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Mine Altinli

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HAL Id: tel-02045939

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Évolution des systèmes infectieux

École doctorale GAIA

Institut des Sciences de l'Évolution-Montpellier (ISE-M)

Ecology and Evolution of *Culex pipiens* – *Wolbachia* – Densovirus Tripartite Interactions

Présentée par Mine ALTINLI
Le 28 Novembre 2018

Sous la direction de
Mathieu SICARD et Bulent ALTEN

Devant le jury composé de

Anne-Nathalie VOLKOFF, Directrice de Recherche, INRA, Université de Montpellier, Présidente du Jury	Examinatrice
Kenneth VERNICK, Chercheur, Institut Pasteur	Rapporteur
Yannick OUTREMAN, Professeur, AGROCAMPUS OUEST	Rapporteur
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UNIVERSITÉ DE MONTPELLIER

DOCTORAT DE L'UNIVERSITÉ DE MONTPELLIER
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Nom et prénom du doctorant : ALTINLI MINE
Titre de la thèse : Écologie et évolution des interactions tripartites entre Culex pipiens, Wolbachia et les Densovirus
École Doctorale : GAIA - Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau
Date de la soutenance : 28 novembre 2018
Président du jury : VOLKOFF AN ..... (À COMPLÉTER)

Membres du jury :

Nom	Signature	Nom	Signature
Mathieu SICARD		**Kenneth VERNICK**	
Yannick OUTREMAN		**Bulent ALTEN**	
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Les membres du jury attestent avoir pris connaissance de l'intégralité du rapport.
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Si le rapport comporte plusieurs pages ou s'il est rédigé sur un document distinct, il devra être paraphé sur chaque page et signé par le Président du jury.

Mme Mine Altinli a exposé les travaux de sa thèse intitulée « Ecology and Evolution of *Culex pipiens* – *Wolbachia* – Densovirus Tripartite Interactions » devant le jury de soutenance le 28 novembre 2018. Le jury a énormément apprécié la clarté de son exposé et la grande qualité du support visuel, mettant en évidence les qualités pédagogiques de Mme Mine Altinli et sa capacité à présenter un sujet complexe tel que celui abordé au cours de sa thèse. La candidate a ensuite répondu avec assurance et honnêteté aux questions du jury, démontrant sa maitrise du sujet, son autonomie intellectuelle ainsi qu'une bonne connaissance de la bibliographie.

Le jury a souligné l'originalité et le caractère innovant du sujet abordé et l'éventail des compétences acquises au cours de ce travail de thèse, qui a abordé différents domaines allant de l'écologie de terrain à des approches mécanistiques. Cette gamme de compétences constituera un atout pour la poursuite de la carrière de Mme Mine Altinli. Finalement, le jury félicite Mme Mine Altinli pour l'importante quantité de travail fourni, pour la ténacité qu'elle a montrée tout au long de son parcours et pour les avancées qu'elle a permises dans le domaine scientifique.

Pour l'ensemble de ces raisons, le jury s'est prononcé favorablement à l'unanimité pour délivrer à Mme Mine Altinli le titre de docteur de l'Université de Montpellier.

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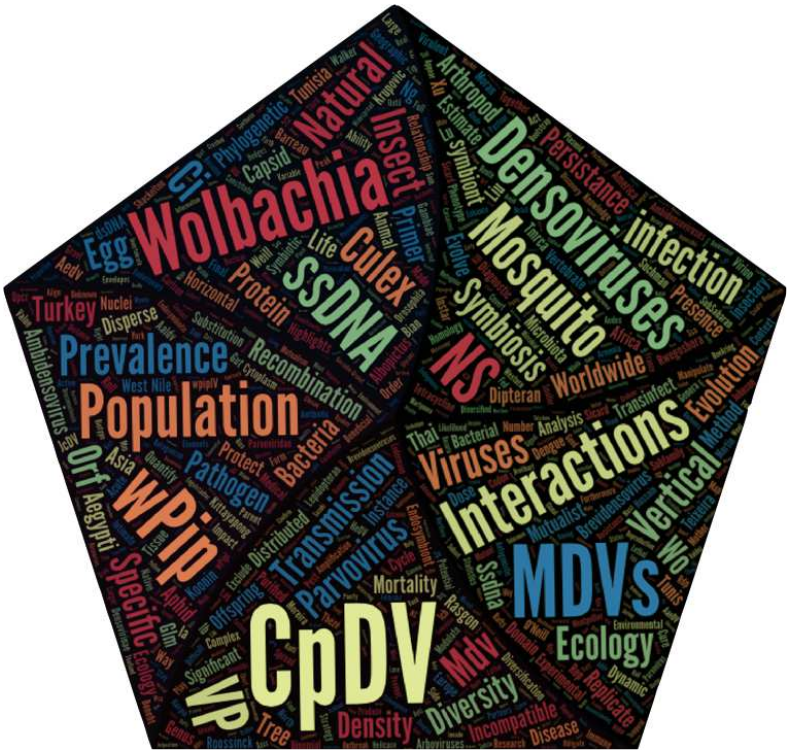

Ecology and Evolution of *Culex pipiens* - *Wolbachia* -Densovirus Tripartite Interactions

Virus discovery has long depended on the observation of outbreaks, and the research to understand their pathogenicity depended on their culture. The advances of sequencing technology and molecular biology techniques allowed researchers to discover the ubiquity and the diversity of viruses. Despite these advances, virus discovery remains mainly focused on vertebrate viruses. Furthermore, even the known diversity of these viruses is poorly studied in term of virus ecology. Arthropods with their ancient evolutionary history represent 80% of animal diversity on our planet. The ubiquity of endosymbiosis in arthropods and their ability to support (sometimes multiple) symbionts suggest that their interactions with their viruses might be of symbiotic nature, intimate long-term associations with outcomes ranging from mutualistic to antagonistic. In this context, this PhD dissertation focuses on a mosquito densovirus, *Culex pipiens* densovirus (CpDV), to investigate their prevalence and diversity in natural populations of *Culex pipiens* mosquitoes and their interactions with endosymbiotic bacteria *Wolbachia* in a natural host-bacteria-virus system (*Cx. pipiens* - *Wolbachia* - CpDV) both in natural populations and laboratory colonies. We reveal a high prevalence and diversity of CpDV in worldwide *Cx. pipiens* populations. Moreover, CpDV persistently infects laboratory lines and are vertically transmitted along with their hosts' endosymbiont *Wolbachia*. We reveal high CpDV prevalence and diversity in Turkey and Tunisia and further focus on these two countries to investigate *Wolbachia*-CpDV interactions in natural populations. In Tunisia, in a narrow contact zone where two genetically distinct *Wolbachia* groups coexist, different *Wolbachia* groups influences CpDV prevalence and diversity. Moreover, we show a positive correlation between *Wolbachia* density and CpDV density. Overall, our results suggests that CpDV-*Cx.pipiens* - *Wolbachia* system is a good model system to study the ecological interactions between these partners and their evolution together both in the nature and in laboratory conditions. Densoviruses with their diversity and long-term evolution with their hosts, and their easily manipulated small genomes, are good models to study complex host-virus-bacteria interactions as well as virus ecology and evolution in a symbiotic context.

Acknowledgements

I would like to start by warmly thanking my supervisor Mathieu Sicard, for believing in me when no one else did and for all his efforts to make this PhD project possible! Thank you Mylene Weill for believing in Mathieu (and later on in me- I hope) and accepting me to the team EVAS and supporting me through my PhD. I also thank my co-supervisor Bülent Alten from Hacettepe University for accepting to collaborate with me and for his support, and to Filiz Gunay for being my co-co-supervisor, friend and inspiration with her invincible optimism.

I would also like to thank all the people that have been directly involved with this work: the members of my PhD jury, Anne-Nathalie Volkoff, Kenneth Vernick, Yannick Outreman and Natacha Kremer for accepting to review my work, and to the members of my different thesis committees, Samuel Alizon, Remy Froissart, Christophe Boete, Mylene Ogliastro, Anne-Sophie Gosselin-Grenet and Mylene Weill for their advice, interesting discussions and support.

Many thanks to Manon – la Duchesse de - Bonneau for suffering through and surviving this PhD with me and for helping me since the first time we have met. I couldn't have asked for a better PhD "sister" to share this experience with and am already missing our morning coffee breaks.

Merci à toute l'équipe EVAS et à l'ISEM. Merci beaucoup à Fabienne Justy, Sandra Unal, Patrick Makoundou, Philippe Clair, Thomas Cantinelli, Frédérique Cerqueira pour avoir toujours été prêts à m'aider à tout l'instant, et surtout leur patience avec mon français.

Many thanks to the whole team EBAL in Hacettepe University for their hospitality, and to Mustafa Akiner, Berna Demirci and Hilal Bedir for including me to their fieldtrips when we had to cancel ours due to political instability in Turkey. Many thanks to the team Christe for accepting to collaborate with me on a nice little project to take my mind away from the PhD during these last months, especially to Romain Pigeault and to Tamara Szentivanyi.

I also kindly thank all of our collaborators. To Anne-Sophie Gosselin-Grenet for her patience answering my many virus-related questions and emails. To Mylene Ogliastro for helpful discussions and her insights. To Julien Soms for his valuable contribution to this PhD project through his hard work during his M1. To Marc Ravallec for his work creating beautiful electron microscopy pictures. To Maxime and Emmanuel Douzery for helping me understand phylogenetics.

Many thanks to my family who supported me through all my questionable life choices including this PhD. Et à ma deuxième famille, Les Chevaliers, merci beaucoup pour tous, pour m'avoir acceptée dans votre famille et pour m'avoir soutenue pendant ces trois années.

Two specific events really picked me up and helped me to move forward during much needed periods of my PhD. I would like to thank all the organizers and participants of the PhD summer Course Host-microbe symbioses 2017, for the inspiration and motivation. Especially to all the members of SymSym team for making me believe in team work. Special thanks to Gui for Kizomba and to Sean for public speech crash courses. I also thank EkoEvoDer for the amazing work they do in Turkey, specifically to all the organizers and participants of Ecology and Evolutionary Biology Symposium in Turkey and Computational Ecology and Evolution Workshop in 2016 for not cancelling on these events despite the political turmoil of 2016 summer.

I thank to all my friends for supporting me despite the distances and turning me into a WhatsApp addict.

Finally, I cannot possibly express my gratitude enough to either of you so this work, however insignificant it might be, is dedicated to you (Son olarak size olan minnettarlığımı yazarak anlatacabileceğimi düşünmediğimden naçizane bu tezi ikinize ithaf ediyorum): Manuel Chevalier and Nesrin Tuncer.

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1.1 Symbiosis

All multicellular organisms evolved in a microorganism rich environment and interacted with them through the course of their evolution. Hence these interactions shaped the evolution of the both partners and continues to do so. While the fact that acquisition of the mitochondrion leading to the formation of the first eukaryotic cells originated from a symbiotic event was discussed for a long time, the important impacts of microbes on all multicellular organisms were not properly appreciated until recently (Sagan, 1967; McFall-Ngai, 2015). For most of the scientific past, microbial research mainly focused on the pathogenic interactions where microorganisms caused diseases especially in humans or other economically important organisms. Associations with non-pathogenic microorganisms was thought to be rare and limited to some specific organisms such as lichens or corals (McFall-Ngai, 2015). This general view started to change in the late 1970s with the use of sequence-based analysis of phylogenetic relationships for the first time, using 16S ribosomal RNA sequences (Woese and Fox, 1977). These new molecular methods coupled with the rapid progress of sequencing technologies i) enlightened the shared evolutionary history between the three "domains" of life ii) led scientists to appreciate the ubiquity and the real diversity of bacteria and archea, as well as the diversity of the interactions and interdependencies they have with their hosts (Woese and Fox, 1977; Woese, 2004; Gilbert *et al.*, 2012; Pace *et al.*, 2012; McFall-Ngai *et al.*, 2013).

Symbiosis term first used by a German botanist to describe the living together of fungi and algae as lichens, is today used to describe the countless forms of long term intimate relationships between organisms, that can range from mutualism to parasitism (Relman, 2008; Combes *et al.*, 2018). Long term intimate symbiotic associations have been described in more than half of the animal phyla so far, especially in arthropods which represents, about 80 % of animal diversity (McFall-Ngai, 2015). While exceptionally rare in vertebrates, in arthropods long-term intimate endosymbioses, *i.e.* where microorganisms colonize inside of the body wall and sometimes even in host cells, are also found widespread (Douglas, 2015, 2016).

As the appreciation of the symbiotic interactions grew, their classification in terms of mutualistic versus parasitic also became more and more difficult. Even though some clearly mutualistic symbionts might exist, for most of the symbionts the nature of their interactions with their hosts are not so clear. Moreover even when they are seen as mutualistic, their interactions with their hosts are context dependent and can change with the ecological context (Keeling and McCutcheon, 2017). As such, many microorganisms causing chronic infections are expected to have both mutualistic and pathogenic properties and they can evolve rapidly into benefiting or harming their hosts (Moran, 2006).

Transmission modes of the symbionts have been demonstrated to affect the nature of these interactions: vertically transmitted, from parent to offspring, are more likely to evolve towards mutualism as their fitness is related to their host fitness, while horizontally transmitted symbionts are expected to evolve towards antagonism (Cressler *et al.*, 2016). In nature most of the symbionts exhibit both of these transmission modes, called mixed-mode transmission (Ebert, 2013).

Symbiotic relationships can also vary in that they are obligate or facultative (Moran, 2007). Many vertically transmitted symbionts are required for their hosts' normal development and kept in a specialized organ called bacteriome. For instance *Buchnera aphidicola*, obligate symbionts of aphids, are unculturable and bacteria-free aphids grow poorly and produce few or no offspring (Douglas, 1998). On the other hand facultative symbionts are not restricted to one tissue in the host (Dobson *et al.*, 1999; Fukatsu *et al.*, 2000). The spread of facultative symbionts in the host populations can be selectively favoured either due to their ability to confer fitness benefits to their hosts (*e.g.* protection against natural enemies, heat tolerance), or alternatively manipulate their host reproduction (Stouthamer *et al.*, 1999; Werren *et*

al., 2008; Engelstädter and Hurst, 2009). Reproductive manipulation seem to have evolved separately in phylogenetically diverse insect symbionts, such as *Arsenophonus nasoniae*, *Spiroplasma*, *Rickettsia* species (*e.g. Wolbachia)* and can include many different strategies such as male killing, cytoplasmic incompatibility, feminization, and parthenogenesis that increases the infected females in the host population (Engelstädter and Hurst, 2009).

Without a doubt, symbionts and their hosts influence each other's phenotypes. This influence can also be observed in their genome evolution. Symbiosis between obligate bacteriome-associated symbionts and their hosts are usually very old where partners co-evolved and co-diversified, as seen in *Buchnera* - Aphid symbiosis (Clark *et al.*, 2000). Interestingly their genomes were found to be more static, showing a lack of recombination, phages or mobile elements and rearrangements and usually gone through a gene reduction (Moran *et al.*, 2008). An extreme example for this has been observed in cicadas (McCutcheon and Moran, 2012). Cicadas carry an obligate nutritional symbiont, *Candidatus Hodgkinia cicadicola*, which has a genome size about 145kb which is thirty times smaller than the genome of a free living bacteria such as *E. coli* (McCutcheon and Moran, 2012). Contrarily facultative symbionts, like *Wolbachia,* do not show a clear pattern of co-diversification with their hosts, as they are able to be horizontally transmitted, and their genomes are more dynamic with rearrangements, recombination, phages and mobile elements (Moran *et al.*, 2008). Additionally such extreme genome-size reduction has not been demonstrated for facultative symbionts (Moran *et al.*, 2008).

1.2 Viruses as endosymbionts

Similar to bacteria, viruses have been seen only as causative agents of diseases, since the start of virology with the discovery of Tobacco Mosaic virus in 1886 (Mayer et al., 1942) until recently. While the sequencing of the 16S ribosomal RNA gene revolutionized the way we see other microbes, study of viruses - lacking a similar common phylogenetic marker - mainly relied on the observation of the disease outbreaks and ability to culture these viruses in cell lines until almost three decades after. Starting with the first study of marine viral communities in 2002, the number of published metaviromes grew significantly during the last decade and with this our understanding of the diversity of viruses, their ecology and the wide-range of environmental conditions they exist in (Breitbart *et al.*, 2002; Breitbart and Rohwer, 2005; Edwards and Rohwer, 2005; Rosario and Breitbart, 2011). This knowledge also highlighted that most viruses do not cause any detectable damages to their hosts contrarily of what is thought (Roossinck and Bazán, 2017).

In fact, viruses can better be described as endosymbionts, given their intimate relationships with their hosts. Similar to bacterial symbionts their interactions with their hosts can be anywhere from mutualism to antagonism on the symbiotic continuum (Roossinck, 2015a). While antagonistic relationships are the most studied for viruses, mutualistic viruses have also been reported notably in plants (Roossinck, 2015b). For instance, several plant viruses have been demonstrated to confer drought and cold tolerance to their hosts (Xu *et al.*, 2008), highlighting the huge impact that viruses can have on their host's ecology.

Studying viruses as endosymbionts, trying to understand ecological context of host-virus interactions rather than only focusing on pathogenic interactions, can improve our understanding of virus-host interactions. Indeed, viruses can be good models to study the evolution of symbiotic relationships (Roossinck and Bazán, 2017). As the virus-host interactions are usually fast evolving and dynamic, study of their evolution and population dynamics can also give insights on how mutualism or antagonism evolve (Roossinck, 2011).

The studies considering viruses in this broad context are so far mainly focused on plant viruses while there are not many studies on arthropod viruses. **Given their ancient evolutionary history, diversity and the fact that they are able to support endosymbionts (sometimes multiple endosymbionts), arthropods are really likely candidates to observe long-term intimate symbiotic relationships with viruses.**

1.3 Symbiont-symbiont interactions

Another change in paradigm that came with the advances in the sequencing techniques was the understanding that the diseases were not caused by only one micro-organism (*i.e.* "the" pathogen) but rather driven or influenced by the interactions between microorganisms (including bacteria, protists, fungi and viruses)(Vayssier-Taussat *et al.*, 2014). Even in the cases of more studied bacterial symbiosis, interactions between symbionts have been poorly studied and research mostly focused on binary host-symbiont interactions (Douglas, 2016). The study of the complex interactions of microbial communities, called microbiota, so far mainly focused on vertebrates (mainly human and mice) and especially on human gut microbiota (Clemente *et al.*, 2012) or on arthropods of medical or economic importance such as mosquitoes and aphids (Morin *et al.*, 1999; Gottlieb *et al.*, 2010; Weiss and Aksoy, 2011; Su *et al.*, 2013; Johnson, 2015a; Jia *et al.*, 2017).

Endosymbionts can interact with each other either directly or indirectly through their extended phenotype (Douglas, 2016; Almand *et al.*, 2017). Between bacteria and viruses, direct interactions are usually seen as exploitation of bacteria by viruses. For instance when orally challenged with poliovirus, mortality in mice with normal gut microbiota was twice as higher compared to mice without gut microbiota due to an increase in viral titers. Such effects were not present when the poliovirus was injected, as the virus did not have to interact with the microbiota before entering the internal organs. It was also hypothesized that bacterial components (called MAMPs for Microbial Associated Molecular Patterns) like LPS and glycans can increase viral binding and shedding (Kuss *et al.*, 2011; Moore and Jaykus, 2018). Other types of direct interactions seem to affect horizontal and vertical transmission of the plant viruses vectored by insects. For instance, a common obligate bacterial symbiont of leafhoppers, *Sulcia*, has been demonstrated to allow rice dwarf virus to vertically transmit to their vector's offspring. Rice dwarf virus achieves this by binding to *Sulcia*'s envelopes due to a specific interaction between the viral capsid protein and the *Sulcia* outer membrane protein (Jia *et al.*, 2017). Horizontal transmission of other plant viruses vectored by aphids and whiteflies could be affected by their hosts microbiota. Notably Potato Leaf Roll Virus (PLRV, luteovirus) transmission by aphids decreased by 70% after antibiotic treatment of aphids' endosymbiont *Buchnera* (van den Heuvel *et al.*, 1994). The interactions between virus capsid protein and *Buchnera*'s GroEL protein is thought to be responsible for this, as *Buchnera* GroEL binds to PLRV *in vitro* but whether it is the mechanism that facilitates virus transmission *in vivo* is still debated (Bouvaine *et al.*, 2011). However a more compelling example of GroEL and virus capsid interaction impact on virus transmission was observed in whitefly *Bemisia tabaci* (Gottlieb *et al.*, 2010; Su *et al.*, 2013). *B. tabaci* has different biotypes harbouring different symbiotic bacteria: both B and Q biotypes harbour *Portiera aleyrodidarum* and *Rickettsia*, additionally B biotype harbour *Hamiltonella* and Q biotype harbour *Wolbachia* and *Arsenophonus*. In nature, B Biotype was more competent for the transmission of Tomato Yellow Leaf Curl Virus (TYLCV) suggesting the involvement of *Hamiltonella* in vector competence (Gottlieb *et al.*, 2010). This involvement has been confirmed by the comparison of Q biotypes with or without *Hamiltonella* infection, where whiteflies infected with *Hamiltonella* transmitted TYLCV more efficiently (Su *et al.*, 2013). Hence the possibility of an interaction between GroEL protein of *Hamiltonella* and the virus transmission has been highlighted.

Indirect interactions can occur either by exploitative competition where different organisms compete for host resources without a direct interaction between them or by apparent competition where one of the organisms modulates the host immune response. In human infections indirect interactions between bacteria and viruses have been shown to generally benefit bacteria when viruses target a particular cell type such as lymphocytes, macrophages and monocytes (Almand *et al.*, 2017). For example influenza virus has been shown to decrease alveolar macrophages which lead to facilitate bacterial superinfections (Almand *et al.*, 2017).

In insect hosts, the endosymbiotic bacterium *Wolbachia* (detailed further in **Chapter 1.5.1 *Wolbachia***) is proposed to do both. Indeed, *Wolbachia* have been reported to indirectly compete with an RNA virus (*Drosophila* C Virus, DCV) by using dietary cholesterol thus increasing *D. melanogaster* life span (Teixeira *et al.*, 2008; Caragata *et al.*, 2013). These effects were reversible by the enrichment of the hosts' diet (Teixeira *et al.*, 2008; Caragata *et al.*, 2013). In mosquitoes, *Wolbachia* are able to confer protection to their hosts against important human arboviruses, such as Dengue and Chikungunya, putatively *via* immune priming (Moreira *et al.*, 2009).

1.4 Single-stranded DNA viruses

Single stranded (ss) DNA viruses constitute a supergroup of economically, medically and ecologically important viruses that have been found in a wide range of environmental conditions: air, soil (Kim *et al.*, 2008; Reavy *et al.*, 2015), fresh and marine waters (Rosario *et al.*, 2009; Labonté and Suttle, 2013; Zawar-Reza *et al.*, 2014) including extreme conditions like hot-spring (Mochizuki *et al.*, 2012) and hypersaline habitats (Pietilä *et al.*, 2009). They can infect eukaryotes - such as plants (Howarth and Goodman, 1982), animals and fungi (Yu *et al.*, 2010), bacteria (Székely and Breitbart, 2016) and archaea (Mochizuki *et al.*, 2012). Furthermore paleovirological studies discovered their existence in the genomes of even wider host range than previously expected as endogenous viral elements (Belyi *et al.*, 2010; Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015). Integrated both in vertebrate and arthropod genomes some ssDNA families are thought to be older than 40-50 million years which highlighted their intimate and long term relationship and evolution with their hosts (Belyi *et al.*, 2010; Katzourakis and Gifford, 2010; Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015).

