a zoom in the egg cytoplasm.
769 Arrows show *Wolbachia* cells at one egg pole.

770 **Electron micrographs of ultrathin sections of dechorionated eggs (B and C).** In B, a large view of
771 an early egg pole (bar scale: 5 μ m), showing the accumulated reserves required for the embryo
772 development (VG, vitellin granule; LD: lipid droplet). In C, a zoom (bar scale: 500 nm) inside the
773 cytoplasm of the early egg showing numerous *Wolbachia* (arrowheads). The virus particles cannot be
774 distinguished from the ribosomes because of their similar size.

775

776 **Figure 5. *Wolbachia* and CpDV amounts in the ovaries and vertical transmission of CpDV in**
777 **different *Cx. pipiens* lines with similar genetic background but different *Wolbachia* strains.** Slab
778 line's genetic background was introgressed into Harash and Tunis lines, to establish BcHarash and
779 BcTunis lines with the same genetic background as Slab (shown in circles) and same *Wolbachia* strain
780 as Harash (shown in orange) or Tunis (shown in green) respectively.

781 ***Wolbachia* amount in ovaries (A).** *Wolbachia* amount in the ovaries was not significantly different
782 between the *Cx. pipiens* lines no matter what the *Wolbachia* strain and host genetic background were
783 (*Wolbachia* strain: glm, df=2, dev=17.703, p=0.875; host genetic background: df=1 dev=0.034,
784 p=0.981).

785 **CpDV amount in ovaries (B)** Ovaries from 15 females per line were dissected and grouped in five
786 pools (three individuals per pool) which have been tested by qPCR assays to quantify CpDV and
787 *Wolbachia* amount. Mixed effect linear model showed no effect of *Wolbachia* amount or genetic
788 background on the CpDV amount in the ovaries (*Wolbachia* amount dev=0.001, df=1, p=0.9, genetic
789 background: dev=1.602, df=2, p= 0.449). *Wolbachia* strain, however significantly affected the CpDV
790 amount (dev=12.236, df=1, p<0.001). **CpDV vertical transmission rate (C)** Females from each line
791 were collected 5 days after their blood meal and isolated to lay eggs. Resulting L1 offspring was tested
792 for CpDV prevalence and vertical transmission rate was calculated per female. A binomial generalized
793 mixed effect model was fitted with host genetic background, *Wolbachia* strain as well as CpDV and
794 *Wolbachia* amount in females' bodies as fixed effects and replicates as random effects. None of these
795 variable had a significant effect on the vertical transmission rate.

796

797 **Figure 6. Effect of curing *Wolbachia* by tetracycline treatment on CpDV amount in *Cx. pipiens*.**

798 We have treated Slab, BcHarash and BcTunis lines with tetracycline (TC) for three generations and
799 dissected the ovaries of 6 days old females. Dissections were performed on the non-treated mosquitoes
800 (Wo+), first and third generation of mosquitoes during tetracycline treatment (during TC treatment) and
801 three generations after the tetracycline treatment was finished (Wo-).

802 **Effect of tetracycline treatment on the amount of *Wolbachia* in ovaries and carcasses (A).**

803 *Wolbachia* amount decreased immediately starting from first generation during tetracycline treatment.
804 Even though *Wolbachia* was detected in the ovaries of some mosquitoes from the first generation of the
805 treatment, there were no *Wolbachia* detected in the subsequent treatment generation neither in ovaries
806 nor in carcasses.

807 **Effect of tetracycline treatment, leading to full *Wolbachia* disappearance, on CpDV amount (B).**

808 CpDV amount in the ovaries significantly decreased starting from the first round of tetracycline
809 treatment but increased when tetracycline treatment stopped and the line "recovered" from the treatment
810 (Wo-)(Dunnett's test with Bonferroni Correction; Wo+ vs. during TC treatment, t=2.302, p=0.025; Wo+
811 vs. Wo-, t= 0.118, p=1). Carcasses had less CpDV compared to ovaries (Wilcoxon rank sum test, W =
812 168, p = 0.021). The effect of the treatment was not significant on the carcasses (Wilcoxon rank sum

813 test with Bonferroni correction; W_{O+} vs. during TC treatment, $W=132.5$, $p=0.08$; W_{O+} vs. W_{O-} , $W=$
814 100 , $p=0.138$; during TC treatment vs. W_{O-} , $W=308$, $p=1$).