Much like their hosts and environmental conditions they exist in, ssDNA families are highly diverse. They are currently split into eight recognized families: the Anelloviridae, Circoviridae, Bidnaviridae and Parvoviridae infecting animals, the Geminiviridae and Nanoviridae infecting plants and the Microviridae and Inoviridae infecting bacteria (https://talk.ictvonline.org/ taxonomy/, ICTV 9th report online). Their genome structures are equally diverse and can be linear or circular, with single or multiple components (Martin *et al.*, 2011). They are usually small viruses (<10 kb, except of an archaeal ssDNA virus with 24.9 kb genome size)(Krupovic, 2013). Their small sized genome is thought to be a result of their high nucleotide substitution rate which is closer to that of RNA viruses than double-stranded DNA (dsDNA) viruses. Indeed this substitution rate can be as high as 10^{-4} to 10^{-3} substitutions/site/year (Shackelton *et al.*, 2005; Firth *et al.*, 2009; Krupovic, 2013), a feature that can lead to the accumulation of deleterious mutations when the genome size gets bigger. It is also possible that their genome size is limited by the encapsidation capacity of their small capsid (a diameter of 18–27 nm) (Krupovic, 2013). Extensive evidence of recombination have also been found for ssDNA viruses and likely to have a big role in the generation of new genera (Shackelton *et al.*, 2007; Lefeuvre *et al.*, 2009; Martin *et al.*, 2011; Koonin *et al.*, 2015; Martynova *et al.*, 2016; Tijssen *et al.*, 2016; Kazlauskas *et al.*, 2018).

Most of the ssDNA viruses code for two types of genes: non-structural (NS/Rep) and structural capsid proteins (VP/CP). NS genes generally play a role in the replication of the viral genome, an endonuclease encoded by viral NS gene starts the replication by nicking the viral DNA. This replication mechanism, named rolling-circle or rolling hairpin mechanism, used by most of the ssDNA viruses is also used by many plasmids and some transposons (Chandler et al., 2013; Krupovic, 2013; Krupovic and Forterre, 2015; Rosario et al., 2012). Surprisingly, ssDNA NS proteins phylogenetically cluster with proteins from different groups of plasmids and are not monophyletic (Koonin *et al.*, 2015). While VP genes of some of the ssDNA families thought to originate from ssRNA viruses on different occasions (Krupovic, 2013). This suggests that current ssDNA virus diversity is of polyphyletic origin, probably resulted from separate recombination events between RNA and DNA donors and highlights that different families might have originated

in different times and evolve further with their hosts (Krupovic, 2013; Koonin *et al.*, 2015). For instance, Parvoviridae family, with their wide host range infecting both vertebrates and arthropods, could have appeared before the rise of major eukaryotic kingdoms (Koonin *et al.*, 2015; Krupovic and Koonin, 2015). On the other hand Bidnaviridae family, lacking the rolling circle/hairpin mechanism, is thought to have evolved much later and possibly by acquiring genes from four different groups of viruses (Koonin *et al.*, 2015; Krupovic and Koonin, 2015).

Both for their ancient origin and wide host range Parvoviridae family is interesting to study in terms of their interactions with their hosts and host microbiota. While the mechanistic aspects of the virus- host interactions and viral pathogenesis of Parvoviridae family are well studied, the ecological interactions between the virus and host as well as the host microbiota and their outcomes are largely unknown. Like it has been observed with bacterial symbionts, the nature of these interactions could be context dependent and they can shape microbial communities and host populations by influencing infection outcomes and can drive the evolution of the different partners involved.

1.4.1 Parvoviridae family

Parvoviridae family consists of small, isometric non-enveloped viruses with linear ssDNA genomes which can range between 4 and 6 kb in length (Kerr *et al.*, 2005). Their genome terminates in hairpin structures at each end, which can either be different from each other in terms of sequence and predicted structure or be the exact same nucleotide sequence inverted, *i.e.* inverted terminal repeat (ITR) (Kerr *et al.*, 2005). These hairpins serve as primer for host's DNA repair mechanism that convert ssDNA to dsDNA (Berns, 1990). Following this, dsDNA is transcribed to viral mRNAs to be translated into viral proteins during host cells' S phase (Berns, 1990). Replication is started by viral endonuclease NS1 (Rep) which cuts between the hairpin structure and coding sequences (Berns, 1990).

Parvoviruses can cause a wide range of acute or chronic diseases; many, however, are not known to be associated with any disease but to cause persistent infections (Kerr *et al.*, 2005; Liu *et al.*, 2011). The family is divided into two subfamilies based on host range: the Parvovirinae infect vertebrates and the Densovirinae infect insects and other arthropods (Tijssen *et al.*, 2016). Further taxonomic classification within the family is made by the construction of phylogenetic relationships using the conserved SF3 helicase domain within NS1 (Cotmore *et al.*, 2014). SF3 helicase domain consists of a contiguous set of functional subdomains, called Walker boxes that stretch between 100 and 103 amino acids of all known parvoviruses, while they are not found in cellular genomes (Kerr *et al.*, 2005). Capsid proteins (VPs) have also been used to understand phylogenetic relationships within parvovirus family (Cotmore *et al.*, 2014; François *et al.*, 2016). While it is not as contiguous as SF3 domains, VPs also have very conserved domain called PLA2 (Phospoliphase A2), which has a role in viral infectivity (Zádori *et al.*, 2001), and the eight-membered ß-barrel that forms the core of the icosahedral capsid shell (Chapman and Rossmann, 1993).

Our knowledge about this family of viruses mainly come from viruses that are pathogens of humans or economically important animals. However recent studies on public transcriptomic and genomic databases highlighted their ubiquity and diversity, especially of densoviruses, in wider range of hosts (Liu *et al.*, 2011; François *et al.*, 2016). This also suggests that the nature of densovirus-arthropod host relationships could also be different than strict antagonism usually observed, making them good candidates to study viruses as endosymbionts of arthropods.

1.4.2 Densoviruses

Densoviruses get their name from the cellular pathology they can cause, "densonucleosis" referring to the electron dense observation of the infected cell's nuclei where the viruses replicate (Meynadier *et al.*, 1964; Gosselin Grenet *et al.*, 2015). They are non-enveloped viruses with icosahedral capsids and

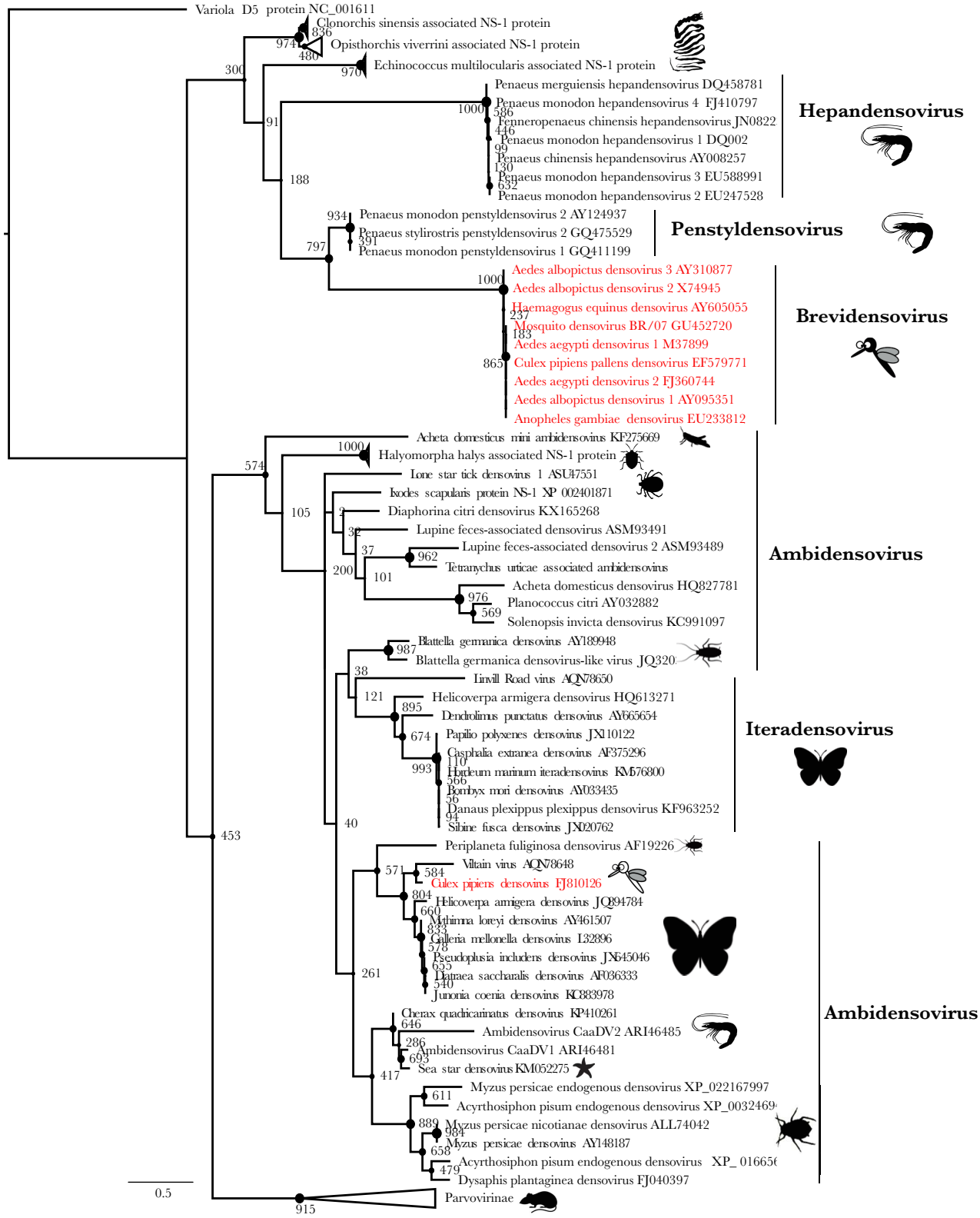

Figure 1. Phylogenetic tree of Densovirus Sub-Family. Densovirus sub-family consists of 5 genera infecting mainly insects from five orders (Lepidoptera, Hemiptera, Blattodea, Diptera, Hymenoptera) but also Crustaceans and Echinoderms, endogenous viral elements and viral sequences found in public transcriptomics and genomics databases are also added. Host species are given as images. Maximum likelihood tree is prepared by **Sarah François** using SF3 helicase domain within NS1, statistical support is given as bootstrap values (François et al. in prep). Variola D5 protein is used as outgroup. CpDV (red) is phylogenetically far from other mosquito densoviruses (red, brevidensovirus) and closer to lepidopteran and blattodean densoviruses. Viltain virus that has been recently detected in genomics studies in *Drosophila* seem to be closely related to CpDV but our knowledge about it limited.

are among the smallest viruses (18-25nm)(Tijssen *et al.*, 2016). Densoviruses have been isolated mainly from insects (orders of Lepidoptera, Diptera, Hymenoptera, Blattodea, Hemiptera, Orthoptera) but also found in malacostraca (shrimps and crayfish) and echinoderms (starfish and sea urchins)(Figure 1)(Tijssen *et al.*, 2016). They are also integrated in many invertebrate genomes and recent studies suggested that densoviruses may be as diverse and ancient as arthropods (Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015; François *et al.*, 2016)(Figure 1).

While this subfamily keeps expending, the categorization of its genera gets more and more challenging. So far there are 5 defined genera (*i.e. Ambidensovirus*, *Brevidensovirus*, *Hepandensovirus*, *Iteradensovirus*, *Penstyldensovirus*) within the Densovirinae subfamily, based on the sequence identity of NS (Rep) gene (Cotmore *et al.*, 2014; Tijssen *et al.*, 2016) (Figure 1).

Densoviruses have originally been discovered and studied following the observation of mass mortality in economically important arthropods, such as silk worms or shrimps (Watanabe and Maeda, 1981; Lightner and Redman, 1985). Hence most of the DVs so far isolated are lethal for their natural hosts, the first symptoms being anorexia and lethargy followed by flaccidity, paralysis, slow melanisation and death (Tijssen *et al.*, 2016). Most of them exhibit a tissue polytropism replicating in many tissues like the fat body, hypodermis, epidermis, central nervous system, muscular and tracheal cells, gut, hemocytes, ovaries and are considered to be lethal for their natural hosts (Tijssen *et al.*, 2016). Once in the population, these infections can spread quickly as observed in farms of arthropods of economic importance such as crickets (Szelei *et al.*, 2011; Weissman *et al.*, 2012) and shrimps (Lightner, 1996). Moreover, the recent outbreak of sea star wasting disease was also caused by a densovirus (Hewson et al., 2014).

Due to their observed pathogenicity, narrow host range and sustainability out of their host-cells in nature, they attracted scientific attention as biological control tools especially against crop pests like lepidopteran pests, and insects of medical and veterinary importance like mosquitoes (Carlson *et al.*, 2006; Mutuel *et al.*, 2010; Gosselin Grenet *et al.*, 2015; Suzuki *et al.*, 2015; Johnson and Rasgon, 2018). For instance, DVs have been successfully used to control Limacodidae larvae in oil palm plantations (Genty and Mariau, 1975; Fédière, 1996). As mentioned until recently their discovery was driven by the observation of epizootics in arthropods of importance and only with the advances in sequencing technologies and growing data from natural populations they have been shown in a wider range of hosts natural populations. Although thus far the research on them still mostly focus on their pathogenicity in experimental infections, virus-host interactions could be much more diverse and complicated than expected in natural populations. **Knowledge of the prevalence and epidemiology of these viruses in nature could help us understand how they evolve and interact with their hosts better, making their control or manipulation easier**

I will further focus on two of the genera of Densovirinae subfamily - Ambidensoviruses and Brevidensoviruses - as they are most relevant for the present dissertation.

1.4.2.1 Brevidensoviruses

General information

Brevidensoviruses have the smallest genomes among the other genera of the Densovirinae subfamily with their genomes about ~4kb in length, (Kerr *et al.*, 2005). Their genome is monosense, Open Reading Frames (ORFs) for the structural (VP) and non-structural (NS) proteins are on the same strand (Kerr *et al.*, 2005)(Figure 2A). Negative strands constitute the majority of the DNA strains encapsidated in the virion populations: 85% negative strand 15% positive strand (Kerr *et al.*, 2005). Unlike the other densoviruses their genome does not contain any recognizable PLA2 domain in their VP gene (Kerr *et al.*, 2005) (Figure 2A).

There are two recognized species within the genus, namely Dipteran Brevidensovirus 1 and 2, but several variants of these distinct species have also been discovered (Cotmore *et al.*, 2014). All the

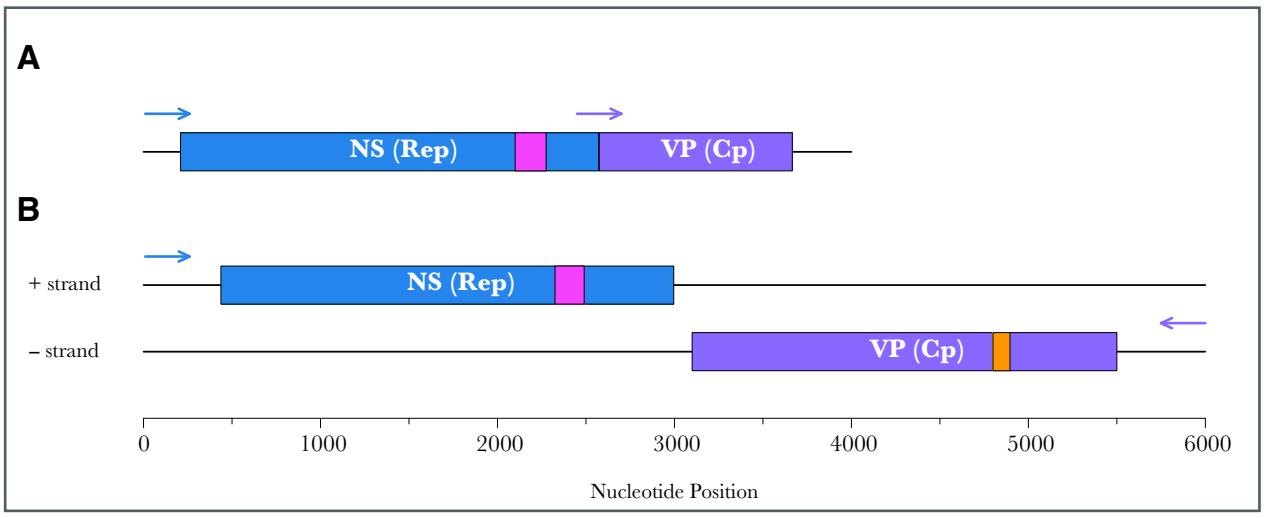

Figure 2. Differences between Ambidensovirus and Brevidensovirus genome structures. Brevidensovirus (A) have smallest genome among densoviruses, about 4kb length. Their genome is monosense, only transcribed from one strand. They lack PLA2 domain that is conserved in most of the Parvoviridae family including Ambidensoviruses. Their DNA strands terminate with hairpin structures but lack inverted terminal repeats. **Ambidensoviruses (B)** have an ambisense genome their ORF of their NS genes are located on plus strand while ORF of VP genes are located on the minus strand. Their capsids encapsidate equal amounts of both strands. Their genome size is larger compared to Brevidensovirus genus (about 6kb). Their genomes are terminated with inverted terminal repeats that forms a hairpin structure. Conserved SF3 domain in NS region and PLA2 domain in VP region are indicated with pink band orange band respectively. Arrows indicate the direction of transcription.

diversity grouped in the Brevidensovirus genus has been isolated from mosquitoes and they are thought to be species specific. In fact, all mosquito specific densoviruses (MDVs) belong to this genus except *Culex pipiens* densovirus, which infects mosquitoes but belong to *Ambidensovirus* genus (Figure 1, *Culex pipiens* densovirus discussed in detail later in section **1.4.3**).

First MDV has been found in Russia, in laboratory colonies of *Aedes aegypti* mosquitoes originated from Sout-East Asia in 1972 (Lebedeva *et al.*, 1972). This MDV, later taxonomically classified as belonging to Dipteran Brevidensovirus 1 species (Cotmore *et al.*, 2014), and other very similar (sequence homology) variants have later been discovered in mosquito cell lines, laboratory colonies or natural populations of *Ae. albopictus* [*Ae. albopictus* densovirus 1, AalDV1, (Chen *et al.*, 2004)],*Ae. aegypti* [*Ae. aegypti* densovirus 1, AaeDV1 (Afanasiev *et al.*, 1991); *Ae. aegypti* densovirus 2, AaeDV2, (Sivaram *et al.*, 2009); *Ae. aegypti* Thai strain (Kittayapong *et al.*, 1999)], *Anopheles minimus* (Rwegoshora *et al.*, 2000), *Anopheles gambiae* [*An. gambiae* densovirus, AgDV, (Ren *et al.*, 2008)], *Culex pipiens pallens* [*Culex pipiens pallens* densovirus, CppDV, (Zhai *et al.*, 2008)]. Dipteran brevidensovirus 2 species consists of AalDV2, then named as AaPV, that have been isolated for the first time from C6/36 cell lines (Jousset *et al.*, 1993). O'Neill et al. (1995) demonstrated similar variants of this species persistently infecting different mosquito cell lines including *Haemagogus equinus* and *Toxorhynchites amboinensis* cell lines [*H. equinus* densovirus, HeDV, (O'Neill *et al.*, 1995)].

Experimental infections

To study the interactions of the MDVs with their hosts, mosquito larvae have been experimentally infected orally by breeding them in the water either where infected crushed larvae or infected cultured cells were added. Both Dipteran Brevidensovirus 1 and 2 species have been studied for their infectivity in experimentally infected mosquito lines. AaeDV1 virus have been shown to successfully infect and cause mortality in *Aedes*, *Culex* and *Culiseta* species (Buchatsky, 1989). *Ae. aegypti* larvae were infected using either infectious clones cultured in C6/36 cells or infected dead larvae. In both cases mortality increased with increased virus dose up to 75% with majority of mortality observed during the larval stage (51%)(Ledermann *et al.*, 2004). Longevity and survival were reduced in the adults that

survived the AaeDV1 infection (Suchman *et al.*, 2006). The effects of AaeDV1 depended on the viral dose and when the larvae were infected. Larvae infected during the 1st instar were affected more than the larvae infected during 3rd instar (Suchman *et al.*, 2006). Infected adults were able to transmit the virus to approximately 70% of the offspring (Suchman *et al.*, 2006). However, vertical transmission was affected by when the larvae was infected at the first place. Indeed, larvae that were experimentally infected during the 3rd instar transmitted the virus less (Suchman *et al.*, 2006). AaeDV virus Thai strain has also been shown to cause 51% and 82% mortality in orally infected *Ae. aegypti* and *Ae. albopictus* mosquitoes (Kittayapong *et al.*, 1999). Surviving adults were also able to transmit the virus to their offspring (Kittayapong *et al.*, 1999).

AalDV2 (dipteran brevidensovirus 2), has been shown to cause mortality when *Ae. aegypti* larvae were infected using naturally infected larvae or viruses cultured in cells (Barreau *et al.*, 1996). The infection method had a large effect on the infection dynamics, larvae fed on the persistently infected cells showed 90% mortality between 6th and 10th day while larvae infected by dead larvae reached this mortality around only on the 20th day. Larval mortality caused by AalDV2 also increased i) when the larvae were infected during first instar larval stage compared to 3rd larval stage, ii) when host population density increased. The fact that mortality usually occurs during life stage change, that requires molting, can possibly be caused by the energetic cost of these changes. Among the mosquitoes that were able to survive the AalDV2 infection and become adults 17% died before laying eggs. However, there was no effect (*i.e.* virulence) of the infection on the fecundity of the females (Barreau *et al.*, 1997). These females were able to transmit the virus vertically to their offspring. The vertical transmission rate depended on the virus titer of the parental female and ranged between 28% -55%. This vertical transmission did not persist beyond the second generation. There was also a low percentage of venereal transmission from infected males to uninfected females. Overall the results highlighted that AaDV2 were transmitted horizontally, both from larvae to larvae and venereally between adult mosquitoes, and vertically to the offspring.

HeDV infection of *Ae. aegypti* larvae on the other hand resulted in low mortality (10%) but higher infection rate in adults where HeDV were still able to replicate (Ledermann *et al.*, 2004). This finding was interesting because HeDV has been shown to be highly cytopathic in C6/36 cell line but was not as pathogenic in *Ae. aegypti* larvae, in contrast with Dipteran Brevidensovirus 1 that can cause persistent infection in the cell lines but is highly pathogenic for larvae (Ledermann *et al.*, 2004; Paterson *et al.*, 2005).

Ecological studies, or lack thereof

Similar to the differences between *in vivo* and *in vitro* studies, experimental infections using these viruses - while useful to understand the virus-host interactions - might not be reflecting how these viruses interact with their hosts in nature. Although there are not many studies focusing on the epidemiology of densoviruses in natural populations, MDVs are thought to be widespread in mosquito populations (Ma *et al.*, 2011; Ng *et al.*, 2011; Zhang *et al.*, 2013). AeDV Thai strain is the most studied MDV in natural populations to date. In *Ae. aegypti* adults collected from Thailand (n=97), AeDV Thai strain prevalence was 44.3% while in *Ae. albopictus* adults tested (n=79) were all negative (Kittayapong *et al.*, 1999). Interestingly AeDV Thai strain have been shown to successfully infect both mosquito species in laboratory conditions, suggesting either the difference between the laboratory and natural conditions or the possibility of a higher virulence in *Ae. albopictus* larvae preventing their development into adults. If true, this would suggest that AeDV Thai strain infection can shape the structuring of *Ae. albopictus* and *Ae. aegypti* populations in nature (Kittayapong *et al.*, 1999). For further investigation on the seasonality of the infection by AeDV in *Ae. aegypti* adults, a total of adults has been collected monthly for a total of 844 individuals. The results showed a monthly variance with a prevalence peak in July (35.1%, n=308) and

lowest prevalence in August (6.5%, n=31) and September (8.0%, n=138). Naturally infected *Ae. aegypti* were also able to transmit the virus vertically to a 57.5% of surviving progeny. Furthermore, vertical transmission in naturally infected lines seems to persist for more generations (up to 6 generations) than it has been seen in experimental infections (2 generations) (Barreau *et al.*, 1997; Kittayapong *et al.*, 1999), highlighting that vertical transmission can play a big role in the persistence of MDV infections in the natural populations. Another study also detected the AeDV Thai strain infection in *An. minimus* adults and larvae, with an overall infection prevalence around 19% in larvae and 15% in adults (Rwegoshora *et al.*, 2000). Larval infection also showed a positive correlation with rainfall two months prior to larval collection (Rwegoshora *et al.*, 2000). Interestingly the infection prevalence in larvae seem to affect the next month's prevalence in adults negatively, suggesting that higher mortality in larvae may cause lower prevalence in adult mosquitoes (Rwegoshora *et al.*, 2000.