815

816 **Figure 7. Effect of curing *Wolbachia* by tetracycline treatment on CpDV vertical transmission.** In

817 BcHarash, BcTunis and Slab lines, females emerged from the larvae that underwent the first tetracycline

818 treatment were isolated five days after bloodmeal to lay eggs. 10 females per line (total $n=30$), 10 L1

819 stage larvae per female were collected and tested for CpDV presence to estimate vertical transmission

820 rate. This procedure was repeated for females emerged after the third generation of TC treatment and

821 for the females W_{O-} (three generation after the end of tetracycline treatment). There was a significant

822 decrease in vertical transmission during tetracycline treatment (Wilcoxon rank sum test with Bonferroni

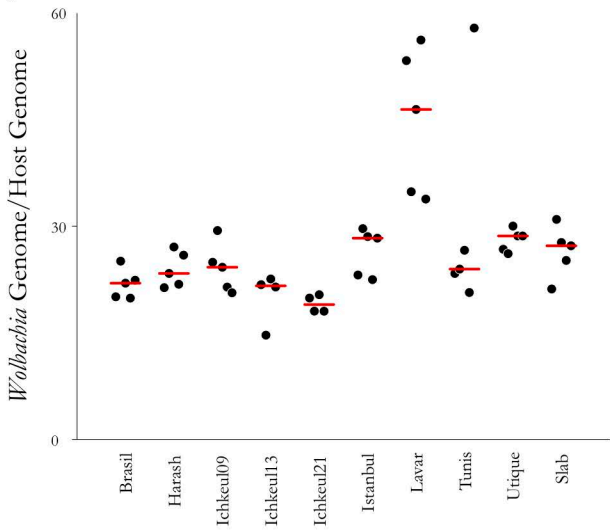
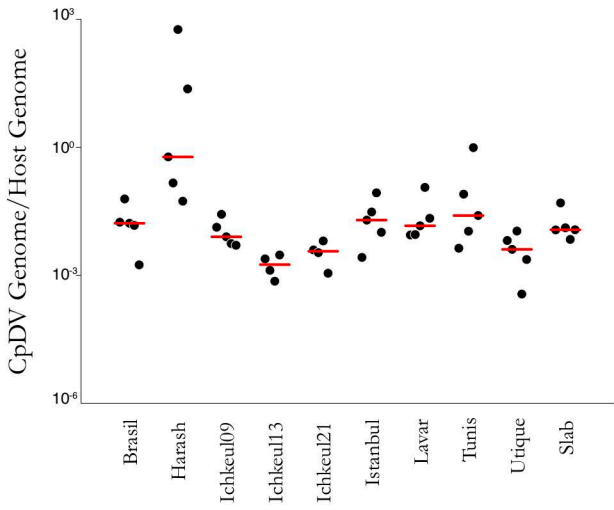
823 correction; W_{O+} vs. during TC treatment, $w=488$, $p=0.004$). Three generations after the end of the