MDVs are really stable in nature and have been shown to stay infective after a year of introduction to a water body (Buchatsky, 1989). MDVs can be already present in the water where female mosquitoes lay their eggs and can infect the hatching larvae. Infected larvae by releasing virions into the water can horizontally transmit the virus to other larvae (Barreau *et al.*, 1996; Ledermann *et al.*, 2004). Depending on the dose they have received and the larval stage when they got infected, larvae can show varying rates of mortality (Barreau *et al.*, 1996; Suchman *et al.*, 2006). Surviving larvae can emerge as adults and vertically transmit the infection to their offspring, or horizontally to new oviposition sites and venereally to other adults (Barreau *et al.*, 1997; Suchman *et al.*, 2006; Wise de Valdez *et al.*, 2010). The infection rates and intensity can possibly be affected by rainfall and host density (Barreau *et al.*, 1996; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000) (Figure 3).

Although it is logical to try infer the general life cycle of MDVs in nature based on the experimental infections there are still a lot of unknown in terms of their interactions with the rest of the microbiota of the host. Especially commonly found endosymbiotic bacteria such as *Wolbachia* and other viruses as insect-specific RNA viruses, the viruses that the mosquitoes are the vectors for like Dengue and Chikungunya, can interact with brevidensoviruses in the host environment.

Experimentally, AalDV and Dengue viruses have been shown to persistently co-infect C6/36 cells. However, AeDV replication has been increased after the infection with Dengue virus, while AeDV presence decreased the Dengue infection (Wei *et al.*, 2006).

1.4.2.2 Ambidensoviruses

Ambidensoviruses get their name from the ambisense organization of their genomes: ORFs of their NS and VP proteins are on the 5' halves of the complementary strands and virion populations encapsidate equal numbers of both single positive and negative strands (Kerr *et al.*, 2005). Their genomes are about 6kb in length and contain about 500 nucleotide long inverted terminal repeats (ITR) (Kerr *et al.*, 2005). Most ambidensoviruses have three non-structural proteins (NS1-NS2-NS3) while other genera of densoviruses have only two (Kerr *et al.*, 2005). NS3 polypeptides are thought to be acquired by horizontal transmission early in the evolution of ambidensoviruses, as similar proteins have been found in other viruses such as *Cryptophlebia leucotreta* granulovirus from different virus families (Baculoviridae) (Kerr *et al.*, 2005). They all have Ca^{++} binding loop and PLA2 motifs in their VP region that are conserved in all ambidensoviruses (Zádori *et al.*, 2001).

Ambidensoviruses have been found in five insect orders Lepidoptera, Diptera, Hemiptera, Blattodea, Orthoptera (Figure 1)(Cotmore *et al.*, 2014). Some of these like GmDV are restricted to their original host, *Galleria mellonella*, some such as AdDV (*Acheta domestica* densovirus) and PfDV (*Periplaneta fuliginosa* densovirus) are restricted to closely related hosts, while others, *e.g.* JcDV (*Junonia coenia* densovirus), MlDV (*Mythimna*

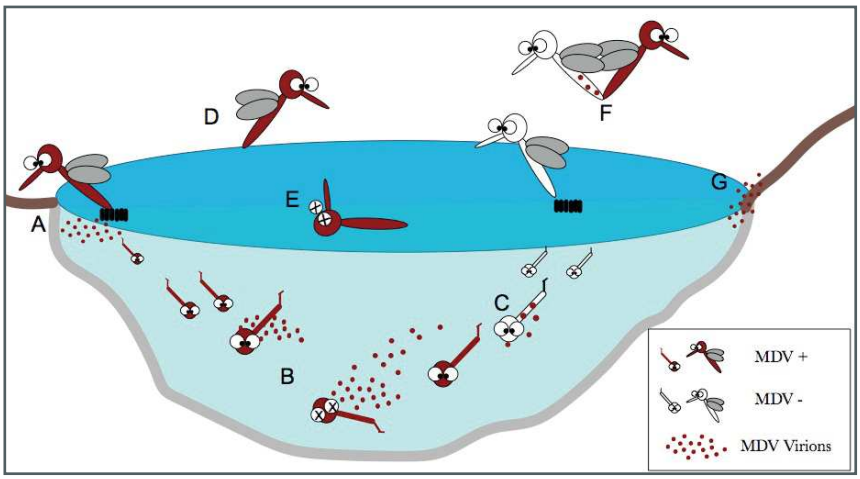

Figure 3 Life Cycle of Mosquito Densoviruses (MDVs): Infected females can vertically transmit MDV to their offspring(**A**). These infected larvae could either survive and become adults or die and in both cases excrete virions to the water body they live in (**B**). Uninfected larvae in the same water body can get infected horizontally(**C**). Surviving larvae can become adults and carry MDVS to other oviposition sites (**D**). MDVs can shorten the lifespan of the adults (**E**) and be transmitted venereally (**F**). MDV virions can stay active in nature for long time (**G**).

loreyi densovirus) can infect many different hosts (Tijssen *et al.*, 2016).

Lepidopteran ambidensovirus strains are generally really closely related and very pathogenic for their hosts. *Diatraea saccharalis* densovirus (DsDV) induced symptoms that starts at the 4th day following the infection of the larvae and mortality reached 100%, 21 days after the infection (Kouassi *et al.*, 2007). In the case of GmDV, the virus is so virulent and contagious that it has been discovered after a population decline in the mass rearing of *Galleria mellonella* larvae for fishing bait (Meynadier *et al.*, 1964). MlDV which shows 95% sequence identity to GmDV, can infect many lepidopteran pests. First isolated from *Mythimna loreyi.* MlDV was also found in field populations of *Agrotis ipsilon, Agrotis spinifera, Autographa gamma, S. exigua* and *S. littoralis* (El-Mergawy *et al.*, 2003). Another ambidensovirus that can infect several lepidopteran species is JcDV and has been shown to replicate in *Aglais urticae, Bombyx mori, Chrysodeixis chalcites, Lymantria dispar, Mamestra brassicae Mamestra oleracea, Scotia ipsilon, Spodoptera exigua, Spodoptera littoralis* (but not in *G. mellonella*) and kill these hosts 18-27 days after infection (Rivers and Longworth, 1968; Mutuel *et al.*, 2010). Remarkably *Helicoverpa armigera* densovirus-1 is the only not pathogenic lepidopteran ambidensovirus. In fact it increases the developmental rates of their hosts both during larval and pupal stages, lengthens their lifespan and increases female fecundity (Xu *et al.*, 2014). Moreover in field studies they have been shown to increase the resistance of their pest host against biological population control tools, such as *Bacillus thuringiensis* and a pathogenic baculovirus (Xu *et al.*, 2014). Interestingly, mutualistic HeDV was found to be vertically transmitted to 100% of the offspring.

Similar mutualistic interactions between ambidensoviruses and their hosts have also been reported for hemipteran ambidensoviruses. *Dysaphis plantaginea* densovirus (Hemipteran ambidensovirus 2) infects rosy apple aphid (Ryabov *et al.*, 2009). Asexually reproducing populations of these aphids, having the same genotype can display two different phenotypes: wingless or winged. While winged aphids are smaller and have lower fecundity than their wingless counterparts, wingless aphids are necessary for the survival of the clonal population in some ecological conditions (Ryabov *et al.*, 2009). For a long time scientists thought crowding of the host plant and poor quality diet were the reason behind the apparition of winged aphids (Wadley, 1923). Instead, Ryabov *et al.* (2009) demonstrated that DplDV caused the apparition of these winged aphids in poor diet and crowded conditions allowing aphids to spread to a new host ensuring the survival of their clonal population (Ryabov *et al.*, 2009). Indeed DplDV-free aphid populations were not able to produce wiged morphs (Ryabov *et al.*, 2009). Moreover, aphids infected with DplDV could still produce some virus-free offspring -hence wingless and with higher fecundity- which allows a fast population growth on the newly

colonized plant (Ryabov *et al.*, 2009). *Myzus persicae nicotianae* densovirus (MpnDV, Hemipteran ambidensovirus 3) on the other hand slightly slowed down the development of the aphids and decreased their body weight which is often correlated with fecundity although they were not highly pathogenic for their hosts. They were also vertically transmitted to the offspring of their hosts. In natural populations of aphids in China, the prevalence of MpnDV was 34%. Phylogenetic analysis revealed that the MpnDV were structured geographically with low level gene flow between locations, suggesting their high vertical transmission and putative co-evolution with their hosts (Song *et al.*, 2016)

Both Blattodean ambidensovirus 1 (*Periplaneta fuliginosa* densovirus) and 2 (*Blattella germanica* densovirus 1) were found highly pathogenic to their hosts (Mukha *et al.*, 2006; Jiang *et al.*, 2008). Similarly Orthopteran ambidensovirus 1, *Acheta domesticus* AdDV, caused outbreaks of very high mortality in rearings in North America (1988, 2009) and Europe (1977, 2004, 2006, 2009) (Szelei *et al.*, 2011). Infected *A. domesticus* were also smaller and less active suggesting that AdDV could impact host ecology in natural populations. Interestingly sequences of AdDV isolates from different outbreaks in North America in 2009 were identical, suggesting the efficient dispersal of the virus between these locations. On the other hand, isolates from European (1977, 2004, 2006, 2007, 2009) and North American (2009) outbreaks differed slightly and are estimated to have diverged in 2006 (Szelei *et al.*, 2011).

1.4.3 *Culex pipiens* densovirus (CpDV)

CpDV, isolated from *Cx. pipiens* mosquitoes (Jousset et al., 2000), is an ambidensovirus, unlike all the other mosquito densoviruses -which are brevidensoviruses. CpDV indeed shares the characteristics of other ambidensoviruses having a larger genome size than brevidensovirus (~6kb compared to ~4kb), an ambisense genome organization and significant sequence homology at both capsid and NS gene levels (Figure2) (Jousset *et al.*, 2000; Baquerizo-Audiot *et al.*, 2009).

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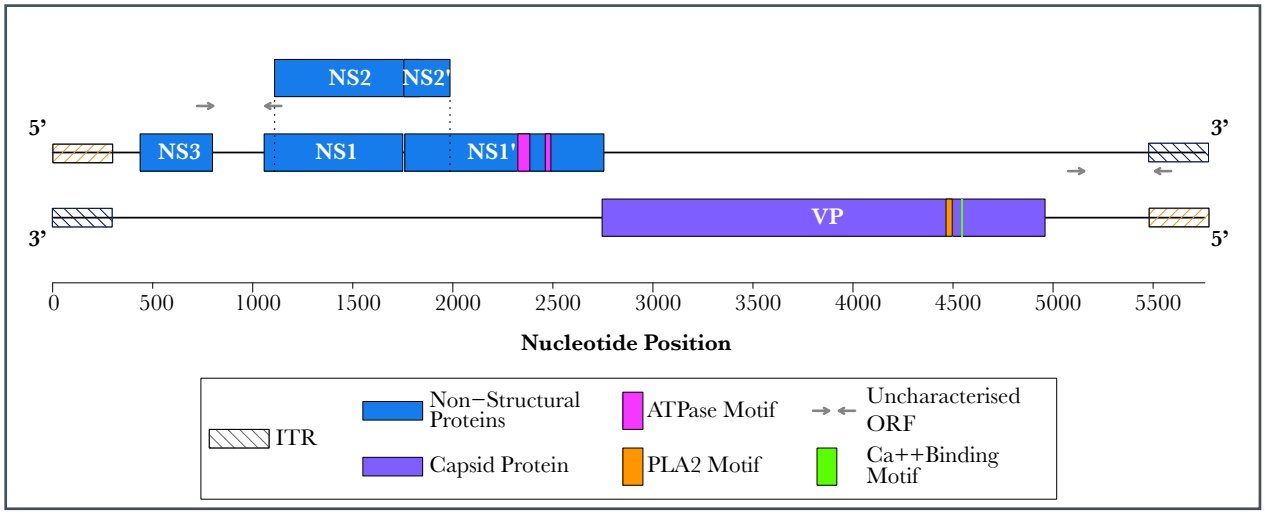

Figure 4 CpDV genome. CpDV genome encodes for two types of proteins: non-structural NS and structural VP. ORFs of NS and VP proteins are situated on different single strands, which makes CpDV genome ambisense. They have 3 putative NS proteins. NS3, NS2, NS1 (ORFs are shown as blue boxes). ORFs of NS1 and NS2 overlap on the same strain (indicated with dashed lines), and split into two ORFs (NS1-NS1', NS2-NS2'). Two ORFs for which homologous proteins were not found, are shown as gray arrows in NS and VP regions. CpDV genome terminates with inverted terminal repeats (ITR, identical sequences repeated on 3' terminal of both strands shown in blue and 5' terminal shown in yellow) that fold into hairpin structures. Conserved SF3 helicase domain consisting of Walker A and B boxes as part of within NS1 is shown in pink (ATPase motif). Conserved PLA2 and Ca^{++} binding motifs in VP have been highlighted as orange and green bands respectively. CpDV has four putative VP proteins (ORFs are shown purple boxes) that are encoded by leaky scanning from single VP ORF, although their location is not certain.

CpDV putatively encodes for three non-structural proteins: NS3, NS1 and NS2 (Figure 4) (Baquerizo-Audiot *et al.*, 2009). Unlike other ambidensoviruses CpDV NS1 and NS2 putative proteins are encoded by four ORFs with introns in between (NS1-NS1, NS2-NS2'; Figure 4)(Baquerizo-Audiot *et al.*, 2009). Another ORF also has been found between NS3 and NS1, however due to the lack of homology with known NS proteins it is not clear whether it is functional (Baquerizo-Audiot *et al.*, 2009) (Figure 4, grey arrows in NS)Their capsid is ~20 nm, similar to other densoviruses, occasionally arranged into paracristalline arrays (Jousset *et al.*, 2000). Like lepidopteran ambidensoviruses, CpDV has four capsid polypeptides (90, 64, 57 and 12 kDa) designated as VP1 to VP4 (Jousset *et al.*, 2000), although there is only two ORFs found (one large 2253 nt shown with purple box and one smaller 443 nt shown with grey arrows in Figure 4) on the 5' side of the complementary strain (Baquerizo-Audiot *et al.*, 2009). Due to lack of homology with known densovirus proteins, it is unknown if the smaller ORF also encodes for VP proteins (grey arrows, Figure 4).

CpDV has been discovered following the observation of mass mortality in laboratory colonies of *Cx. pipiens* kept in the insectaries of ISEM in Montpellier University. Symptoms, like distorted and comma shaped bodies, appeared in 3rd instar larvae and usually ended up in death in 4th instar larvae (Jousset *et al.*, 2000). Thick sections of infected larvae showed hyperthrophied nuclei, a clear sign of densonucleosis (Jousset *et al.*, 2000). Insect cell lines were infected with CpDV and replication of it was checked by immunofluorescence (Jousset *et al.*, 2000). No CpDV-positive cells were observed in *D. melonagaster* and *S. littoralis* cells (Jousset *et al.*, 2000). The number of fluorescent cells were low in *Ae. albopictus* but 1% of *An. gambiae* and *Culex* cells showed immunofluorescence, suggesting *Ae. gambiae* larvae could also be susceptible to CpDV (Jousset *et al.*, 2000). After serial passages, CpDV-positive cell amount decreased and no positive cells were observed at the third passage (Jousset *et al.*, 2000).

Recently a genetically similar densovirus have been found in genomic studies of natural populations of *D. melonagaster* (Figure 1)(Kapun *et al.*, 2018). Our knowledge about it is so far limited although this suggested there might be similar viruses to CpDV in nature that have not been detected so far. Unfortunately, this discovery has been made too late for this thesis and thus could not be included in the phylogenetic analysis of CpDV in the following chapters.

1.5 *Culex pipiens - Wolbachia* Model System

1.5.1 *Wolbachia*

Among the endosymbiotic bacteria that infect arthropods and influence their phenotype to a large extend, *Wolbachia* are the most widespread as they may infect up to 50% of the arthropod species (Hilgenboecker *et al.*, 2008; Zug and Hammerstein, 2012; Weinert *et al.*, 2015). They are vertically transmitted from mother to offspring. Hence the success of their transmission relies on the increase of infected females' proportion in the host populations. Such increase can theoretically be achieved either by conferring some fitness benefits to their hosts or by manipulating the reproduction of their hosts in different ways. *Wolbachia* can use both strategies.

Wolbachia manipulate the host-sex ratio directly or indirectly increasing its spread by inducing cytoplasmic incompatibility, parthenogenesis, male killing or feminizing males (Figure 5)(Werren *et al.*, 2008). Most common of these strategies, Cytoplasmic Incompatibility (CI), has been described in several arachnids, isopods and insect orders (Werren *et al.*, 2008). The mechanism of *Wolbachia* induced CI is conceptualized as resulting from a modification factor secreted by the bacteria, possibly a toxin, deposited in the sperm that can be rescued in embryos resulting from eggs infected with a compatible strain (Serbus et al., 2008). Infected females can reproduce successfully with both infected and uninfected males, while uninfected females can only have offspring with uninfected

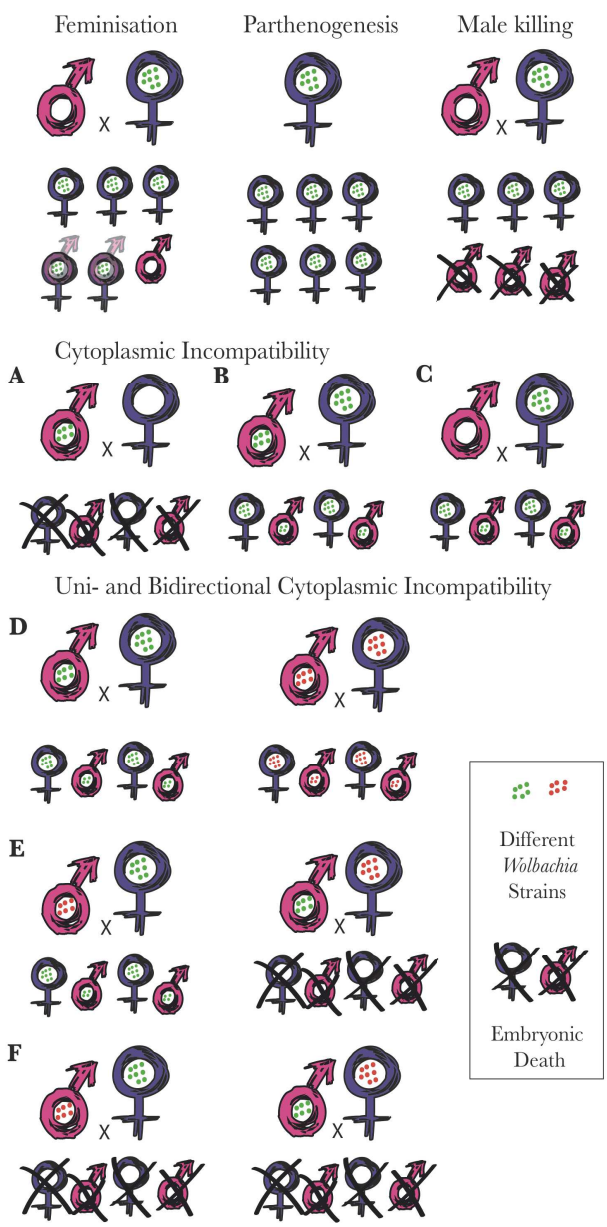

Figure 5 Reproduction Manipulation by *Wolbachia*. In arthropods, *Wolbachia* induce four different reproductive phenotypes. **Feminization** leads infected genetic males to develop as females. By inducing **Parthenogenesis** *Wolbachia* can exclude males from reproduction. **Male killing** results in higher survival of infected female siblings. **Cytoplasmic incompatibility (CI)** prevents uninfected females to reproduce with infected males (**A**) while infected females can reproduce with both infected (**B**) and uninfected males (**C**). More complex CI patterns are observed in *Cx. pipiens* complex mosquitoes. Mosquitoes with the same or compatible *Wolbachia* strain can reproduce together (**D**). **Uni-directional incompatibility** occurs when mosquitoes harbouring different *Wolbachia* strains are compatible in one cross while reciprocal cross results in embryonic death (**E**). **Bi-directional incompatibility** is when both of the reciprocal crosses between mosquitoes infected with different *Wolbachia* strains are incompatible **(F).**

males (Figure 5). Another manipulation called feminization consists for *Wolbachia* of forcing the development of infected genetic male embryos into functional females, thus becoming able to transmit *Wolbachia*, resulting in a female bias in the population (Figure 5)(Bouchon et al., 2008).

In addition to the ability of the *Wolbachia* to manipulate their hosts reproduction, some interactions have been reported to be detrimental on various host life history traits, including body size (Hoffmann and Turelli, 1988), fecundity (Hoffmann *et al.*, 1990; Fleury *et al.*, 2000), survival (Fleury *et al.*, 2000; Tagami *et al.*, 2001), larval competitiveness (Huigens *et al.*, 2004), mating choice (Rigaud and Moreau, 2004) as well as the hosts' immune parameters (Fytrou *et al.*, 2006; Braquart-Varnier *et al.*, 2008; Sicard *et al.*, 2010).

Although reproduction manipulation strategies appear efficient for *Wolbachia* to become widespread in host populations, conferring beneficial traits to their hosts and increasing their host's fitness could give complementary advantage to invade populations. It has been theoretically shown that mutualistic strains should outcompete non-mutualistic ones (Turelli, 1994). In accordance with these predictions, recent studies showed that *Wolbachia* is not always conflictual and manipulative but may also act as a mutualist with its hosts by being "protective" (*i.e.* increasing their host's survival to pathogenic challenge) (Teixeira *et al.*, 2008; Gross *et al.*, 2009; Bian *et al.*, 2010; Glaser and Meola, 2010; Zélé *et al.*, 2012; Eleftherianos *et al.*, 2013) or improving nutrition (Hosokawa *et al.*, 2010). The first "protective" mutualistic effect of the presence of a native *Wolbachia* (*w*Mel, *w*MelPop, *w*MelCS) against several viruses has been demonstrated in *D. melanogaster* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). However, this protection seems limited to RNA viruses (Teixeira *et al.*, 2008; Rottschaefer and Lazzaro, 2012; Ye *et al.*, 2013). On the other hand, in mosquitoes, in addition to the reports of a protective effect against viruses (*i.e.* Dengue, Chikungunya in *Ae. aegypti* and *Ae. albopictus*) (Moreira *et al.*, 2009), protection against protozoans (Moreira *et al.*, 2009), filarial nematodes (Kambris *et al.*, 2009), as well as two bacteria species (Kambris *et al.*, 2009; Ye *et al.*,

2013) have been linked to the presence of *Wolbachia*.

1.5.1.1 *Wolbachia* Pathogen Inference in Mosquitoes

Wolbachia natural infections are common in mosquitoes including several arbovirus vectors such as *Cx. pipiens* complex (Rasgon and Scott, 2003; Dumas *et al.*, 2013), *Ae. albopictus* and some *An. gambiae* (Baldini *et al.*, 2014; Gomes *et al.*, 2017). Furthermore it is possible to create mosquito lines stably transinfected with *Wolbachia* either from other mosquito species or *Drosophila* (Hughes and Rasgon, 2014). In fact, *Ae. aegypti* (Walker *et al.*, 2011), *Ae. albopictus* (Blagrove *et al.*, 2012), *Cx. pipiens*, and *An. stephensi* (Bian *et al.*, 2013) have been successfully transinfected this way. Mosquitoes can also be transiently transinfected with *Wolbachia* by injection of adult mosquitoes (Kambris *et al.*, 2010; Dodson *et al.*, 2014).

In the natural *Wolbachia*-host associations, *Wolbachia* is usually found in lower density, and rarely interferes with pathogens (Johnson, 2015b; Zug and Hammerstein, 2015). For instance, native *Wolbachia* of *Ae. albopictus*, *w*AlbA or *w*AlbB, did not affect viral replication of Dengue or Chikungunya. However dengue dissemination to salivary glands was slightly reduced (Mousson *et al.*, 2010, 2012). On the other hand, when *Ae. albopictus* were stably infected with *w*Mel from *D. melanogaster* both Dengue and Chikungunya transmission were reduced (Blagrove *et al.*, 2012, 2013). Interestingly when *w*AlbB was used to stably transinfect naive *Ae. aegypti* colonies, it reduced the Dengue infection rate, virus load and transmission (Bian *et al.*, 2010), suggesting that pathogen interference was not a characteristic of the host or *Wolbachia* but rather their interaction. Similarly, both *w*Mel-Pop and *w*Mel, *Wolbachia* strains from *Drosophila,* reduced the infection rate and virus load of Dengue, Chikungunya, West Nile and Yellow Fever viruses in stably transinfected *Ae. aegypti* lines (Moreira *et al.*, 2009; Walker *et al.*, 2011; Hussain *et al.*, 2012; van den Hurk *et al.*, 2012). This important distinction between the protective phenotypes that native and non-native *Wolbachia* cause, is thought to be related to the higher density that the bacteria reaches when transinfected. In contrast, in natural *Wolbachia*-host associations bacteria stay at lower densities. Indeed *Wolbachia* density and protective phenotype correlation have also been shown in mosquito cells against Dengue and West Nile virus (Hussain *et al.*, 2012; Lu *et al.*, 2012; Frentiu *et al.*, 2014) and in *Drosophila* against RNA viruses (Martinez *et al.*, 2017). Naturally infected *Cx. pipiens* and *Cx. quinquefasciatus* that differed in terms of their resistance to West Nile virus also differed in their *Wolbachia* density. Indeed, recently caught *Cx. quinquefasciatus* had lower *Wolbachia* density, compared to previously established *Cx. pipiens* laboratory lines, and *Wolbachia* pathogen blocking was only observed in the latter (Glaser and Meola, 2010; Micieli and Glaser, 2014). In contrast transient transinfection of *Wolbachia* into adult mosquitoes enhanced the West Nile Virus infection rate in *Cx. tarsalis* mosquitoes.

Wolbachia have also been studied for their interactions with *Plasmodium* parasites. Stably transinfected *An. stephensi* with *w*AlbB conferred resistance against *Plasmodium falciparum* (Bian *et al.*, 2013) and reduced *Plasmodium yoelii* oocyst intensity at 28°C while there was no effect of *w*AlbB at 20°C (Murdock *et al.*, 2014). In natural *Wolbachia-Cx. pipiens* system, *Wolbachia* increased the mosquitoes' susceptibility to *Plasmodium relictum* infection (Zélé *et al.*, 2014). Recently *Wolbachia* infected *Anopheles* mosquitoes have also been reported in Burkina Faso and Mali (Baldini *et al.*, 2014; Shaw *et al.*, 2016; Gomes *et al.*, 2017). In field studies a lower prevalence of *Plasmodium* infection in *An. coluzzii* infected with *Wolbachia* have been reported (Shaw *et al.*, 2016; Gomes *et al.*, 2017). Interestingly in this case this negative correlation between native *Wolbachia* and *Plasmodium* seem to occur despite the very low density of *Wolbachia* (Gomes *et al.*, 2017).

Though interesting, many of these situations do not represent natural symbiotic systems where *Wolbachia* would have evolved towards being mutualistic by conferring protection to their host against their natural enemies. *Wolbachia* conferred protection in mosquitoes have mostly been investigated against human pathogens (that mosquitoes

are vectors for, typically arboviruses), owing to the prospective applications in disease control. However, **the interactions between *Wolbachia* and pathogens for which the mosquitoes are the main hosts could be really different, although these are not very well studied**. The insect-specific flaviviruses only recently gained attention following the appreciation of their ubiquity in mosquitoes and the discovery of the possible impact on arbovirus transmission (Bolling *et al.*, 2012; Hobson-Peters *et al.*, 2013; Kenney *et al.*, 2014; Vasilakis and Tesh, 2015). However, their basic ecology in nature, their potential effects on mosquitoes and arbovirus transmission is largely unknown. On their interactions with *Wolbachia*, there are only two recent studies. Newly discovered insect specific virus, Anphevirus (AeAV) infection have been enhanced by *Wolbachia* in *Ae. aegypti* cells (Parry and Asgari, 2018). Similarly, field studies in release sites of stably transinfected *Ae. aegypti* mosquitoes were associated with higher insect specific flavivirus infection rates and density compared to the *Wolbachia*-free mosquitoes (Amuzu *et al.*, 2018). Much like insect-specific flaviviruses (RNA viruses), effects of DNA viruses on the vector competence of mosquitoes and their interactions with host associated *Wolbachia* is poorly investigated. However, *Wolbachia* have been shown to enhance DNA virus infection in *Drosophila* (Teixeira *et al.*, 2008) and in *Spodoptera exempta* (Graham *et al.*, 2012).

Although mechanistic bases of *Wolbachia*'s pathogen interference are not well understood, it is thought to be either caused i) indirectly by modulation of the host immune system or modification of host cellular structures resulting in a less hospitable environment for viral replication or ii) by direct competition between *Wolbachia* and pathogens for host resources (Terradas and McGraw, 2017). Enhancement of the pathogen replication can similarly be caused by the modulation of the immune system (*i.e.* reduction in resistance) of the host by *Wolbachia* or to a better tolerance (*i.e.* survival to higher pathogens load) when they are already infected with *Wolbachia*. Possibly it could also mean that once infected with a deadly pathogen, host loses the control of their endosymbiont hence causing an increase in *Wolbachia* density. In the case of insect-specific viruses *Wolbachia* could also be interacting directly with the viruses during their co-vertical transmission. **Further studies on these interactions are needed both to understand ecology and evolution of different partners of these multi-partite systems and for the use of *Wolbachia* and insect specific viruses in natural host populations as vector or disease control strategies.**

1.5.2 *Wolbachia* in *Culex pipiens* mosquitoes

Consisting of several species, including important disease vectors with worldwide distribution (*e.g.* *Cx. pipiens* and *Cx. quinquefasciatus*)(Farajollahi *et al.*, 2011), mosquitoes of *Cx. pipiens* species complex have a unique relationship with their endosymbiont *Wolbachia* (*w*Pip). Indeed, *w*Pip is fixed in natural *Cx. pipiens* s.l. populations where they induce the most complex CI relationships yet described among arthropods, including uni- and bidirectional incompatibility (Laven *et al.*, 1967; Duron *et al.*, 2006; Wolfgang *et al.*, 2009; Atyame, Delsuc, *et al.*, 2011; Atyame *et al.*, 2014).

In contrast to this observed diversity of CI patterns, *Cx. Pipiens'* *w*Pip strains are closely related and all belong to a clade within *Wolbachia* B super group (Guillemaud *et al.*, 1997; Baldo *et al.*, 2006; Atyame, Delsuc, *et al.*, 2011). However, recent studies of fast evolving markers showed the presence of many genetically distinct *w*Pip strains in *Cx. pipiens* s.l. mosquitoes (Duron *et al.*, 2006, 2007; Atyame, Delsuc, *et al.*, 2011) distributed in five distinct phylogenetic groups (*w*Pip-I to V) (Atyame, Delsuc, *et al.*, 2011). Using a PCR/RFLP assay based on pk1 gene, encoding proteins with ankyrin motifs, a *w*Pip strain can be assigned to one of these five groups (Atyame, Delsuc, *et al.*, 2011; Dumas *et al.*, 2013). A study of the *w*Pip worldwide distribution showed an important spatial structure of *w*Pip groups (Dumas *et al.*, 2013). For instance, only *w*Pip-I was found in Sub-Saharan Africa, South America and Southeast Asia, while *w*Pip-III was mainly observed in North America. Strains belonging to *w*Pip-II group were mostly found in Western Europe and *w*Pip-V in Asia. *w*Pip-IV group strains exhibit a patchy distribution

in Europe, North Africa and Asia (Dumas *et al.*, 2013). In addition, *Wolbachia* genetic diversity - especially diversity of a loci called cida/cidB (Atyame *et al.*, 2014; Bonneau *et al.*, 2018) - strongly correlate with their CI patterns. Most *w*Pip strains from the same group render their host compatible with each other (with the exception of few unidirectional incompatibilities) whereas those from different groups often lead to unidirectional or bidirectional incompatibilities (Atyame *et al.*, 2014). No effect of host genetic background on the CI patterns (Duron *et al.*, 2012) and no multiple infections by several strains have ever been shown (Atyame, Delsuc, *et al.*, 2011; Atyame, Duron, *et al.*, 2011; Dumas *et al.*, 2013).

1.6 Objectives of this thesis

As mentioned before, bacteria have been studied for some time now as intimate partners of their hosts. This perspective, taking microorganisms into account, revolutionized many aspects of science including applied and medical sciences. Recent advances of sequencing technologies also showed the ubiquity and diversity of viruses. It is thus now possible to imagine that viruses can have long-term intimate relationships with their hosts and these relationships can evolve to become antagonistic or mutualistic, or anywhere on the symbiotic spectrum. A lot of factors can shape this evolution including abiotic factors and biotic factors, such as the host or the interactions with the rest of the microbiota. Hosts can be seen as habitats where ecological interactions happen in different scales (Douglas, 2016).

In this context arthropods represent really interesting study models not only because they are ancient and really diverse but also they are able to establish symbiotic relationships with endosymbionts and sometimes to support multiple endosymbionts in their cells. Densoviruses, as ancient and diverse as their arthropod hosts, are also ideal candidates to investigate host-virus-bacteria relationships.

During my PhD, I studied these interactions in a natural host-bacteria-virus system, that is *Cx. pipiens*-*Wolbachia*-CpDV, both in natural populations and laboratory colonies. Interactions within *Cx. pipiens*, *w*Pip and CpDV could be intricate (Figure 6). Mosquito hosts could control the density of their endosymbiont *Wolbachia* and *Wolbachia* in return can modulate host immune system, affect host fitness positively or negatively, and manipulate host reproduction (Figure 6). Host immune system stimulated/supressed by *Wolbachia* could suppress/enhance CpDV. CpDV could also have fitness effects on their hosts or modulate host immune system hence indirectly influencing *Wolbachia* density. *Wolbachia* and CpDV could also compete for resources within the host resulting in the decrease in density of one of them (Figure 6). Alternatively costs of co-infection could be too high and cause disease leading to higher densities of both (Figure 6). Vertically transmitted *Wolbachia* could possibly have an effect on the transmission of CpDV (Figure 6).

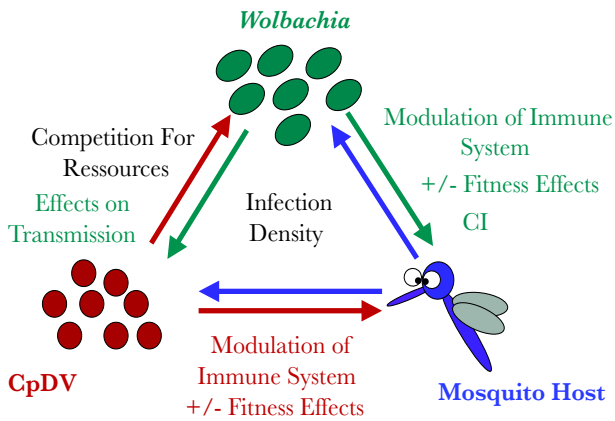

Figure 6 Possible CpDV-*Wolbachia*-*Cx. pipiens* interactions. CpDV and *Wolbachia* can affect each other's density either by competing for resources or modulating host immune system. They can both cause positive or negative fitness effects on their hosts and beneficial variants could get selected. *Wolbachia* might also influence CpDV transmission and infection outcomes.

In natural populations, the interactions between *Wolbachia* and CpDV can be diverse. One major parameter would be the interaction outcome between CpDV and their hosts which could range from mutualism to antagonism. If CpDV are harmful for their hosts, *w*Pip groups which can confer a better protection to their hosts could invade populations easier. On the other hand, CpDV could be mutualistic or commensal with its host. In this case

*w*Pip strain which supports the CpDV infection could be favoured. *w*Pip could also be allowing or facilitating vertical transmission of CpDV in which case it might be possible observe their co-divergence together.

First of all, as the knowledge on CpDV was limited to its first discovery in ISEM's insectary in Montpellier University (Jousset *et al.*, 2000), we investigated their prevalence in natural populations of *Cx. pipiens* worldwide to understand their relevance for *Cx. pipiens*-*w*Pip system in natural populations (**Chapter 2**). For this I first established a specific PCR diagnostic test. This approach made the use of many DNA samples, that have been collected by our team (EVAS) over the years for different studies, possible (**Chapter 2**). I then further studied their diversity and evolution in natural populations(**Chapter 2**).

Secondly, given their fundamental differences with the other mosquito densoviruses described so far, I have tried to understand their life cycle and dynamics in our laboratory colonies naturally infected with both *Wolbachia* and CpDV (**Chapter3**). Due to the persistent CpDV infection in our mosquito lines, it was difficult to assess the horizontal transmission of CpDV. Therefore, I have mainly focused on their vertical transmission. As *Wolbachia* is also vertically transmitted I have further investigated whether they might interact during their journey from mother to offspring(**Chapter3**).

Finally, I explored *Wolbachia*-CpDV interactions in natural *Cx. pipiens* populations by assessing their prevalence and microevolution together (**Chapter4 and 5**). First, I have investigated the prevalence and diversity of *w*Pip and CpDV in Turkey where the *Cx. pipiens* diversity is high as populations from Europe, Middle East and Africa are likely to meet (**Chapter 4**). Secondly, I have focused on a previously determined contact zone where two *w*Pip strains co-exist in Northern Tunisia to investigate the interactions between *w*Pip and CpDV in *Cx. pipiens* populations (**Chapter 5**).

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Chapter 2:
Prevalence and evolution of CpDV

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2.1 Diversity and dispersal of *Culex pipiens* densovirus a worldwide highly prevalent ambidensovirus

Altinli M., Courcelle M., François S., Justy F., Gosselin-Grenet A.S., Ogliastro M., Weill M., Sicard M., in prep., invited for the special issue 'New Insights into Parvovirus Research' in *Viruses*. To be submitted before 31/12/2018.

Diversity and dispersal of *Culex pipiens* densovirus; a worldwide, highly prevalent ambidensovirus

Abstract Single stranded DNA (ssDNA) viruses are thought to have several independent origins resulting from recombination events between bacterial plasmid and RNA virus genomes, which places them in an evolutionary continuum between DNA and RNA viruses. Many ssDNA viruses exhibit indeed evolutionary rates closer to RNA viruses than to double stranded DNA (dsDNA) viruses. Mosquito densoviruses (MDVs) are ssDNA viruses from Parvoviridae family. They have been found to persist in mosquito cell lines and detected in many mosquito populations although their worldwide prevalence and diversity have never been investigated. While most MDVs are closely related and belong to brevidensovirus genus, *Culex pipiens* densovirus (CpDV) constitutes an exception as it is closely related to ambidensoviruses, which infect lepidoptera and blattodea. Using samples collected between 1975 and 2018, we have investigated the prevalence of CpDV in natural populations of *Culex pipiens* mosquitoes. Phylogenetic reconstructions based on partial sequencing of both non-structural (NS) and viral particle (VP) genes, representing 40% of their genomes, demonstrated three well-supported CpDV clades worldwide. Phylogeographical model reconstructed based on sequences of 39 CpDV variants showed their significant dispersal worldwide. Our results reveal high substitution rates, while we did not detect any sign of acute infections based on their population dynamics. Combined with their high prevalence this could mean that CpDV might be responsible for persistent infections in *Cx. pipiens* populations rather than acute infections. This study improves our knowledge on the evolution of arthropod virus infections in natural insect populations.

2.1.1 Introduction

Single-stranded DNA (ssDNA) viruses infect hosts in all domains of life and environmental conditions (Pietilä *et al.*, 2009; Rosario and Breitbart, 2011; Mochizuki *et al.*, 2012; Krupovic, 2013). They can also be found integrated in their host chromosomes, as endogenous viral elements, in many vertebrates and invertebrates suggesting their long-term evolution with animals (Belyi *et al.*, 2010; Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015). The ssDNA virus genome size, typically below 10kb, is thought to be constraint by their small capsid size (a diameter of 18–27 nm) (Krupovic, 2013). The evolution of their genomes is shaped by high punctual mutation rates (Shackelton *et al.*, 2005)- that might also restrain their genome size due to fast accumulation of deleterious mutations- and frequent recombination both by DNA and RNA donours (Shackelton *et al.*, 2007; Martin *et al.*, 2011; Martynova *et al.*, 2016; Tijssen *et al.*, 2016). Indeed, ssDNA multiple origins are thought result from independent recombination events between bacterial plasmids and RNA viruses (Krupovic, 2013; Koonin *et al.*, 2015).

Parvoviridae family represents one of the most widely distributed ssDNA virus families in animals (Cotmore *et al.*, 2014). They infect a wide range of metazoans including vertebrates (Parvovirinae subfamily) and invertebrates, mostly arthropods (Densovirinae subfamily) (Cotmore *et al.*, 2014). The study of their diversity has been largely biased towards medically and economically important viruses, especially the ones affecting mammals (Cotmore *et al.*, 2014). With the advances in viral metagenomics and next generation sequencing techniques, their high diversity and prevalence in wider host range, particularly in arthropods, have been unravelled (François *et al.*, 2016). Densovirinae indeed are expected to be more diverse than their vertebrate counterparts (Parvovirinae subfamily), as their arthropod hosts, are also more diversified and ancient. Densovirinae subfamily so far consists of 5 genera (*i.e. Iteradensovirus, Brevidensovirus, Ambidensovirus, Penstyldensovirus,*

Hepandensovirus) that have been isolated mostly from insects (e.g. lepidopteran, dipteran, orthopteran, and dictyopteran) and malascostracean (e.g. prawns and crayfish) (Kerr, J. and Cotmore, S. and Bloom, 2005; Tijssen *et al.*, 2016). They are usually pathogenic for their hosts causing epizootics in the infected host populations. Therefore they have long been considered as possible population control tools against agricultural pests such as lepidopterans or as human disease vectors such as dipterans (Carlson *et al.*, 2006; Mutuel *et al.*, 2010; Gosselin Grenet *et al.*, 2015; Suzuki *et al.*, 2015).

Mosquitoes being the vectors of many human diseases, their associated viruses have been studied, originally with *a priori* diagnostic tests designed for human RNA arboviruses (Kuno, 1998). With the recent advances in viral metagenomics, mosquito virome could now be explored without *a priori*. Nevertheless mosquito virome studies are yet still mostly focused on RNA viruses transmissible to humans and lately on insect specific RNA viruses while DNA virus diversity in mosquitoes stayed even less explored (Bolling *et al.*, 2012; Junglen and Drosten, 2013; Blitvich and Firth, 2015). However, the only study which focused on the discovery of mosquitoes' DNA viruses revealed their contribution to mosquito's viromes, especially highlighting the importance of Densovirinae (Ng *et al.*, 2011). Mosquito densoviruses (MDVs) have been isolated from several natural populations and laboratory colonies of different species of mosquitoes and found to persistently infect many mosquito cell lines (Afanasiev *et al.*, 1991; Jousset *et al.*, 1993, 2000; Boublik *et al.*, 1994; O'Neill *et al.*, 1995; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000; Ng *et al.*, 2011), although MDVs have never been investigated in terms of their general prevalence, diversity and putative impact on vector ecology in natural populations.

Most of the MDVs discovered so far are closely related (82-99% sequence homology) and considered to be different strains from two distinct species (so called Dipteran Brevidensovirus 1 and 2) belonging to the genus *Brevidensovirus,* except *Culex pipiens* densovirus (CpDV) (Cotmore *et al.*, 2014). CpDV has been isolated from *Culex pipiens* laboratory colonies following high larval mortality periods (Jousset *et al.*, 2000). With its ambisense genomic organization and larger genome size (~6kb) compared to brevidensoviruses (~4kb), CpDV belongs to *Ambidensovirus* genus (Baquerizo-Audiot *et al.*, 2009; Cotmore *et al.*, 2014). Like other members of the *Parvoviridae* family, CpDV genome only encodes two types of proteins: nonstructural proteins (NS or Rep for replication proteins) that have a role in the viral gene expression and DNA replication, and structural proteins (VP for viral proteins or Cap for capsid proteins) that constitute the components of the viral capsids (Figure 1) (Kerr *et al.*, 2005).

First isolated two decades ago following unusually high larval mortality in *Culex pipiens* colonies (Jousset *et al.*, 2000), CpDV has later been detected to persist in seemingly healthy laboratory colonies in all life stages of mosquitoes (**Chapter 3**). Their covert infection, in low amounts, combined with vertical transmission explained their long-term persistence in laboratory colonies and suggested that these viruses could also be prevalent in natural populations of mosquitoes (**Chapter 3**). To test this hypothesis, we designed a CpDV specific PCR-based diagnostic test to investigate CpDV prevalence in *Culex pipiens* mosquitoes collected worldwide for more than forty years in natural populations (Dumas *et al.*, 2013; Atyame *et al.*, 2015; Altinli *et al.*, 2018). Sequencing amplicons from Open Reading Frames (ORF) encoding for partial VP and NS allowed us to analyse the worldwide evolution of CpDVs in natural populations and laboratory colonies.

2.1.2 Methods

2.1.2.1 Sample collection and DNA extractions

Samples from natural *Cx. pipiens* (s.l.) populations have been collected between 1975-2018 (**Appendix** Table 1). Samples that have been sequenced were chosen among the different populations to cover the diversity in different continents. Slab, laboratory mosquito line, that has been initially collected from USA in 1954 (Georghiou *et al.*, 1966) has been reared in our insectary from 1985 to

present . We used individuals kept in liquid nitrogen between 1990 and 2018 to follow genetic variations between CpDVs through this time period (**Appendix** Table 1).

2.1.2.2 CpDV genomes partial sequencing

CpDV have three putative NS genes (Figure 1). NS1 and NS2 genes are putatively encoded by four ORFs (NS1-NS1', NS2-NS2')(Figure 1) (Baquerizo-Audiot *et al.*, 2009). Four different VP proteins have been identified for CpDV and thought to be encoded by a single ORF via leaky scanning (Figure 1) (Baquerizo-Audiot *et al.*, 2009). To detect the presence of CpDV and to further infer the within species diversity, we designed CpDV specific primers in NS2, NS1', VP regions in a maximum number of samples coming from diverse mosquito populations worldwide. To do this we used an alignment of the reference genome (Baquerizo-Audiot *et al.*, 2009) with a partial consensus genome obtained by next-generation sequencing of CpDV from six different mosquito populations (data not shown).