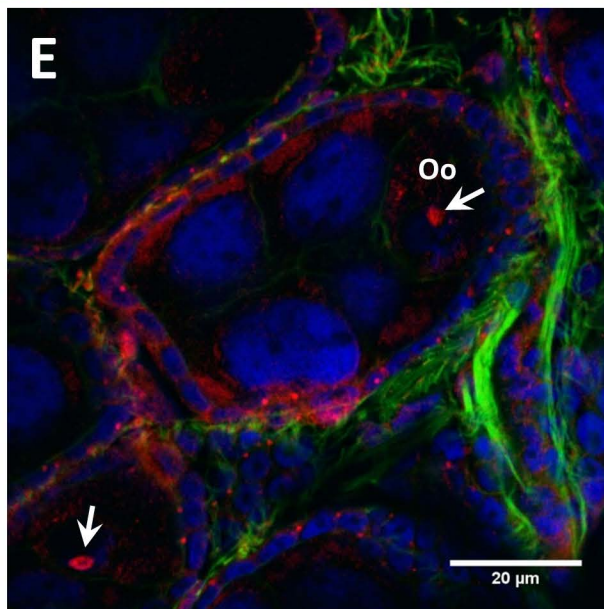
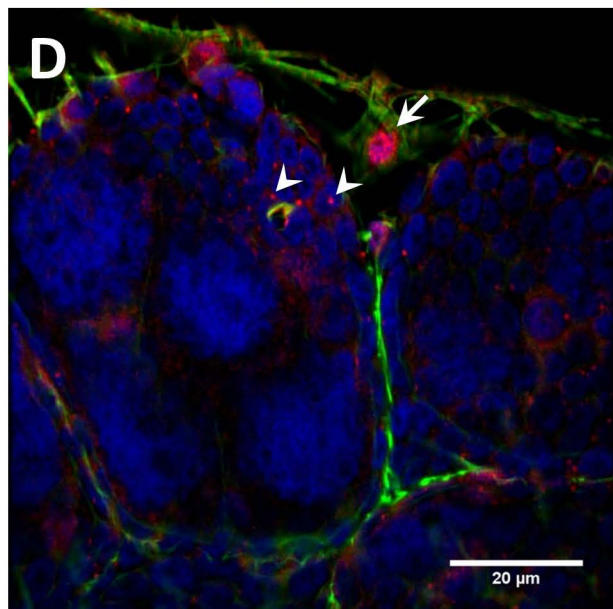
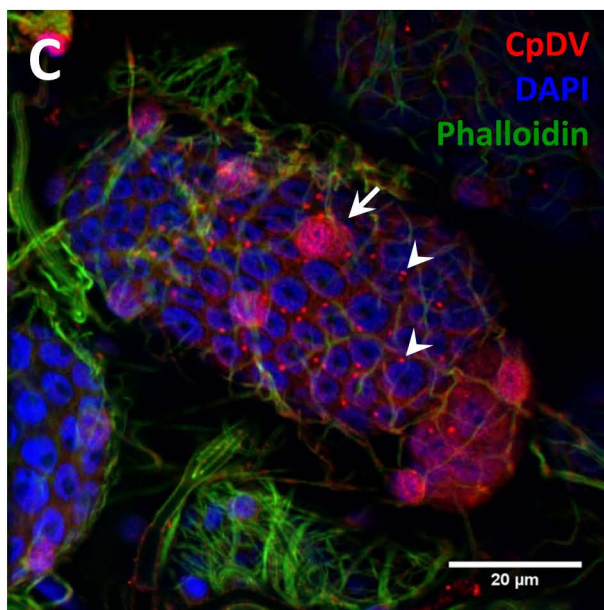
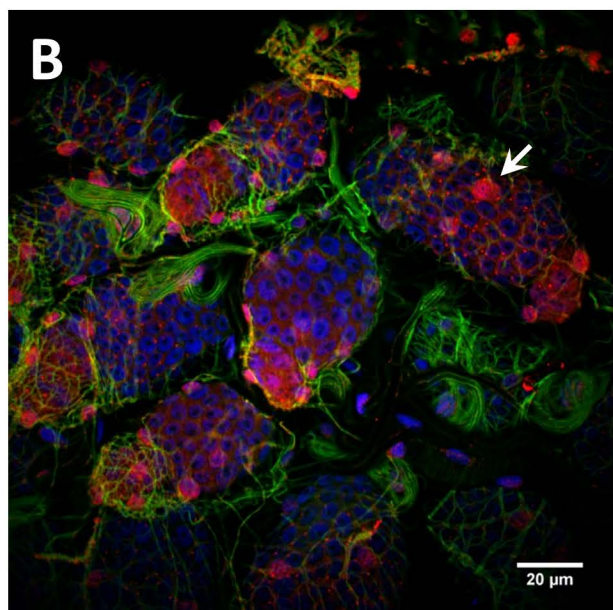
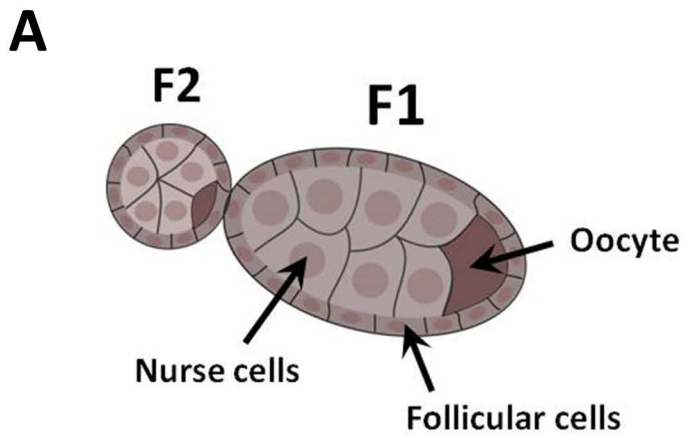
824 treatment the vertical transmission rate increased but still was marginally significant from the non-