For the detection of CpDV, CpDV specific set of primers (**Appendix** Table 2) was designed from position 1606 to 1843 (238pb) in NS2 and NS2' overlapping ORFs that are expected to be CpDV specific and very conserved (Figure 1). A PCR diagnostic test using these primers was established (**Appendix** Table 2).

In NS1 region we designed the primers to amplify the whole NS1' (1681-2648; 967nt, Figure 1) which encompasses conserved SF3 helicase domain used for taxonomic classification of densovirus genuses (Cotmore *et al.*, 2014). We also sequenced partial VP ORF including conserved PLA2 domain and stretches for 1389 bp (3570-4959; 1389 bp, Figure 1). With both of these NS and VP fragments amplified, we covered about 40% of the CpDV genome (2.356 over 6kb). A total of 54 samples were amplified and sequenced for NS1 gene, and 39 samples were amplified and sequenced for both genes.

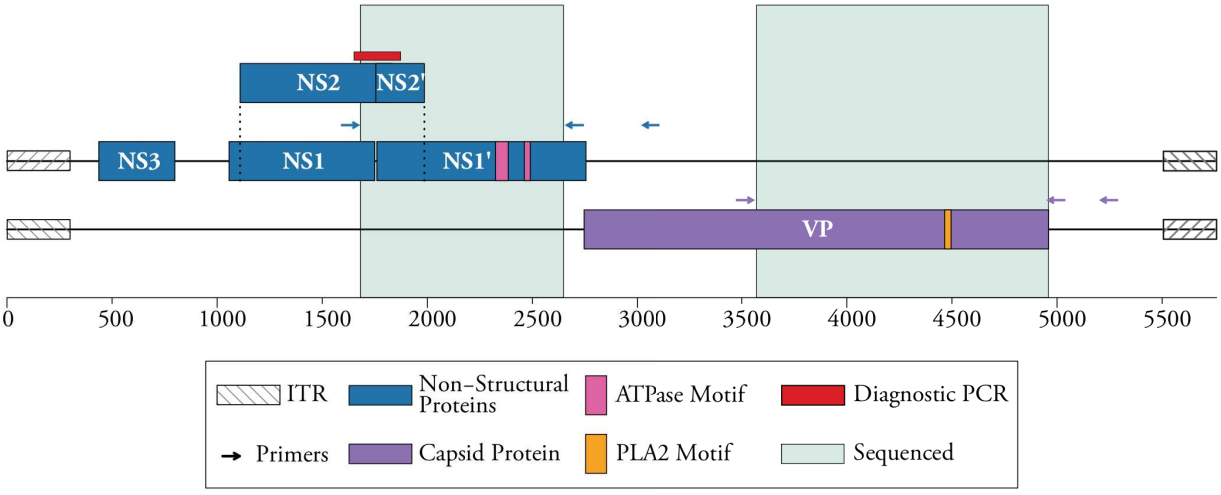

Figure 1 CpDV Genome encodes for two types of proteins: non-structural NS and structural VP. ORFs of NS and VP proteins are situated on different single strands, which makes CpDV genome ambisense. CpDV hs 3 putative NS proteins. NS3, NS2, NS1 (ORFs are shown as blue boxes). Conserved SF3 helicase domain consisting of Walker A and B boxes as part of within NS1 is shown in pink (ATPase motif). Conserved PLA2 in VP have been highlighted in orange CpDV has four putative. Location of primers used in semi-nested PCRs have been shown as arrows. Red part has been amplified for the diagnostic test and green parts are sequenced to infer CpDV diversity.

To improve the amplification of both NS1 and VP regions semi-nested PCRs were conducted with two PCR steps (**Appendix** Table 2). PCR products were purified using AMPure (Agencourt) with Biomek 4000. Following the purification, sequencing reaction has been performed using 100ng of DNA. After the completion of sequencing reaction (96°C for 5min, 30 cycles of 96°C 15sec, 50°C 10sec and 60°C 4mn) products have been purified using CleanSEQ (Agencourt) with Pipeteur Biomek 4000.

2.1.2.3 Selection, recombination and probabilistic phylogenetic analyses

All sequences were aligned using MAFFT v7.273 (Katoh and Standley, 2013). In order to avoid misleading phylogenetic signal due to recombinant parts of sequences, signatures of recombination were searched with help of RDP, GENECONV, Bootscan, MaxChi, and LARD methods with default parameters, as implemented in the RDP4 software (Martin *et al.*, 2015). Six sequences were identified as potential recombinants by more than two methods. These sequences were removed from the dataset.

To build maximum likelihood phylogenetic tree of CpDV, we started by using modelfinder (Kalyaanamoorthy *et al.*, 2017) in order to find the model which best fits our dataset while avoiding over-parameterization (Lemmon and Moriarty, 2004). The HKY85 + \Gamma model of sequence evolution using RAxML v8.2 was chosen (Stamatakis, 2014). This model estimates different substitution rates for transitions and transversions (Hasegawa *et al.*, 1985), and allows for a Gamma distribution of evolutionary rates among sites (Yang, 1994). Statistical support of the phylogenetic nodes was computed using a bootstrap procedure, 100 iterations (Felsenstein, 1985). Both concatenated (*i.e.* VP and NS) and individual gene trees were computed using the same protocol.

Coding regions of our sequences including, ORF of NS1' (1764-2512; 750 bp) and VP (3545-4867; 1326 bp), have been analysed for selection using DATAMONKEY web server (Weaver *et al.*, 2018). Individual sites have been tested for i) episodic positive or negative selection using MEME (Mixed Effects Model of Evolution) (Murrell *et al.*, 2012) and ii) pervasive positive or negative selection using FEL (Fixed Effects Likelihood)(Kosakovsky Pond and Frost, 2005). Distance between CpDV variants and different clades were calculated using Jukes-Cantor distance model implemented in Mega7.

2.1.2.4 Phylodynamics and dispersal of CpDV

Bayesian Markov Chain Monte Carlo approach implemented in BEAST v1.8.4 package (Suchard *et al.*, 2018) was used to estimate the evolution rate, the time to the most recent common ancestor (tMRCA) and internal nodes, and the geographic movement of CpDV. For these analyses we only considered samples where both VP and NS1' amplicons were obtained (n=39, length=2076bp). Both concatenated, and separate NS1'-VP data sets were analysed as these two parts of the genome could be evolving differently.

Exponential growth model has been used as the tree prior with a relaxed uncorrelated lognormal molecular clock model (Drummond *et al.*, 2006) and HKY85 substitution model. The symmetric substitution model, a standard continuous-time Markov chain (CTMC) in which the transition rates between locations are symmetric, has been used as the discrete trait substitution model to infer migratory events. As we had many sampling sites from some geographical areas(*e.g.* four in Sub-Saharan Africa) and less samples from others (*e.g.* New York one location), we have grouped the samples from the same geographic area to decrease the sampling bias and inferred the major dispersion routes between these areas (i.e. Turkey and Armenia as Western Asia; Porto Rico, Haiti and Venezuela as Central America; China, Vietnam, Thailand as Eastern Asia; Sub-Saharan African countries as Sub-Saharan Africa; Montpellier natural populations and laboratory, as well as one sample from Moscow as Europe).

The Bayesian stochastic search variable selection (BSSVS) approach was used to identify well-supported rates using Bayes Factors test using Spread3 (Bielejec *et al.*, 2016). Markov Chain Monte Carlo was ran for 100 million generations and convergence (Effective Sample Size >200) of

all parameters was verified with TRACER v1.6.0 (Rambaut *et al.*, 2018). TreeAnnotator v1.8 was used to obtain the maximum clade credibility tree after discarding 10,000,000 states (10%) as burn-in. The estimated uncertainty of the results is reported as 95% highest probability density (HPD) values. For each node posterior probability have also been reported to . We verified that our results were not shaped only by the priors by running the MCMC chains on the data sets with the same prior parameters but without the sequence data.

2.1.3 Results

2.1.3.1 CpDV prevalence in *Culex pipiens* populations worldwide

To investigate the general prevalence of CpDV in *Cx. pipiens* populations, a total of 2843 individual larvae samples from 136 different locations that have been collected between 1975-2017 were subjected to a diagnostic CpDV specific PCR test (Figure 1, **Appendix** Table 1). 48% of the samples were CpDV positive and displayed an amplicon at expected size (Figure 2, **Appendix** Table 1). In only 29 localities out of 136, no CpDV was detected. No apparent spatial or temporal structuration of CpDV prevalence were observed.

2.1.3.2 Worldwide diversity and evolution of CpDV

Primers within NS1 and VP genes have been designed to infer the diversity of CpDV genomes worldwide as well as their diversity and evolution within one laboratory line through a 28 years period. A total of 54 samples were selected due to their location, amplified and sequenced for NS1 gene, then 39 of them were successfully amplified and sequenced for VP gene. As for some samples it was not possible to amplify VP gene fragment, putatively because of i) the higher diversity of this region that prevents primers to be suitable for all the samples, or ii) VP, which is a longer DNA fragment compared to NS, was more difficult to amplify due to poor DNA conservation over the years.

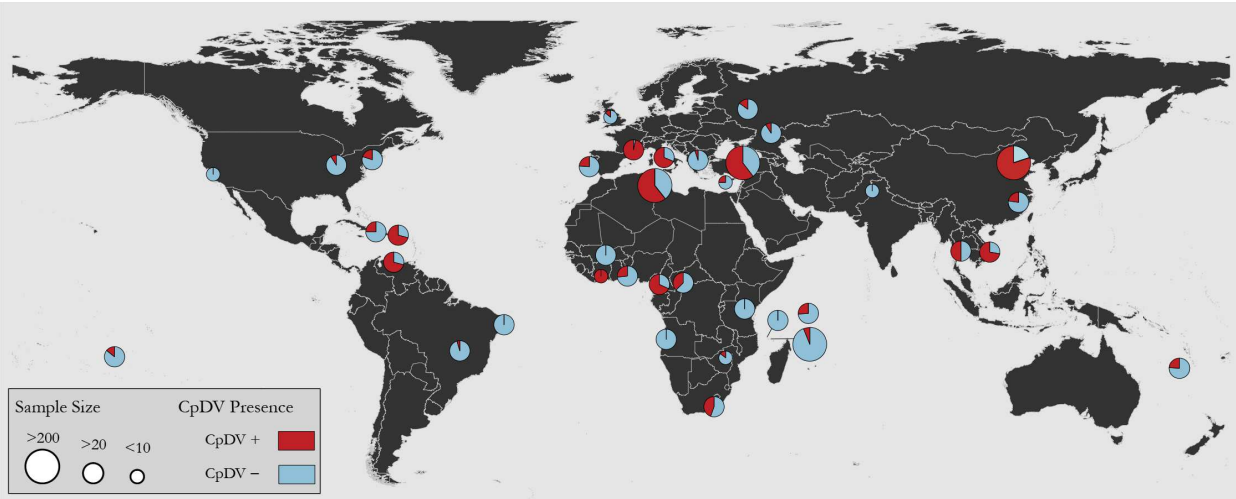

Figure 2 Worldwide prevalence of CpDV in *Culex pipiens s.l.* populations A total of 2843 individual larvae samples collected from 136 different locations worldwide have been tested with a PCR detection test using conserved Ns2 gene in CpDV. As we had too many sampling sites for some specific areas such as Turkey, Tunisia and Mayotte we were not able to show all 136 populations on the map separately and rather grouped these sampling sites together. (see for the full **Appendix**, Table 1 for the list of sampling sites and CpDV prevalence). 48% of these samples showed a PCR amplification. Only in 29 out of 136 locations there was no CpDV amplification in any of the individuals.

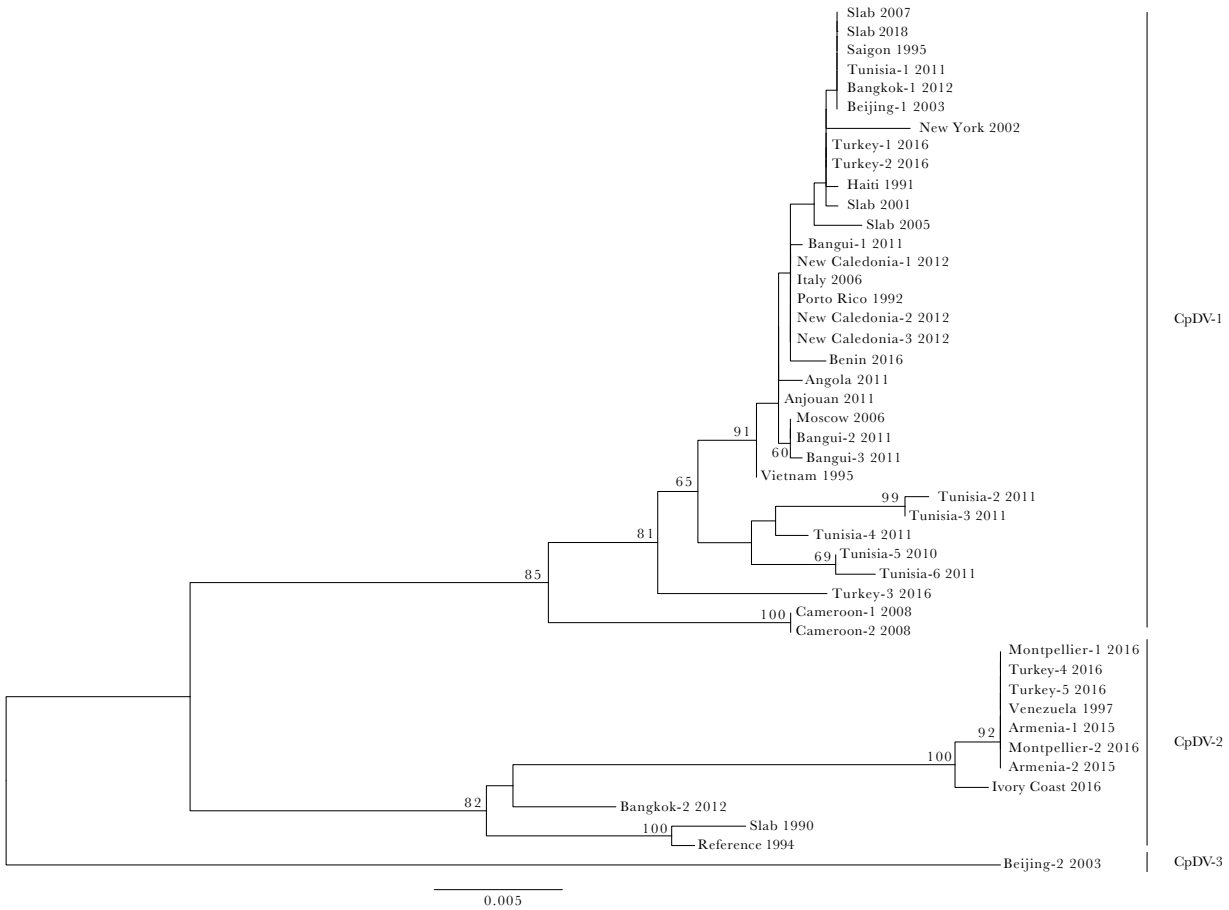

Figure 3 Phylogenetic tree of CpDV variants. Midpoint rooted Maximum Likelihood tree (RaxML) was built using NS1-VP concatenated sequence data set after the exclusion of recombinant sequences. 100 Bootstraps have been made to test the statistical significance of the nodes and nodes that were supported with more than 60% are indicated. There were three well-supported clades within CpDV; CpDV-1, CpDV-2, CpDV-3. CpDV-3 was represented by only one sample from Beijing while the other clades were more populated and diverse.

Recombination events

Several studies highlighted the negative impact of recombination on phylogenetic reconstruction of viruses evolutionary histories (Scheffler *et al.*, 2006; Arenas and Posada, 2010; Pérez-Losada *et al.*, 2015). We thus checked recombination in both NS1 and VP gene datasets, as well as in the concatenated one. There was no recombination detected within the sequenced NS1 gene fragment. Within VP region, we have detected 2 different recombinant sequences (between Haiti 2 and Bangui 3, **Appendix** Figure 1). However, within the concatenated dataset, we observed 4 other recombinant sequences. Breakpoints of recombination were similar in many of the recombinants (**Appendix** Figure 1 shown as New York 2, but also found in samples from Venezuela 2, Karaoke, Porto Rico 2) generally located around 900 nt position. As these positions correspond to where NS1 sequence ends and VP sequence starts it highlighted the phylogenetic incongruence between these two parts of the CpDV genome (**Appendix** Figure 1).

Phylogeny

We have constructed a midpoint rooted maximum likelihood phylogenetic tree with the concatenated dataset of NS1' and VP sequences after the removal of the recombinant sequences (n=48). Three distinct and well supported groups were

discriminated within CpDV: CpDV1, CpDV2 and CpDV3 (Figure 3). One of the groups was represented only by one sample from Beijing collected in 2003. The other two groups were mixed in terms of their collection date and geographical location. We have also built phylogenetic trees using NS1' and VP sequences separately. While most of the phylogenetic relationships were well resolved in both of the gene trees, and generally bootstraps values were better than it is for the concatenated tree. General topology of the trees was similar to the concatenated tree, in terms of the two well supported clades, CpDV1-CpDV2, although trees were not totally congruent. Overall mean distance between variants calculated for the concatenated data was 0.02 (± 0.005) nucleotides. Distance between CpDV1 and CpDV2 clades was 0.05 (± 0.01) nucleotides, confirming all the variants were closely related and belong to CpDV species.

Selection

The purifying (negative) and diversifying (positive) selection putatively acting on both NS1' ORF and VP ORF have been tested. Both pervasive selection (selection detected across the whole phylogeny) and episodic selection (selection detected only at a subset of branches) were also investigated.

Within NS1' 23 individual sites and within VP 22 individual sites were subjected to pervasive purifying selection (**Appendix** Figure 2 and 3 respectively, FEL, $p<0.05$).

No positive selection was detected at any individual sites of NS1'. In VP only one site was subjected to episodic positive selection which only occurred on the branch between Slab 1990 and Reference and caused by one non-synonymous nucleotide change in site 342 (MEME, $p=0.01$, Glycine to Threonine change, **Appendix** Figure 3). Contrarily there was no pervasive diversifying selection detected.

Additionally, both Walker A and Walker B sites were fully conserved as well as putative start and termination codons of other predicted ORFs within NS1'. Similarly, in VP, Ca++ binding or PLA2 motifs were highly conserved: there was no nucleotide change in Ca++ binding motif while there was only one synonymous nucleotide change in Slab 1990 for PLA2 motif.

Phylodynamics of CpDV

To understand the evolution, population dynamics and dispersal of CpDV, we used concatenated coding sequence matrix of the samples. As recombination analysis showed a breakpoint between NS1 and VP sequences we have also analysed these genes separately.

The resulting tip-dated phylogenetic trees using both gene fragments and concatenated datasets (with and without recombinant regions) were similar in topology to the maximum likelihood tree (Figure 4, **Appendix** Figure 4). Only major difference is that as we were missing Beijing 2 sample which represented CpDV3 in probabilistic phylogenetic tree. The two well-supported clades in maximum likelihood tree were present in the tip dated Bayesian tree as well, however CpDV-2 showed lower Bayesian posterior probability (Figure 4).

We have detected major differences between the estimated most recent common ancestor (tMRCA) of different gene trees and concatenated datasets. This was mainly caused by the incongruence between NS1 and VP coding regions. This incongruence could be a result of recombination that were mainly identified in NS1 region (**Appendix** Figure 6). Hence the exclusion of these regions meant exclusion of NS1 fragment from the dataset. Models were run with and without data to make sure that the results were not only shaped by the prior parameters and that the data had enough temporal information. Both NS1 and concatenated datasets showed sufficient temporal structure while it was less true for VP dataset. With the concatenated dataset time to most recent common ancestor (tMRCA) was estimated to be 71 years before present (1947; HPD 95% 40-108 years before present, Table 1) and mean substitution rate was 6.18×10^{-4} (Table1). The mean substitution rate was lower when the analysis was based on only NS1

	Mean Substitution Rate (substitution/site/year)	HPD Substitution Rate (95%CI)	tMRCA (Years)	HPD tMRCA (95%CI)	Growth Rate	HPD Growth Rate (95%CI)	Sequence Length
NS1	$9.3x10^{-5}$	$1.27x10^{-5}$ -$2.16x10^{-4}$	245	50-540	$2.36x10^{-3}$ - $5.15x10^{-4}$	-0.01- 0.02	750
VP	$3.33x10^{-5}$	$1.92x10^{-6}$- $6.59x10^{-5}$	1103	133-2391		-0.01-0.003	1326
Concatenated	$6.18x10^{-4}$	$2.22x10^{-4}$- $1.41x10^{-3}$	71	40-108	- $1.25x10^{-2}$	-0.05- 0.02	2076

Table 1. Nucleotide substitution rates, time to most recent common ancestor (tMRCA), population dynamics of CpDV estimated using concatenated data set and separate NS1 (ORF2) and VP gene fragments.

region (Table 1, $9.3.10^{-5}$ substitution/site/year). tMRCA for this gene was approximately 245 years before present although the confidence of this estimate was lower compared to concatenated dataset (HPD 95%, 50-540 years before present). Due to the lack of temporal information VP region a lower substitution rate and higher uncertainty for the estimation of tMRCA (Table 1).

The NS1', VP and concatenated datasets were also analyzed using exponential growth model to determine the fluctuations in growth of the effective population size between the 1990-2018. We have observed a constant population size through time for both NS and VP fragments while for the concatenated dataset, there was a slight decrease in population size (see **Appendix** Figure 5).

BF (Posterior Probability)	**Sub-Saharan Africa**	**Tunisia**	**Eastern Asia**	**New Caledonia**	**New York**	**South America**	**Western Asia**
Europe	NS	NS	NS	NS	NS	NS	NS
Sub-Saharan Africa		NS	**94.73 (0.97)**	NS	**283.61(0.99)**	**11.70 (0.81)**	**24.57 (0.90)**
Tunisia			NS	NS	NS	**9.06 (0.77)**	NS
Eastern Asia				NS	NS	NS	NS
New Caledonia					NS	**7.17 (0.73)**	NS
New York						NS	NS
South America							NS

Table 2. Significant Dispersal Routes of CpDV. Bayesian Factors (BF) >5 and their posterior probability (in brackets) are given. NS: BF < 5

CpDV-1 clade was found in *Cx. pipiens* from all the sampled areas while CpDV-2 was only found in the samples from Europe (Montpellier), Western Asia (Turkey and Armenia) and South-Central America (Venezuela and Haiti). To infer the dispersal of CpDV, we have only used NS1 gene fragment due to the uncertainty due to the recombinants in concatenated dataset. CpDV1 and CpDV2 clades are likely to be diverged in either South-Central America or Sub-Saharan Africa (SSA) and dispersed to worldwide mosquito populations from here. Sub-Saharan Africa appears to be a hub for CpDV 1 clade distribution as a meeting point of highly significant dispersal routes (Table 2; SSA-Eastern Asia, SSA-New York, SSA- South America, SSA- Western Asia). Variants of the CpDV 2 clade seem to have colonized Europe and Eastern Asia in two different occasions although we did not observe any significant dispersal route between these locations (Table 2, Figure 4).

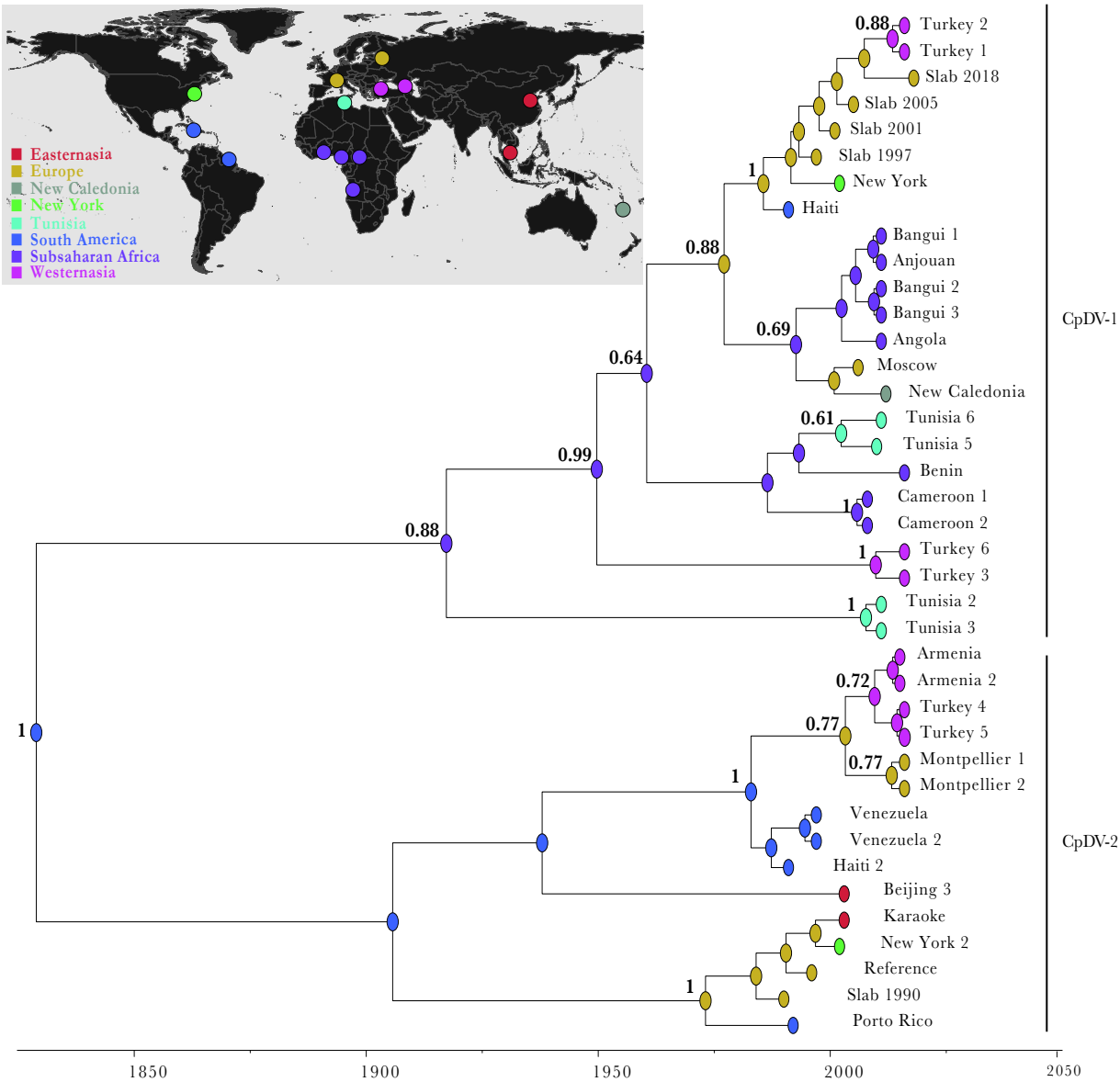

Figure 4 Phylodynamics of CpDV. Bayesian maximum clade credibility phylogenetic tree of tip dated NS1 sequence data set was built under an exponential coalescent tree model and HKY substitution model. Tips are colored by the location of the sample collection. As shown in the map some locations were grouped together to decrease sampling bias. the internal nodes are also coloured based on the reconstructed ancestral state as estimated by a symmetric discrete phylogeographic model. x axis shows the time of divergence (for nodes) and sample collection (for tips). Posterior probability >0.5 were given on the left side of the nodes.

2.1.4 Discussion

Prevalence and diversification of insect ssDNA viruses have never been studied at a worldwide scale. Our study revealed that one of them, the CpDV, is detected in 78% of the 136 *Cx. pipiens* populations and, on average 48% of larvae belonging to these populations. While it is possible this high prevalence is due to the detection of endogenized CpDV sequences in the mosquito genome rather than detection of a CpDV infection, CpDV endogenization has never been reported (Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015; François *et al.*, 2016). Furthermore, the absence of CpDV in some of the samples within the same location would require this endogenization to be really recent and an on-going process. In the laboratory lines, we demonstrated variations in vertical transmission and were able to isolate CpDV particles demonstrating that CpDV is not endogenized (**Chapter 3**). MDVs had been previously detected in mosquito's natural populations but reports on their prevalence are scarce (Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000). Indeed, to our knowledge, two studies on *Aedes aegypti* Thai strains densovirus showed that 6% to 35% of *Ae. aegypti* adults were infected in natural populations (Kittayapong *et al.*, 1999) while the same densovirus was later found at 19% prevalence in natural populations of *Anopheles minimus* larvae (Rwegoshora *et al.*, 2000). The prevalence of the latter *Brevidensovirus* was influenced by seasonal environmental changes and rainfall, probably due to variations in host population density and consequently transmission rate of the virus (Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000). Here, we did not statistically test the influence of environmental factors. However, the 2843 individuals analysed were collected in 33 different countries in quite contrasted environments, the presence of CpDV in half of them, independent of latitudinal or longitudinal distributions, suggests the persistence of CpDV in natural *Cx. pipiens* populations living in quite different environmental conditions.

The phylogeographic diffusion analysis further demonstrated a significant dispersal of CpDV in *Cx. pipiens* populations worldwide, with highly significant dispersal routes especially meeting in Sub Saharan Africa. This dispersal of CpDV could be achieved by the migration of mosquitoes of the *Cx. pipiens* complex by planes and by boats, as either larvae or adults (Farajollahi *et al.*, 2011). Mosquito eggs and larvae can also be dispersed in used tyres (Schaffner, 2003). Emergent infected adult mosquitoes can transmit densoviruses to new larval/oviposition sites horizontally (Wise de Valdez *et al.*, 2010) or to their offspring vertically (Barreau *et al.*, 1997; Ren *et al.*, 2014)(**Chapter3**). Densoviruses have also been shown to stay active in natural conditions for long amount of time (Buchatsky, 1989) so they could have also been carried environmentally by human activity alone. Another possibility for densovirus dispersal could be via migratory birds. Migratory birds have been associated with emergence of many viruses including West Nile Virus (Reed and Medical, 2003), they also could possibly carry CpDV in low titers. Although densoviruses have previously been found in insectivorous bats faeces (Ge *et al.*, 2012) and in a great tit (Yang *et al.*, 2016), their transmission to mosquitoes through birds have yet never been tested.

In addition to their prevalence and dispersion, we inferred the worldwide CpDV diversity in natural populations using NS and VP markers that cover 40% of the CpDV genome. We demonstrated that CpDV strains are polymorphic on these markers and that all CpDV strains sequenced form three distinct well-supported clades (CpDV 1, CpDV 2, CpDV 3). These clades are closely related suggesting that all CpDV strains reported here belong to one single species within *Ambidensovirus* genus. Indeed in *Densovirinae* sub-family, viruses in a species required to show >85% amino acid sequence identity in NS1 protein (Cotmore *et al.*, 2014). Other MDVs, all belonging to *Brevidensovirus,* have been so far grouped in two species: Dipteran Brevidensovirus 1 (Lebedeva *et al.*, 1972; Chen *et al.*, 2004; Ren *et al.*, 2008; Zhai *et al.*, 2008; Sivaram *et al.*, 2009) and Dipteran Brevidensovirus 2 (Jousset *et al.*, 1993; Boublik *et al.*, 1994; O'Neill *et al.*, 1995; Paterson *et al.*, 2005) with several really closely related (98% sequence similarity) variants within these species. However, phylogenetic

analyses conducted on NS and VP separately, even if they both demonstrated the same clades, exhibited some incongruencies. These incongruencies explained by the recombination breakpoint that has been highlighted by our analyses right at the end of NS coding region. Further investigation of the recombinants indicated that NS and VP fragments were more closely related to different parental recombinants. Recombination is known to be fundamental both in the origin and the evolution of ssDNA viruses such as densoviruses (Krupovic, 2013; Koonin *et al.*, 2015). An extreme example of is the origin of ssDNA viruses that is thought to result from ancient recombination events resulted in acquisition of NS from DNA donors (such as bacterial plasmids) and VP from RNA viruses (Krupovic, 2013; Koonin *et al.*, 2015). Moreover, recombination have also been shown to play an important role in microevolution of densoviruses, especially in *Ambidensovirus* and *Iteradensovirus* genera (Martynova *et al.*, 2016). Indeed, phylogenetic trees using different gene fragments (NS3, NS1 and VP) had also lead to incongruences in phylogenetic relationships between lepidopteran *Ambidensovirus* species, such as *Junonia coenia* densovirus JcDV, *Pseudoplusia includens* densovirus (PiDV), *Diatraea saccharalis* densovirus (DsDV), *Helicoverpa armigera* densovirus 1 (HaDV1) which are closely related to CpDV (Martynova *et al.*, 2016). This suggested that recombination events between ancestral variants could be the major cause of divergence between new "species" (Martynova *et al.*, 2016). As an important evolutionary mechanism that shape virus genomes putatively resulting in convergent evolution, we also tracked positive (diversifying) and negative (purifying) selection signals. In both NS1 and VP ORFs, we mainly detected purifying selection, while there was one site subject to diversifying selection in VP region. In agreement with our results, Martynova et al., (2016) also reported strong signal of purifying selection in other *Ambidensovirus* species (*i.e.* JcDV, PiDV, MlDV, GmDV, DsDV, HaDV1) (Martynova *et al.*, 2016).

Phylodynamics methods allowed us to estimate the evolutionary rate of CpDV. NS1 region and concatenated dataset had sufficient temporal structure that allowed an estimation of tMRCA based on these datasets, while it was not the case for VP region. As we have also detected by the analysis of recombination such discordance could result from acquisition of NS or VP parts from different variants of CpDV. However, recombination while obviously one of the main drivers of virus evolution cannot be taken into account by phylodynamics method and constitutes one of the biggest challenge for these methods (Frost *et al.*, 2014). For these reasons, it is difficult to totally rely on our results based on the concatenated dataset. However, while NS1 region dataset (which does not include recombination events) showed slower substitution rate than concatenated one, it still shows high substitution rate compared to most DNA viruses. Variations in evolutionary rates between viruses are usually explained by whether they use the high fidelity DNA polymerases or low-fidelity RNA polymerases (Elena and Sanjuán, 2007; Duffy *et al.*, 2008). Indeed, DNA viruses that replicate using host DNA polymerases have lower mutation rates closer to what is reported for their hosts' genome (10^{-9}- 10^{-8}) (Duffy *et al.*, 2008). However, the difference between polymerases used during viral replication is not the only explanation of substitution rates variations. Selection also acts on substitution rates (Duffy *et al.*, 2008). For instance, a very high substitution rate (10^{-3}) is reported during host-switching of Canine parvovirus (CPV) from dogs, when it emerged as feline panleukopenia parvovirus (FPLV) in cats and has been explained by high positive selection pressure (Shackelton *et al.*, 2005). Studies on other temporally sampled ssDNA belonging to parvovirus have also shown a high substitution rate (10^{-3} to 10^{-5}) nearing the one observed for RNA viruses (10^{-2} – 10^{-5}) (Shackelton *et al.*, 2005, 2006; Firth *et al.*, 2009; Streck *et al.*, 2011).

First isolated from laboratory lines kept in ISEM's insectary following high larval mortality, CpDV has since been shown to persist in these lines (**Chapter 3**). Here, we monitor CpDV persistence during almost 30 years from 1990 to 2018 in one laboratory line (Slab). This approach also allowed to monitor CpDV diversification during this period in laboratory conditions. The reference strain

(isolated from our laboratory lines in 1994), while exhibiting some differences, is closely related to the strain present in Slab 1990 both belonging to CpDV-2 clade. However, in the following years we observe a swift change, from CpDV-2 to CpDV-1, in CpDV variants infecting Slab line. Indeed, CpDV samples from 2001 to present belong to the same clade within CpDV-1. This change could be explained either by the evolution of the existing virus or by its replacement by another variant possibly arriving to the insectary with newly established mosquito lines. Our data seems to favour both of these hypotheses, because of the recombination event we have detected in the Slab 1997 sample, collected during this shifting period (**Appendix** Figure 1). Indeed, its VP belongs to CpDV-2 while its NS region belonged to CpDV-1 (**Appendix** Figure 6). This suggested that during this period CpDV-1 and a CpDV-2 variant could have co-existed in the laboratory and co-infected Slab line resulting in a recombination event between them. The fact that CpDV-1 clade strains i) have since then not been replaced in the laboratory line and ii) are more frequent in nature might also reflect that CpDV-1 clade could be more adapted to their hosts and more competitive.

Viruses are generally seen as pathogens and most of the research focuses mainly on medically or economically important pathogenic viruses. Therefore our understanding of Parvovirus evolution is biased towards pathogenic viruses -such as canine parvovirus, porcine parvovirus (Shackelton *et al.*, 2005; Hoelzer *et al.*, 2008; Firth *et al.*, 2009; Streck *et al.*, 2011). As virus evolution and population dynamics can also be influenced by the nature of their interactions with their hosts (Duffy *et al.*, 2008), considering viruses as symbionts of their hosts (including other possible host-virus interactions than strict antagonism) can deeply improve our understanding of virus and disease evolution as a whole. Overall our results on CpDV evolution showed no significant change in exponential growth and a r low genetic diversity compared to their relatively high substitution rate, high prevalence and vertical transmission (**Chapter 3**). Such characteristics might be pointing their persistence in their hosts instead of causing acute infections (Roossinck, 2011). Our current knowledge of the persistence on virus population dynamics is very limited (Roossinck, 2011). In this context CpDV-*Culex pipiens* system is a good model to study host-virus interactions and their evolution together both in nature and laboratory conditions. More generally, due to the ability of arthropods to host endosymbionts including vertically transmitted ones, the study of arthropod-virus systems can give valuable insights on the effect of this continuum of interactions on the virus population dynamics, and on the emergence of pathogenic or mutualistic viruses.

2.1.5 Acknowledgements

We would like to thank Vincent Robert for the samples collected in Armenia, Benoit Assogba for the samples from Benin and Beniamino Caputo for samples from Italy. We also thank Sandra Unal and Patrick Makoundou for technical support. Sequencing data were produced at the LabEX "Centre Méditerranéen de l'Environnement et de la Biodiversité" in the GENSEQ platform.

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Appendix

Figure.1 Recombination in CpDV. Bootscan plots of the significant recombination events within the concatenated NS1-VP dataset detected by RDP4 software. Y-axis represents the bootstrap support of each pair of sequences, x-axis shows their position in the alignment. Recombinant regions are shown in pink. Triangles indicate where NS1 region ends and VP starts approximately at 900 nucleotide position. Recombination break points coincided with this point in most of the samples, except Haiti 2 which showed a slightly different recombination pattern.

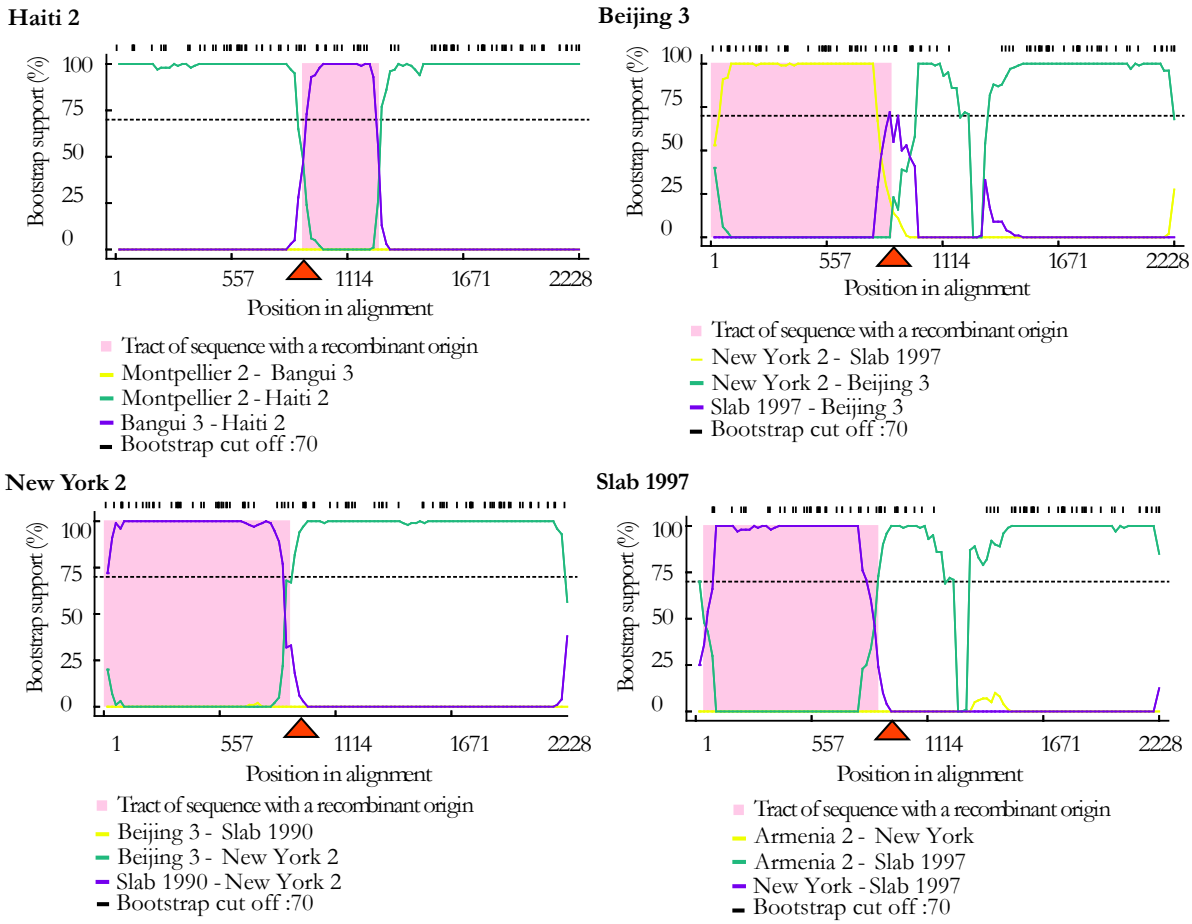

Figure. 2. Selection on NS1'. Subscript under each nucleotide represents how many variant had a given nucleotide. $N_{0.5}$ indicates double peaks. Information on only 25 sequences are shown after clonal sequences were removed. Numbers on the right side represents the nucleotide position within the sequenced fragment. Figure generated using classic.datamonkey.org. Codons under purifying selection are indicated in red boxes.

Figure. 3. Selection on VP. Subscript under each nucleotide represents how many variant had a given nucleotide. $N_{0.5}$ indicates double peaks. Information on only 25 sequences are shown after clonal sequences were removed. Numbers on the right side represents the nucleotide position within the sequenced fragment. Figure generated using classic.datamonkey.org. Codons under purifying selection are indicated in red boxes and one codon under diversifying selection is shown in green.

760
800
840
880
920
960
1000
1040
1080
1120
1160
1200
1240
1280
1320
1325

Figure 4. Phylodynamics of CpDV concatenated dataset including recombinant sequences. Ancestral locations (nodes) and collection locations (tips) are represented by the colours as given in the map. Posterior probability > 0.5 for the nodes are reported. X-axis shows the timeline for the divergence of the nodes and collection time for the tips.

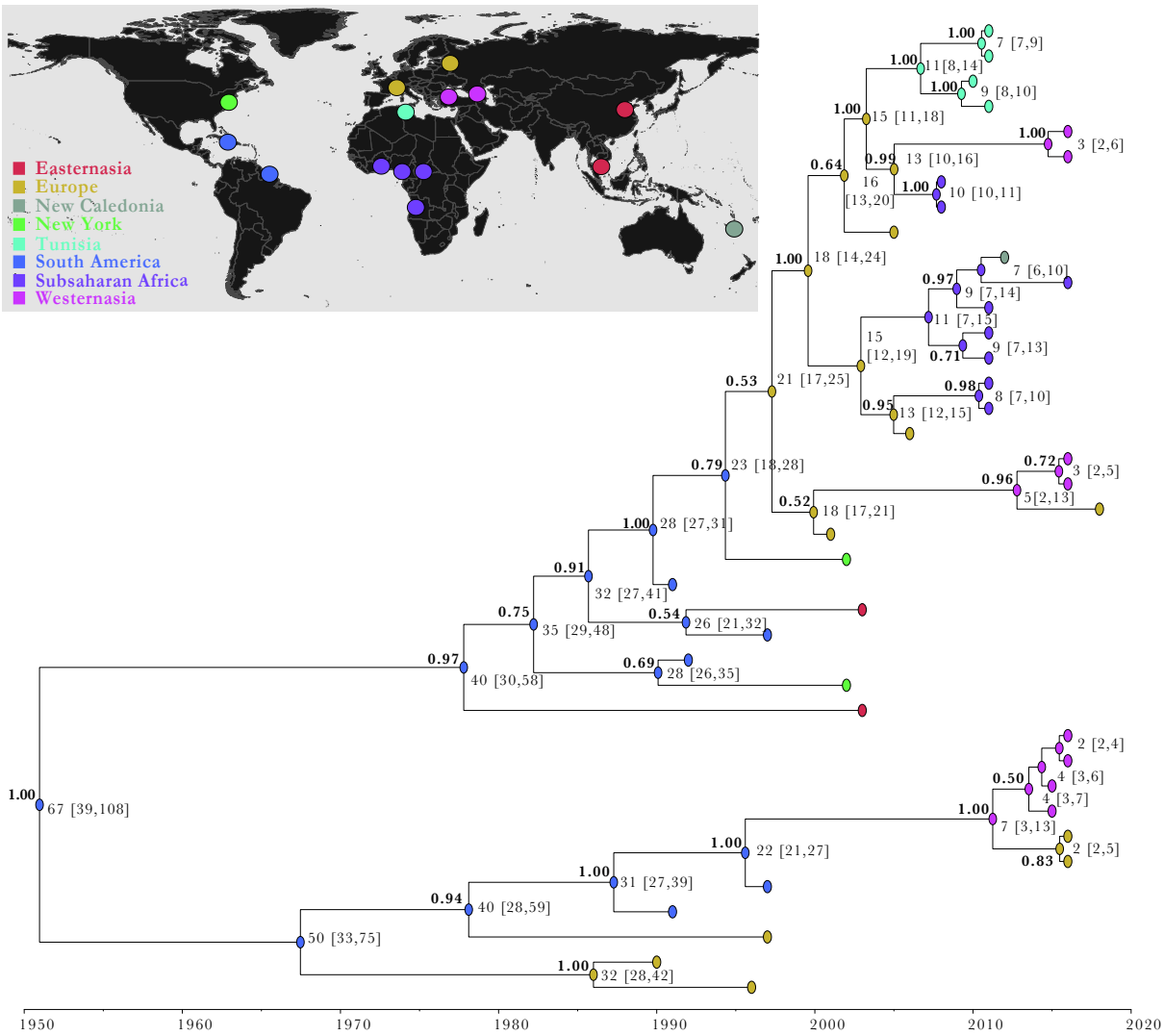

Figure 5. Population dynamics of CpDV estimated for partial NS1(A), VP (B) codons and concatenated dataset (C) using exponential growth model as implemented in BEAST. Y-axis represents the exponential growth line for the median growth rate through time and the 95% HPD intervals for this growth are shown as a solid area.