825 treated group (Wilcoxon rank sum test with Bonferroni correction; W_{O+} vs. W_{O-} , $w=576.5$, $p=0.05$).

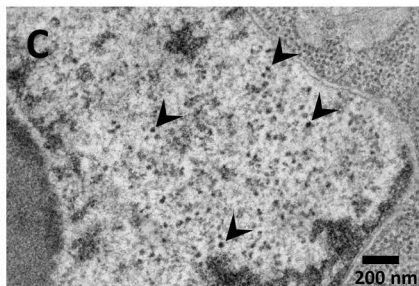
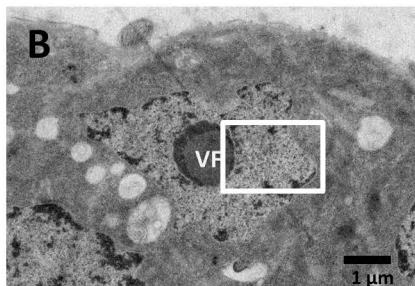
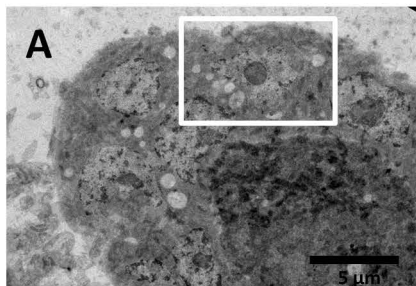
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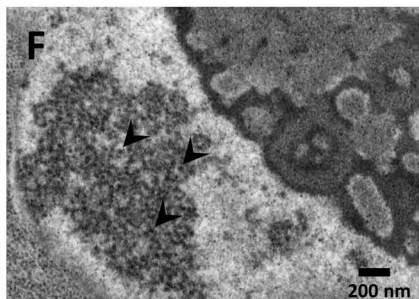
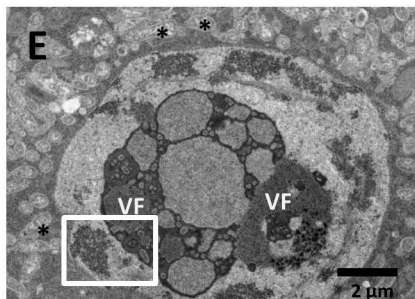
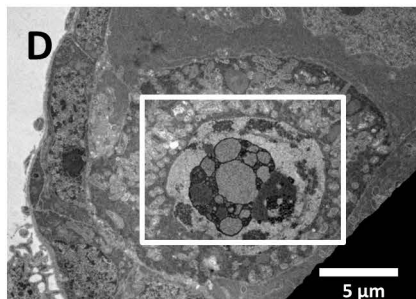
A**B**