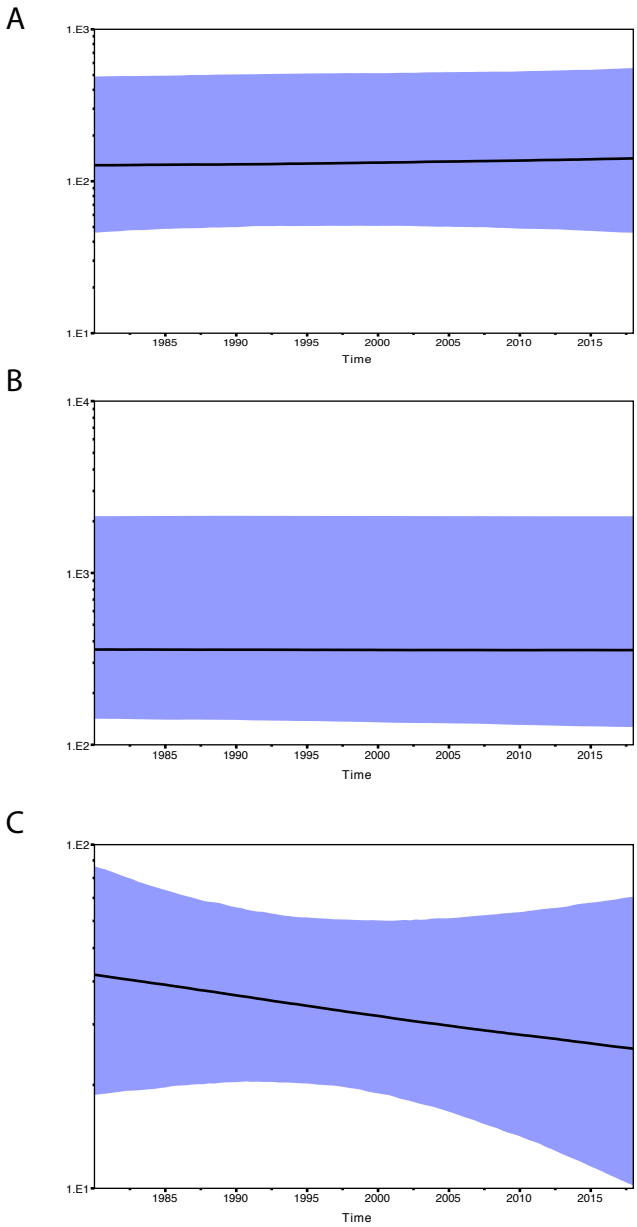

Figure.6. Phylodynamics of CpDV using partial NS1 (A) and VP (VP) codons. Ancestral states are represented by the colours as given in the map. Posterior probability > 0.5 for the nodes are reported. X-axis shows the timeline for the divergence of the nodes and collection time for the tips.

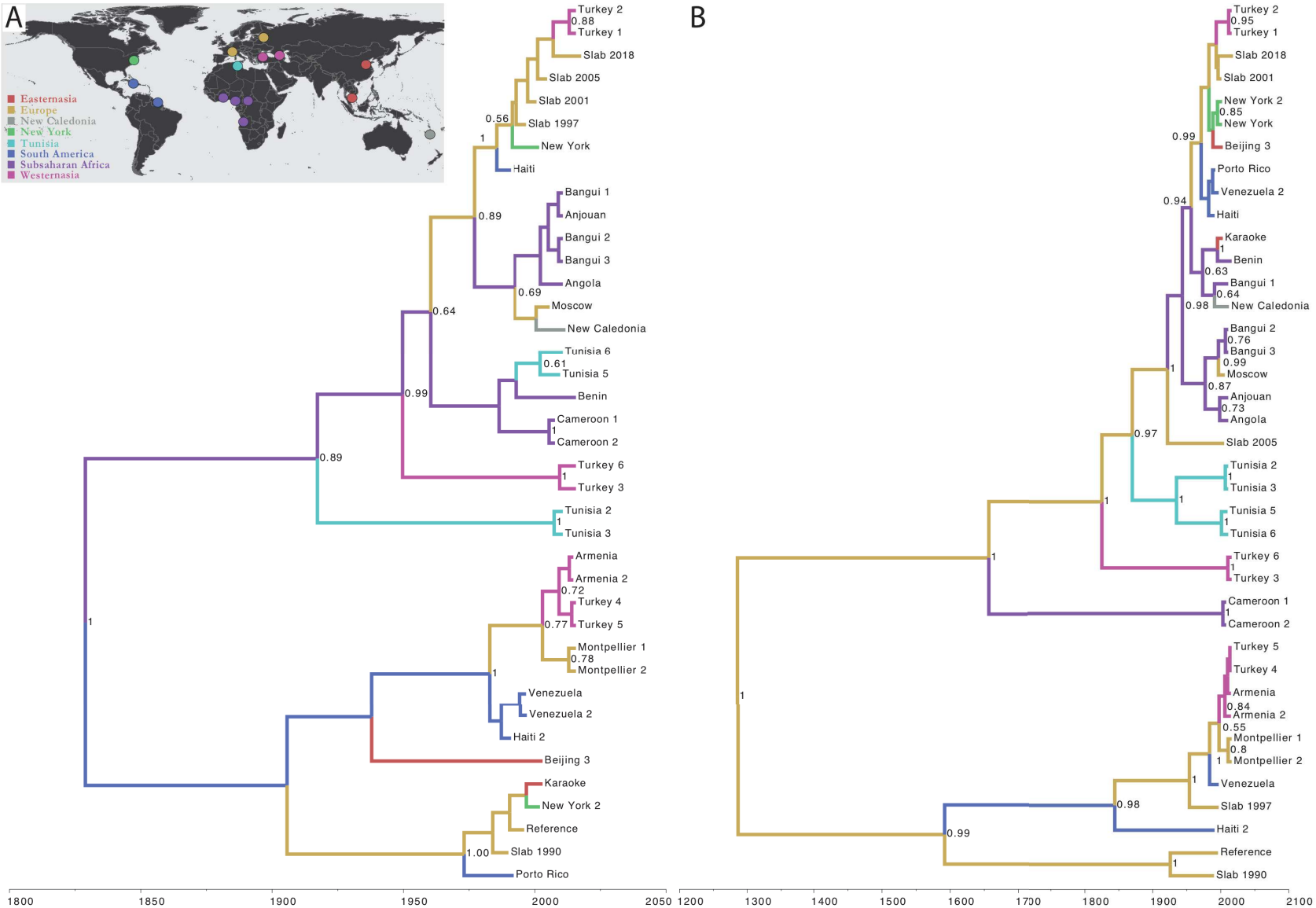

Table.1. Samples that have been used in the study. Collection country, population, coordinates, collection year, CpDV presence, total sample size (N) for a given population and the reference study that the samples were taken from are given.

	Country	Location	Latitude	Longitude	Collection Year	CpDV+	N	Reference
1	Angola	Lobito	-12.36	13.53	2011	0	24	Dumas et al.2013
2	Armenia	Am109VR	41.22	44.85	2016	9	9	This study
3		Am51VR	39.20	46.44	2016	10	10	This study
4	Benin	Ouidah	6.36	2.08	2016	4	15	This study
5	Brazil	Brasilia	-15.77	-47.92	1993	1	22	Guillemaud et al. 1997
6		Recife	-8.05	-34.88	1995	0	18	Duron et al. 2005
7	BurkinaFaso	Bobo Dioulassabo	11.17	-4.29	2011	0	15	Dumas et al. 2013
8	Cameroon	Yaounde	3.86	11.51	2008	15	22	Dumas et al. 2013
9	Central African Rep.	Bangui	4.36	18.55	2011	9	24	Dumas et al. 2013
10	China	Karaoke	27.29	119.92	2003	12	12	Duron et al. 2005
11		Taiqui	34.10	116.29	2003	0	24	Dumas et al. 2013
12		Zhuchang	26.8	105.11	2003	3	30	Dumas et al. 2013
14		BJBJT	35.98	119.39	2003	8	10	Duron et al. 2005
15	Comoros	Anjouan	40.11	-8.26	2011	0	24	Dumas et al. 2013
16	Cotedivoire	BouA_1	7.68	-5.01	2016	1	1	This study
17	Cyprus	Nene	34.75	32.40	2004	2	8	Duron et al. 2005
18	France	m73	43.69	3.88	2016	30	31	Altinli et al 2018
19	French Polynesia	Moorea	-17.52	-149.82	1992	3	21	Pasteur et al. 2005
20	Greece	Thessaloniki	40.64	22.93	2010	1	20	Dumas et al. 2013
21	Haiti	Gonaives	19.44	-72.68	1991	6	24	Dumas et al. 2013
22	Italy	Latina	41.46	12.90	2011	4	24	Dumas et al. 2013
23		Roma	41.89	12.51	2011	0	6	Dumas et al. 2013
24		Rome	41.89	12.51	2017	55	56	This study
25	Mayotte	Acoua	-12.80	45.20	2013	2	24	Poquet et al. 2013
26		Bouyouni	-12.8063889	45.20	2013	2	24	Poquet et al. 2013
27		Dembeni	-12.80	45.20	2013	0	24	Poquet et al. 2013
28		Kahani	-12.80	45.20	2013	1	24	Poquet et al. 2013
29		Kawen	-12.80	45.20	2013	4	24	Poquet et al. 2013
30		Mramadodou	-12.80	45.20	2013	5	23	Poquet et al. 2013
31		Mtsamoudou	-12.80	45.20	2013	0	58	Poquet et al. 2013

32		Mtsangamouji	-12.80	45.20	2013	1	24	Poquet et al. 2013
33		Sada	-12.80	45.20	2013	0	23	Poquet et al. 2013
34		Tsounozou	-12.80	45.20	2013	2	31	Poquet et al. 2013
35	**New Caledonia**	Poindimie	-20.93	165.34	2012	6	25	Dumas et al. 2013
36	**Pakistan**	Lahore	31.54	74.34	1988	0	6	Beyssat-Arnaouty et al. 1989
37	**Portugal**	Bodes	38.56	-27.91	1994	0	2	Duron et al. 2005
38		Ferreira	41.26	-8.39	1993	0	9	Duron et al. 2005
39		Mitra	38.73	-9.14	1993	4	9	Duron et al. 2005
40		Praias	38.73	-9.14	1993	2	5	Duron et al. 2005
41	**Porto Rico**	Rico	18.46	-66.10	1992	17	24	Dumas et al. 2013
42	**Russia**	Krasnodar	55.74	37.60	2006	2	10	Dumas et al. 2013
43		Moscou	55.74	37.60	2006	1	10	Dumas et al. 2013
44		Volgograd	48.65	44.49	2006	1	11	Dumas et al. 2013
45	**Seychelles**	Mahe	-4.68	55.48	2011	6	23	Dumas et al. 2013
46	**Southafrica**	Bed	-32.31	27.65	1993	1	2	Dumas et al. 2013
47		BSQ	-32.31	27.65	1993	1	7	Weill et al. 2003
48		Killcare	-33.16	151.10	1993	1	2	Guillemaud et al. 1997
49		Wellington	-33.64	19.00	2011	5	7	Dumas et al. 2013
50	**Tanzania**	Arusha	-3.36	36.68	2011	0	22	Dumas et al. 2013
51	**Thailand**	Bangkok	13.75	100.50	2012	12	24	Dumas et al. 2013
52	**Tunisia**	Ariana	36.54	10.13	2011	18	24	Atyame et al 2015
53		Azib	37.13	9.56	2011	21	22	Atyame et al 2015
54		Beja_Gare	36.43	9.11	2009	0	19	Atyame et al 2015
55		Beja_Oued	36.43	9.12	2011	0	24	Atyame et al 2015
56		Bizerte	37.16	9.50	2011	20	24	Atyame et al 2015
57		Boussalem2	36.36	8.58	2011	18	24	Atyame et al 2015
58		Briss	37.02	9.53	2008	20	47	Atyame et al 2015
59		Buses	36.22	9.24	2009	0	15	Atyame et al 2015
60		Chardon	36.36	9.38	2011	3	24	Atyame et al 2015
61		Dougga	36.23	9.14	2011	4	17	Atyame et al 2015
62		El_Alia	37.09	10.02	2010	1	24	Atyame et al 2015
63		El_Battan	36.48	9.53	2009	0	24	Atyame et al 2015
			36.48	9.53	2011	24	24	Atyame et al 2015
64		El_Manar	37.017	9.52	2010	61	61	Atyame et al 2015
			37.01	9.52	2011	24	24	Atyame et al 2015
65		Elevage	36.33	9.00	2009	0	6	Atyame et al 2015
66		Font_Mjez	36.39	9.28	2010	33	33	Atyame et al 2015

67		Fontaine	36.32	9.24	2009	0	23	Atyame et al 2015
			36.32	9.24	2010	29	54	Atyame et al 2015
			36.32	9.24	2011	52	90	Atyame et al 2015
68		Goubellat	36.31	9.40	2011	24	24	Atyame et al 2015
69		Hamra	37.02	9.39	2011	19	23	Atyame et al 2015
70		Ichkeul	37.07	9.44	2011	24	24	Atyame et al 2015
71		Jedaida	36.48	9.56	2011	21	24	Atyame et al 2015
72		Khetmine	37.08	9.59	2010	0	24	Atyame et al 2015
73		Mateur	37.02	9.38	2010	0	23	Atyame et al 2015
74		Mjez_el_bab	36.39	9.36	2011	23	24	Atyame et al 2015
75		Nofrancaoui	36.25	9.19	2011	8	14	Atyame et al 2015
76		Oued_Melah	36.35	9.30	2011	22	22	Atyame et al 2015
77		Pompe	36.54	10.13	2010	43	43	Atyame et al 2015
78		Ras_Jebel	37.13	10.07	2011	20	22	Atyame et al 2015
79		Ras_Rajel	36.57	8.52	2010	39	66	Atyame et al 2015
80		Riadh	36.50	9.58	2009	0	24	Atyame et al 2015
			36.50	9.58	2010	17	48	Atyame et al 2015
81		Si_Thabet	36.57	10.02	2010	20	20	Atyame et al 2015
82		Sloughia	36.32	9.24	2008	0	24	Atyame et al 2015
83		Tabarka	36.56	8.45	2010	13	16	Atyame et al 2015
84		Tebourba	36.52	9.5187	2011	20	21	Atyame et al 2015
85		Utique	37.04	10.00	2011	14	14	Atyame et al 2015
86		Utique_pont	37.02	10.02	2011	19	22	Atyame et al 2015
87		Zerga	36.4014	9.26	2011	23	24	Atyame et al 2015
88	**Turkey**	m1	40.62	29.17	2016	19	45	Altinli et al 2018
89		m11	41.38	41.43	2016	19	36	Altinli et al 2018
90		m12	41.49	41.53	2016	0	5	Altinli et al 2018
91		m16	41.38	41.57	2016	2	5	Altinli et al 2018
92		m17	41.36	41.68	2016	15	21	Altinli et al 2018
93		m18	41.39	41.69	2016	19	24	Altinli et al 2018
94		m19	41.36	41.66	2016	0	3	Altinli et al 2018
95		m21	41.37	41.62	2016	16	25	Altinli et al 2018
96		m25	41.31	41.35	2016	12	12	Altinli et al 2018
97		m26	41.31	41.34	2016	5	10	Altinli et al 2018
98		m27	41.32	41.30	2016	34	36	Altinli et al 2018
99		m3	40.60	29.20	2016	6	42	Altinli et al 2018
100		m32	40.89	39.71	2016	33	34	Altinli et al 2018

101		m33	41.83	32.71	2016	0	10	Altinli et al 2018
102		m34	41.99	35.09	2016	6	6	Altinli et al 2018
103		m35	41.88	32.99	2016	5	5	Altinli et al 2018
104		m37	41.74	32.38	2016	0	5	Altinli et al 2018
105		m4	40.64	29.09	2016	19	40	Altinli et al 2018
106		m42	41.93	34.58	2016	6	6	Altinli et al 2018
107		m45	41.41	32.08	2016	9	10	Altinli et al 2018
108		m46	41.35	32.09	2016	15	15	Altinli et al 2018
109		m47	41.45	31.82	2016	7	7	Altinli et al 2018
110		m5	40.94	29.30	2016	19	38	Altinli et al 2018
111		m52	41.28	31.42	2016	6	6	Altinli et al 2018
112		m54	41.07	30.96	2016	10	10	Altinli et al 2018
113		m55	41.07	30.84	2016	5	5	Altinli et al 2018
114		m6	40.94	29.30	2016	29	44	Altinli et al 2018
115		m61	40.688217	30.279797	2016	7	15	Altinli et al 2018
116		m64	39.795018	30.497299	2016	14	27	Altinli et al 2018
117		m65	41.613471	26.965631	2016	8	27	Altinli et al 2018
118		m66	41.673115	26.980916	2016	1	1	Altinli et al 2018
119		m67	41.673115	26.980916	2016	10	10	Altinli et al 2018
120		m69	41.663512	26.507806	2016	9	17	Altinli et al 2018
121		m7	40.979638	29.055797	2016	14	32	Altinli et al 2018
122		m70	41.845866	27.806536	2016	10	10	Altinli et al 2018
123		m9	39.873036	32.737012	2016	19	27	Altinli et al 2018
124		tr501	39.7098832	30.403559	2016	0	5	Altinli et al 2018
125	**UK**	Liv	53.40964	-2.907825	2007	1	2	Dumas et al. 2013
126		Willow	53.372494	-2.578072	2001	0	5	Duron et al. 2005
127	**USA**	NewYork_FT	40.797478	-73.776042	2002	4	10	Dumas et al. 2013
128		NewYork_Sewer	40.797478	-73.776042	2002	0	10	Dumas et al. 2013
129		Madeira	39.189836	-84.364155	2010	1	11	Dumas et al. 2013
130		EDIT	36.778259	-119.417931	2005	0	1	Guillemaud et al. 1999
131		Selax	36.778259	-119.417931	2005	0	1	Duron et al. 2005
132		Transp	36.778259	-119.417931	1975	0	1	Priester & Georghiou 1978
133	**Venezuela**	CimetierePrintemps	10.4679018	-67.4509141	1997	17	24	Dumas et al. 2013
134	**Vietnam**	HotToc	10.830359	106.691428	1995	6	10	Pasteur et al. 2001
135		Saigon	10.76262	106.660172	1995	7	8	Pasteur et al. 2001
136	**Zimbabwe**	Harare	-17.827047	31.067834	2001	1	7	Weill et al. 2003

Table.2. Primers and PCR protocols for CpDV detection (NS2) and the sequencing for partial NS1, VP codons

NS1'			
step1		step2	
primer F	primer R	primer F	primer R
CpDV_1681F	CpDV_3087R	CpDV_1681F	CpDV_2648R
CACCACCACGGA CTGGTA	TTGGGGAGCACAA GATCC	CACCACCACGGA CTGGTA	TAAGACGGGAGT TCTGGTTACATG
PCR program		PCR program	
	95C 5min		95C 5min
35 cycles	94C 30sec	35 cycles	94C 30sec
	60C 30sec		58C 30sec
	72C 2min		72C 2min
	72C 10 min		72C 10min
sequencing			
CpDV_1681F & CpDV_2648R			

diagnostic test- NS2	
primer F	primer R
Nsq1F	CpdvR2
CATTGGAGGAGAAAGT TGGAA	TCCCCATTATCTTGCTG TCG
PCR program	
	95C 5min
33 cycles	94C 30sec
	56C 30sec
	72C 45sec
	72C 5min

VP			
step1		step2	
primer F	primer R	primer F	primer R
CpDV_3570F	CpDV_5281R	CpDV_3570F	CpDV_4959R
ATACCCAACCTCC CCAGGAA	TAGCTAATGCTGG TGCAAAGTGG	ATACCCAACCTCC CCAGGAA	GTATGTCTTCAAC ACTTCGTCGTC
PCR program		PCR program	
	95C 5min		95C 5min
35 cycles	94C 30sec	35 cycles	94C 30sec
	57C 30sec		58C 30sec
	72C 2min 45sec		72C 2min
	72C 10min		72C 10min
sequencing			
CpDV_3570F & CpDV_4959R			

Chapter 3:
CpDV horizontal and vertical transmission

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3.1 CpDV persistence in the laboratory lines

Observation of high prevalence of CpDV worldwide (**Chapter 2**) brought up several questions: How they can persist in natural populations? How do they transmit from host to host and what is the nature of their relationship with their hosts?

CpDV was isolated for the first time from mosquito lines kept in the insectaries of ISEM, following an episode of high mortality during 4th instar larvae. Even though at the time it was discovered, CpDV has been shown in really high amount in the larvae and clear signs of densonucleosis were observed, it was not possible to replicate this infection experimentally to date. Hence the nature of the interactions between CpDV and its host is still not clear.

To investigate CpDV-host interactions and whether there is a CpDV-*Wolbachia* interaction within host, experimental infections were necessary. However, CpDV specific diagnostic PCR test, based on the amplification of partial NS2 gene revealed the presence of CpDV in all the lines that were kept in the insectary. CpDV infection seemed to be mostly in low amounts, displaying weak PCR bands, nevertheless seemed to persist. Further investigation of adults and larvae from some of the insectary lines, showed i) CpDV presence both in larvae and adults, ii) larvae were 40% more likely to be CpDV positive (20-54%, **Appendix A** Figure 1), iii) adult males and females displayed similar CpDV prevalence (**Appendix A**, Figure 2). Attempts to establish CpDV-free mosquito lines by separating females or bleaching egg rafts failed.

3.2 Experimental Infections

There were two major problems on the way to carry on with the experimental infections i) the lack of CpDV free lines and the unpredictable nature of the persistent infection, ii) low amount of CpDV infection in the transinfected cells.

Despite these problems, we have conducted pilot studies for experimental infections were using two common infection techniques i) using crushed infected larvae (**Appendix A**, Figure 3) ii) using CpDV positive cell cultures (Appendix A, Figure 4). While the results looked promising with the latter, it was not possible to tackle all of these problems during the course of my PhD.

However, we have managed to improve CpDV cell transinfection and isolation protocols, which made it possible to test synthetic CpDV and natural CpDV (isolated from Ich13 lines) both on *Ae. aegypti* and *Cx. pipiens* larvae. First trials showed that CpDV (synthetic with reference genome or isolated current variant) did not cause any mortality in *Ae. aegypti* larvae. Interestingly, in *Cx. pipiens* larvae synthetic CpDV with ancestral genome caused higher mortality than negative control while larvae survived better than negative control when the isolated current variant added. If true these results could suggest either that synthetic CpDV is more infectious than the natural one or that the pathogenic CpDV has been replaced by a less infectious variant in the insectary populations. Although the effects of co-infection might be at play due to the already established persistent CpDV infection in the tested larvae (we would be testing persisting CpDV infection + synthetic CpDV vs. persisting CpDV infection + natural isolate), these results looked promising.

Impossibility of cleaning the lines from CpDV after bleaching egg rafts, highlighted the possibility of transovarial vertical transmission of CpDV. Therefore, we have tested vertical transmission of CpDV in naturally infected *Cx. pipiens* mosquito lines and investigated the effect of their endosymbiotic bacteria, *Wolbachia*.