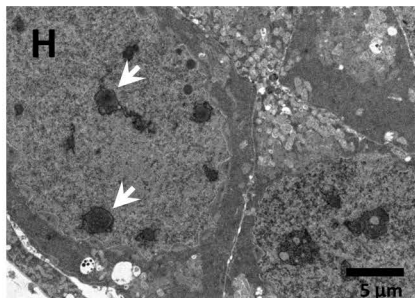
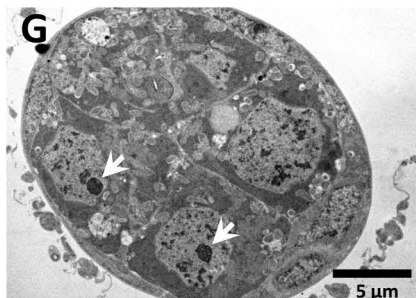
Follicular cells



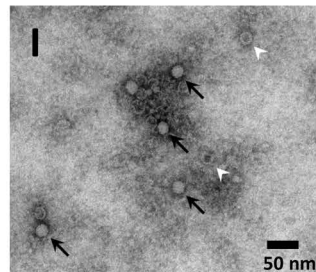
Oocyte

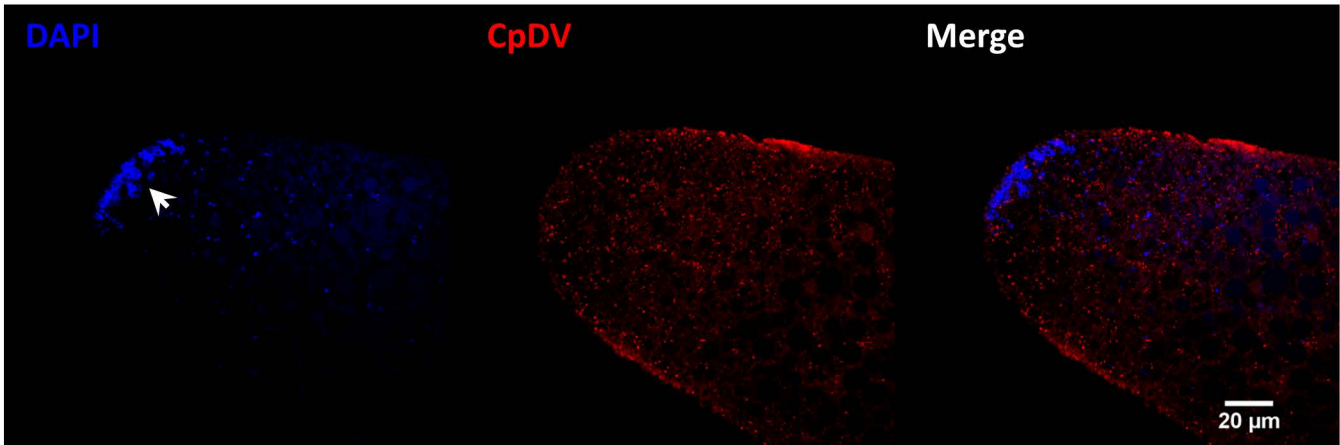
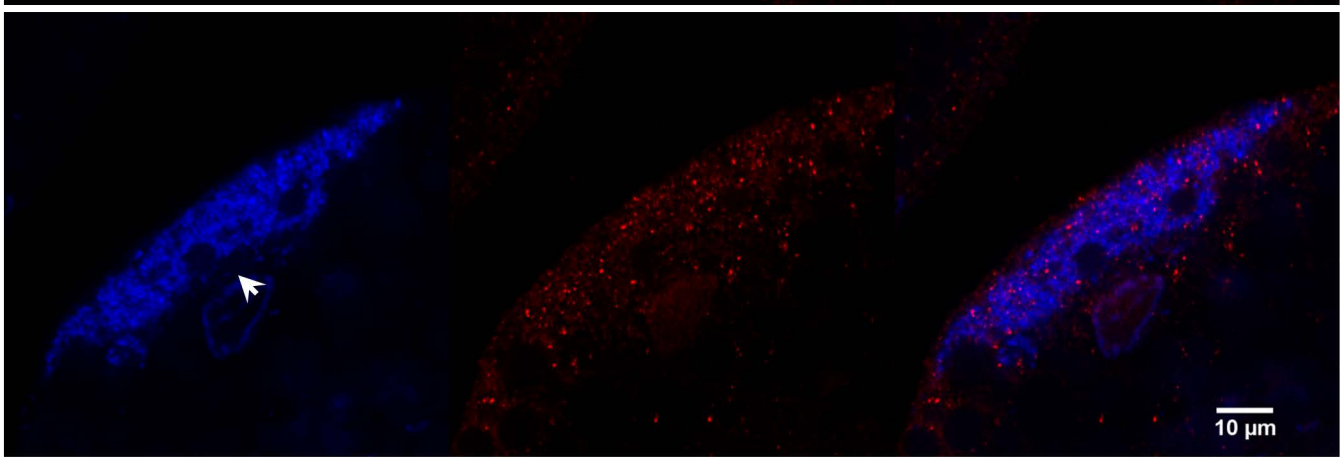
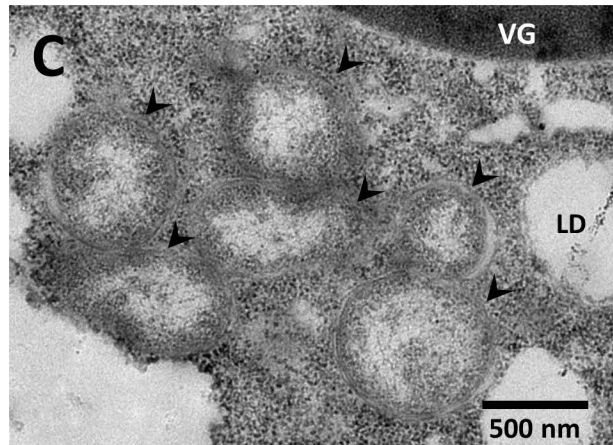
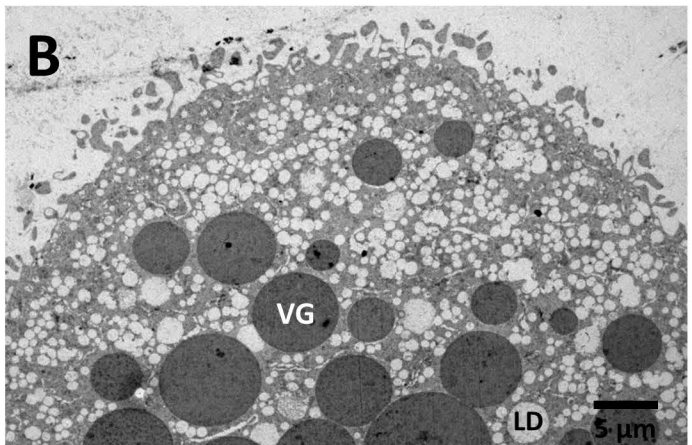
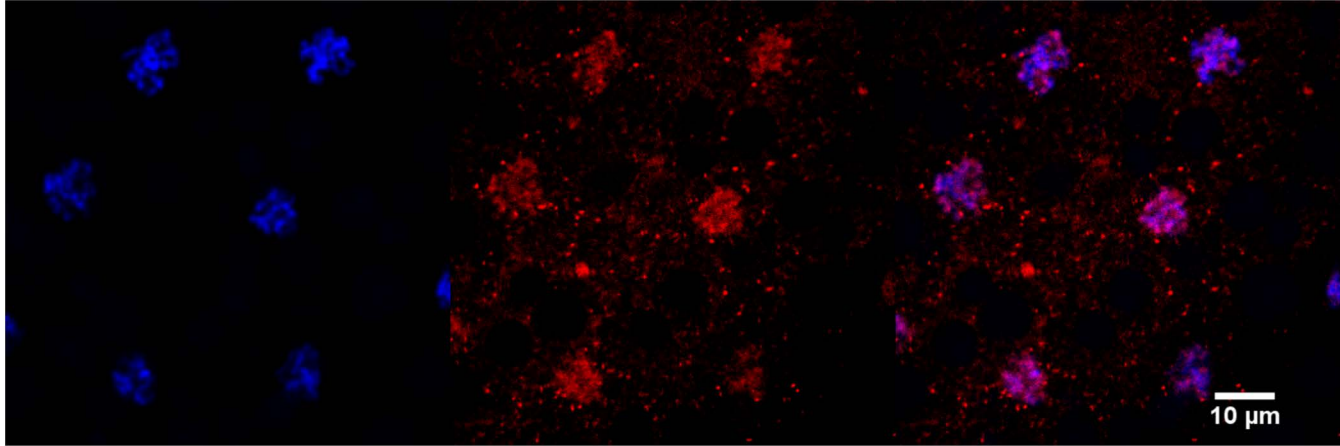
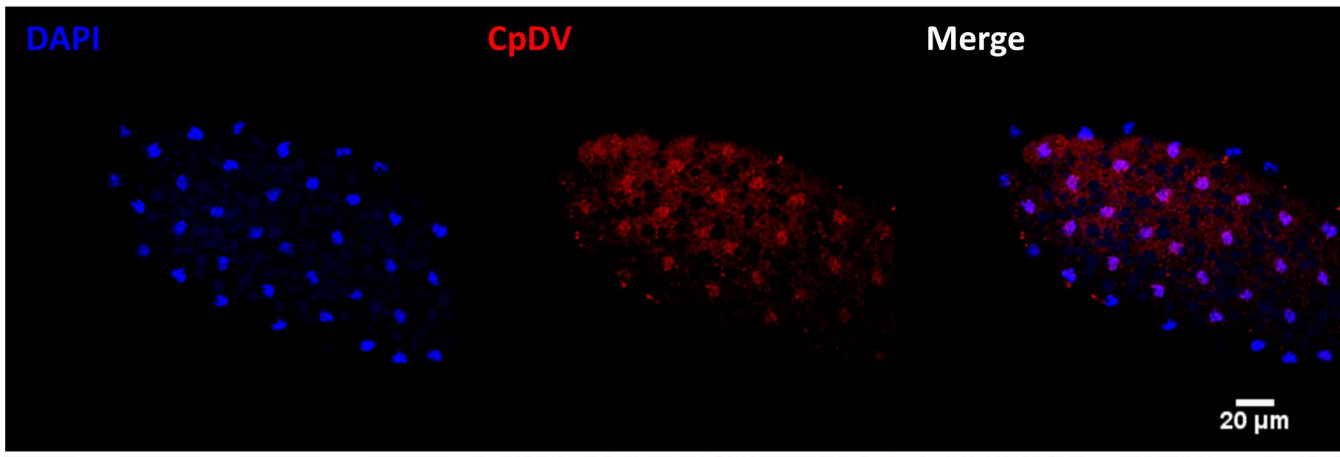


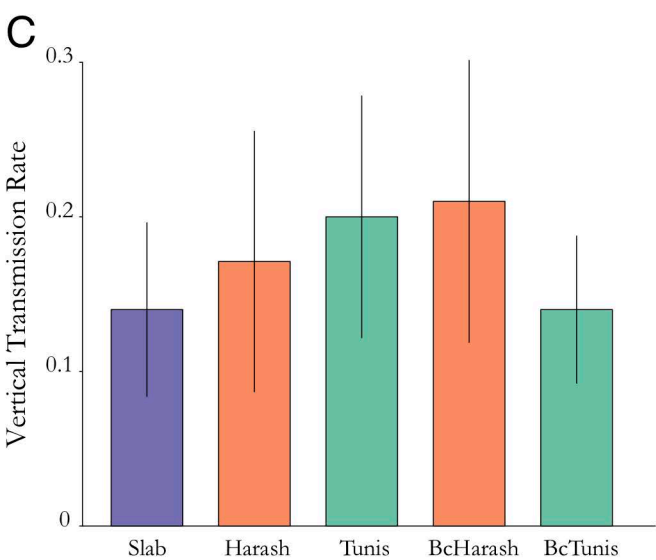
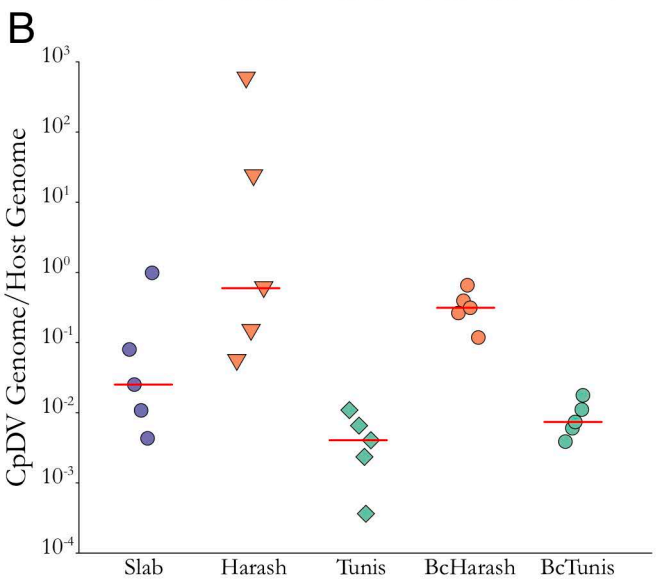
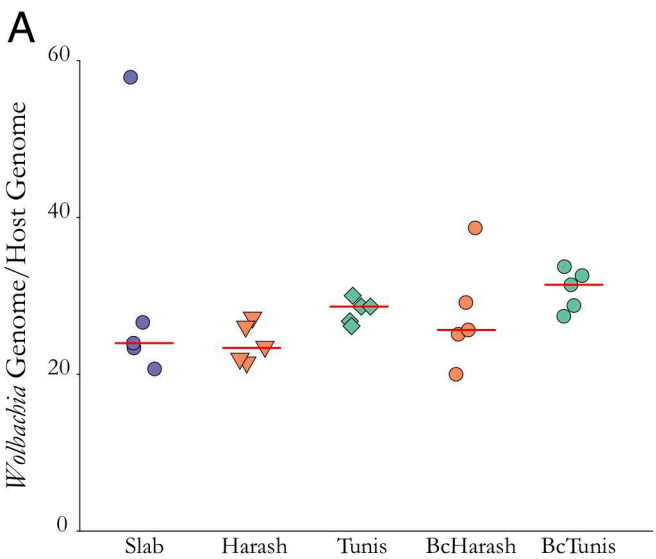
Nurse cells



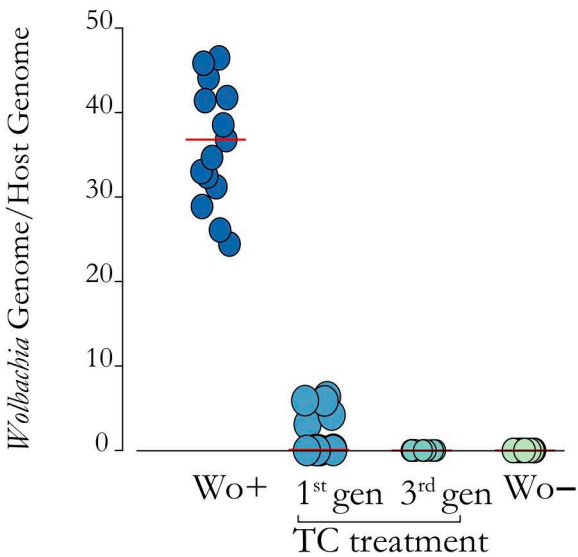
Ovarian homogenate



A**Early stage****Late stage**



OVARIES



CARCASSES