3.3 Vertical transmission of *Culex pipiens* Densovirus

This study has been submitted for review as: **Altinli, M.,** Soms J., Ravallec M., Justy F., Bonneau M., Weill M., Gosselin-Grenet A.S., Sicard M. **Sharing cells with *Wolbachia*: the transovarian vertical transmission of *Culex pipiens* densovirus.**

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

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

3.3.1 Introduction

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

Mosquito densoviruses (MDVs) have been described in natural populations (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000; Ng *et al.*, 2011), laboratory colonies (Jousset *et al.*, 2000) and established mosquito cell lines (Jousset *et al.*, 1993; O'Neill *et al.*, 1995). The first described mosquito densoviruses were *Aedes aegypti* densovirus (AeDV) (Lebedeva *et al.*, 1972) and *Aedes albopictus* densovirus (AalDV) (Jousset *et al.*, 1993). Thereafter many other MDV strains, namely *Toxorhynchites amboinensis* densovirus (TaDV), *Haemagogus equinus* densovirus (HeDV), *Ae. aegypti Thai strain* densovirus (AThDV), *Ae. Peruvian densovirus* (APeDV) have been identified (O'Neill *et al.*, 1995; Kittayapong *et al.*, 1999; Rwegoshora *et al.*, 2000; Ledermann *et al.*, 2004). Studies on MDV pathogenicity and transmission modes have generally shown a dose and age dependent infectivity

and virulence (Barreau *et al.*, 1996; Kittayapong *et al.*, 1999; Ledermann *et al.*, 2004; Rwegoshora and Kittayapong, 2004; Becnel and White, 2007). Vertical transmission of these viruses has only been observed when using sub-lethal doses that reduced the adult mosquitoes' longevity and fertility but did not kill them (O'Neill *et al.*, 1995; Barreau *et al.*, 1996, 1997; Kittayapong *et al.*, 1999; Rwegoshora and Kittayapong, 2004; Becnel and White, 2007). In contrast, *Anopheles gambiae densovirus* (AgDV), a recently discovered MDV, which exhibits a negligible pathogenic effect on its host *A. gambiae* mosquitoes (Ren *et al.*, 2008, 2014) has been shown to replicate in its host and to be vertically transmitted to the offspring highlighting its potential as transducing agents for mosquito control (Ren *et al.*, 2008; Suzuki *et al.*, 2014).

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

Two decades after its first isolation from *Cx. pipiens* laboratory colonies, due to an unusually high larval mortality, we detected the presence and persistence of CpDV in all life stages of seemingly healthy *Cx. pipiens* lines by a PCR diagnostic test (data not shown). This long-term persistence of CpDV in laboratory colonies through generations certainly relies on an efficient transmission. Transmission modes are generally categorized as i) horizontal, transmission from host to host via the environment, ii) vertical transmission from the host parent to offspring, iii) mixed-mode, both horizontal and vertical transmission (Ebert, 2013). Transmission modes have long been investigated in many symbiotic systems especially to understand their impact on virulence evolution (Ewald, 1983, 1987; Alizon *et al.*, 2009; Cressler *et al.*, 2016; Le Clec'h *et al.*, 2017). Empirical studies mostly showed that increased horizontal transmission led to increased virulence, while dominance of vertical transmission mode mostly reduced the virulence [reviewed in (Cressler *et al.*, 2016)]. Therefore transmission modes can have a major impact on the dynamics of virus infection, their persistence and spread in populations.

To understand CpDV infection dynamics, especially its maintenance through generations, we investigated the ability of *Cx. pipiens* mosquitoes to transmit CpDV vertically. *Cx. pipiens* are all naturally infected with *Wolbachia* strains belonging to one of the five genetically distinct phylogenetic group *w*PipI to *w*PipIV (Atyame et al. 2011). These *w*Pip strains induce a wide diversity of cytoplasmic incompatibility phenotypes in their mosquito host (Atyame et al. 2014). In addition to their ability to manipulate host reproduction to benefit their own vertical transmission (Stouthamer *et al.*, 1999; Werren *et al.*, 2008; Sicard *et al.*, 2014), *Wolbachia* are also known to interact with several of their hosts' viruses (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Zug and Hammerstein, 2015). While putative difference in protective ability between the *w*Pip groups identified in *Cx. pipiens* has not yet been investigated, a general protection by *w*Pip has been shown against West Nile Virus infection (Micieli and Glaser, 2014). To date, no *Wolbachia*-mediated "protection" against DNA viruses have been shown in any insects. Contrarily, one study demonstrate that *Wolbachia* makes their lepidopteran

host more susceptible against a Baculovirus (Graham *et al.*, 2012).

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

3.3.2 Results

3.3.2.1 CpDV presence in ovaries and eggs

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

To confirm the presence of virus particles -and not only of the viral genome (*i.e.* as detected by qPCR)- and to investigate their localization in the ovaries, we dissected ovaries from 6 days old Harash females and performed immunolabelling of CpDV using an anti-capsid antibody for confocal observation (Figure 2 and **Appendix B** Figure 1). The labelling of CpDV was mainly observed in follicular cells surrounding the follicles (Figure 2A-B-C) and occasionally in some muscle cells of the sheath enclosing the ovariole (Figure 2D). In both cell types, the labelling is mainly nuclear with some dots present in the cytoplasm of follicular cells

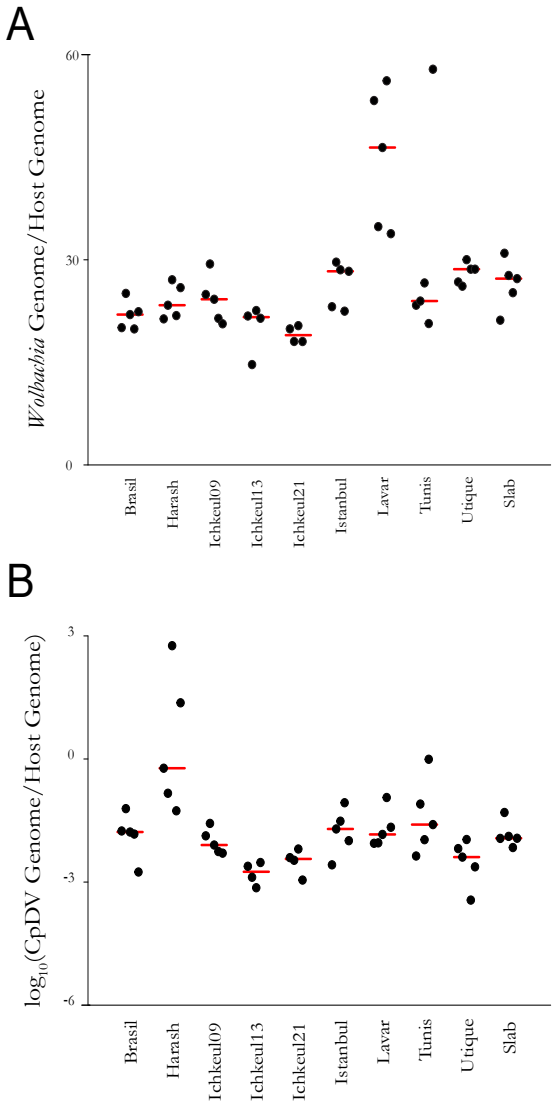

Figure 1. The two symbionts *Wolbachia* and CpDV coexist in *Cx. pipiens* ovaries of ten laboratory lines *Wolbachia* amount **(A)** Ovaries from three females per line were dissected and pooled. *wsp* copy number/*ace-2* copy number was quantified for 5 pools per line by qPCR assays. CpDV amount **(B)**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

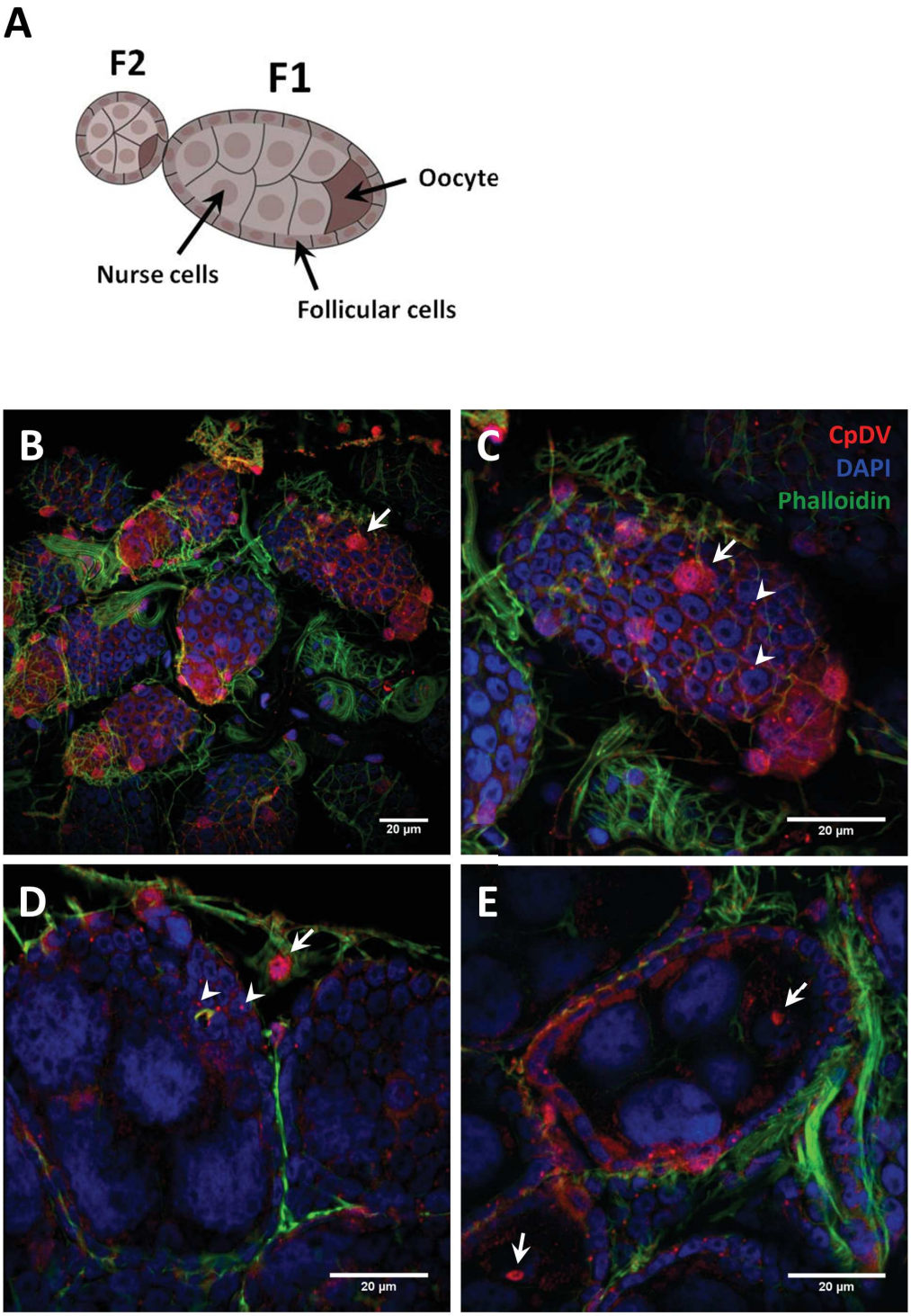

Figure 2. Immunolabeling of CpDV in *Cx. pipiens* ovaries. Structure of a *Cx. pipiens* ovariole **(A)**. The mosquito ovaries are paired and composed of functional units called ovarioles, containing primary (F1) and secondary follicles (F2) (Clements, 1992). Each primary follicle contains seven nurse cells and one oocyte, both surrounded by follicular epithelium. The secondary follicle is composed of undifferentiated cells surrounded by follicular epithelium. The secondary follicle will turn into a primary follicle after oviposition of the oocyte contained in the previous primary follicle (Raikhel and Lea, 1983). The sheath that enclosed each ovariole is a thin membrane with an outer muscle layer composed of isolated cell bodies interconnected by muscle fibers. Confocal images of ovaries isolated from 6 days old *Cx. pipiens* females (**B, C, D, E**). Immunolabeling of CpDV (in red) was performed with an anti-capsid antibody, actin was labeled with phalloidin-FITC (green) and nuclei with DAPI (blue). Arrows show CpDV-infected cells and arrowheads point cytoplasmic dots of CpDV labelling. Images of the surface of an ovary and an ovariole (composed of a primary and a secondary follicles) are shown in **B** and **C**, respectively. Internal views of primary follicles are shown in **D** and **E**.

(Figure 2B to E). Within the follicles, the labelling of CpDV in the nurse cells was not as apparent and rather similar to the background immunofluorescence (Figure 2D-E). Although we cannot entirely exclude a lack of permeability and accessibility of the nurse cells to the antibodies, a clear labelling was nevertheless observed in a well-defined area of the nucleus of the oocyte (Figure 2E). As all the *Cx. pipiens* lines were infected, the specificity of the CpDV labeling was tested on ovaries isolated from PCR tested CpDV-free *Ae. albopictus* mosquitoes and no labelling was detected (**Appendix B** Figure 2).

To examine the CpDV replication, which takes place in the nucleus, we prepared ultrathin sections of ovaries and analyzed the nuclear ultrastructure with transmission electron microscopy (TEM) (Figure 3). Although no unequivocal densonucleosis was observed, follicular and nurse cells showed hypertrophied nuclei (Figure 3A and G). Intranuclear structures that could be interpreted as viral factories were observed in several follicular cells (Figure 3B), nurse cells (Figure 3E) and oocytes (Figure 3H). Particles with the expected diameter of CpDV (about 25 nm) were also present in the nucleoplasm (Figures 3C and F).

To confirm the presence of viral particles in ovaries, we prepared filtered homogenate of the full organ for observation using TEM. Micrographs revealed the presence of both icosahedral empty capsids and CpDV virions with the expected diameter (Figure 3I).

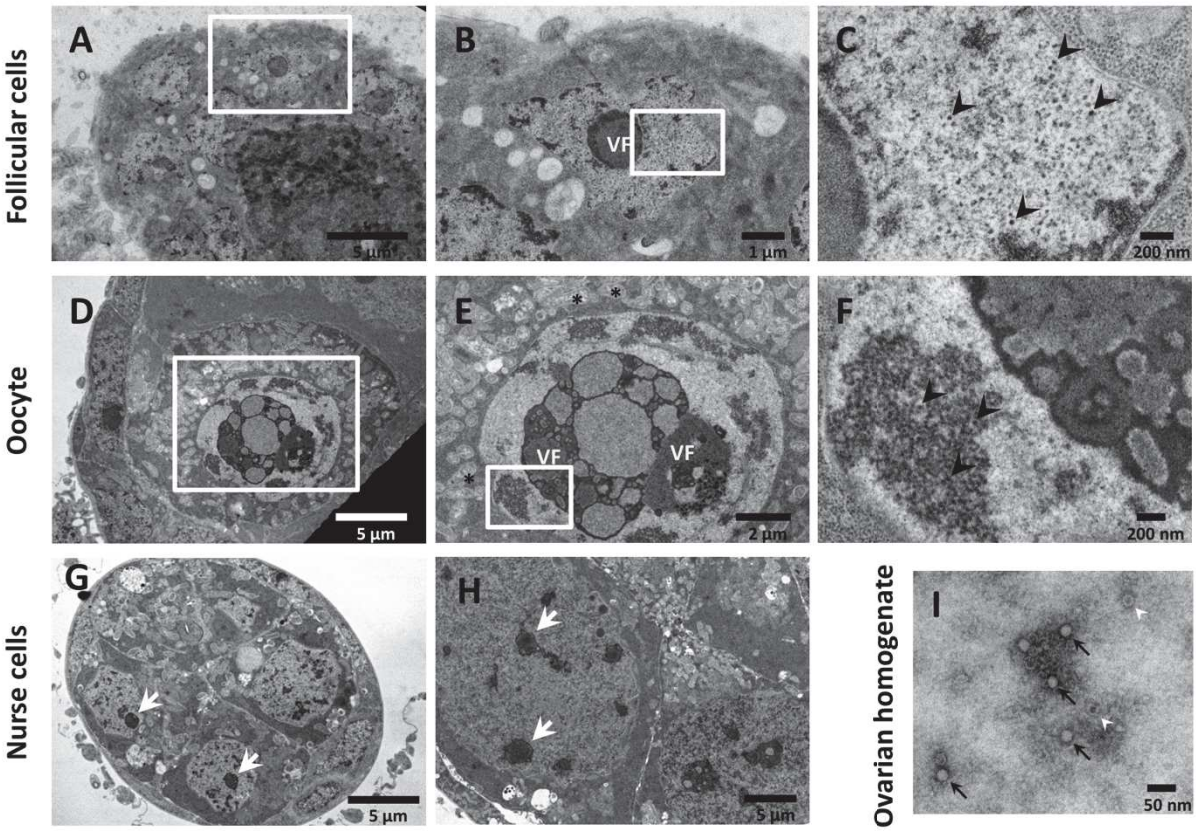

Figure 3 Ultrathin sections of ovaries isolated from 6 days old females (Harash line) (A to H). Large views of an ovariole (primary follicle) in **A, D** and **G** (bar scale: 5 µm). Ultrastructure of the hypertrophied nuclei and putative virus factories in a follicular cell (**A** and **B**), in an oocyte (**D** and **E**) and in a nurse cell (**G** and **H**). Zooms in the nucleoplasm of the follicular cell (**C**) and in the oocyte (**F**) show particles with the expected size of CpDV (25 nm). Virus particles observed in a filtered homogenate of ovaries (**I**). Arrows show icosahedral virus particles and arrowheads point empty particles with the expected size of CpDV (bar scale: 50 nm).

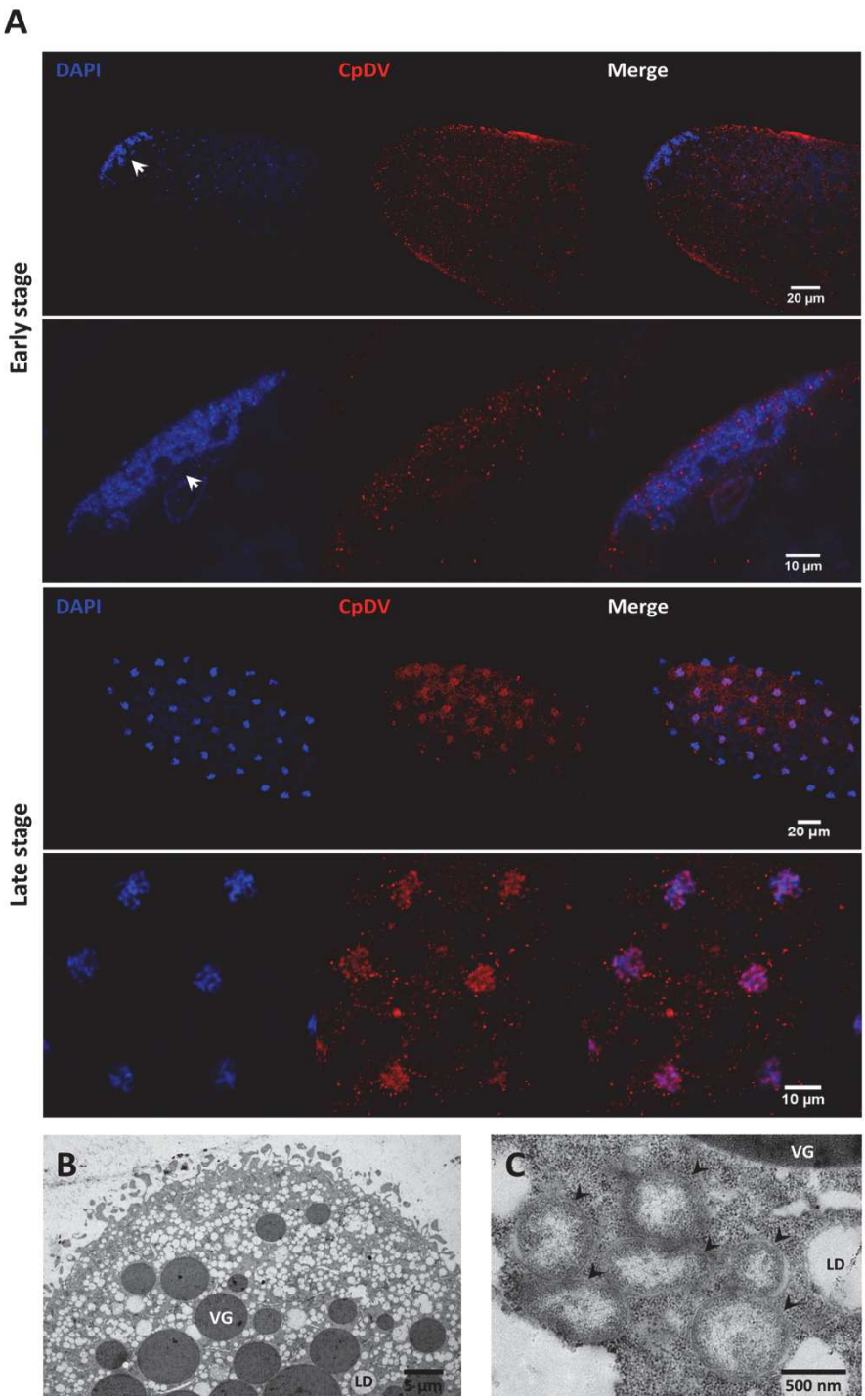

Figure 4. Localization of CpDV in *Cx. pipiens* eggs. Confocal images of dechorionated eggs from *Cx. pipiens* females (Harash line) (**A**). CpDV (in red) were immunolabeled with an anti-capsid antibody; *Wolbachia* and nuclei (in blue) were labeled with DAPI. Top panel shows eggs in the first hour after laying (early developmental stage) and bottom panel eggs 2 hours after laying (late developmental stage). The second part of the Top panel is a zoom of one egg pole in the first hour after laying; the second part of the Bottom panel is a zoom in the egg cytoplam. Arrows show *Wolbachia* cells at one egg pole. Electron micrographs of ultrathin sections of dechorionated eggs (**B** and **C**). In **B**, a large view of an early egg pole (bar scale: 5 µm), showing the accumulated reserves required for the embryo development (VG, vitellin granule; LD: lipid droplet). In **C**, a zoom (bar scacle: 500 nm) inside the cytoplasm of the early egg showing numerous *Wolbachia* (arrowheads). The virus particles cannot be distinguished from the ribosomes because of their similar size.

The same experiments were performed on freshly laid dechorionated eggs from Harash females (Figure 4). Immunolocalization of CpDV and DAPI staining of *Wolbachia* cells at early stage of the embryo development showed a *Wolbachia* localization especially in the poles, and a CpDV

localization both inside and in the periphery of the eggs suggesting no co-localization of these two (Figure 4A, Top Panel). At the syncytial blastoderm stage, CpDV were co-localized with the nuclei, presumably starting viral replication (Figure 4A, Bottom Panel). *Wolbachia* were easily detected in the cytoplasm in the ultrathin sections of fertilized eggs even at an early stage of development (Figure 4C). However, at the early stage of development nuclei were rare, meaning CpDV could only be observed in the cytoplasm where the ribosomes are also present (Figure 4C). As ribosomes and CpDV have the same diameter, it is not possible to firmly distinguish them (Figure 4C).

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

To assess the effect of *Wolbachia* (strains and density) on the amount on CpDV in the ovaries, we have established mosquito lines, with a backcrossing protocol, with the same host nuclear genetic background but different *Wolbachia* strains (BcTunis, Slab genetic background harbouring *w*Pip I from Tunis line; BcHarash, Slab genetic background harbouring *w*Pip IV from Harash line, Table 1). We then quantified the amount of *Wolbachia* and CpDV in these lines as well as in Harash, Tunis and Slab lines (Figure 5). *Wolbachia* amount in the ovaries was not significantly different between the *Cx. pipiens* lines no matter what the *Wolbachia* strain and host genetic background were (*Wolbachia* strain: glm, df=2, dev=17.703, p=0.875; host genetic background: df=1 dev=0.034, p=0.981, Figure 5A). Indeed, there was no significant effect of the *Wolbachia* amount in the ovaries on CpDV density (lme, dev=0.001, df=1, p=0.9). However, *Wolbachia* strain (*i.e.* the genotype) infecting the mosquito lines explained a

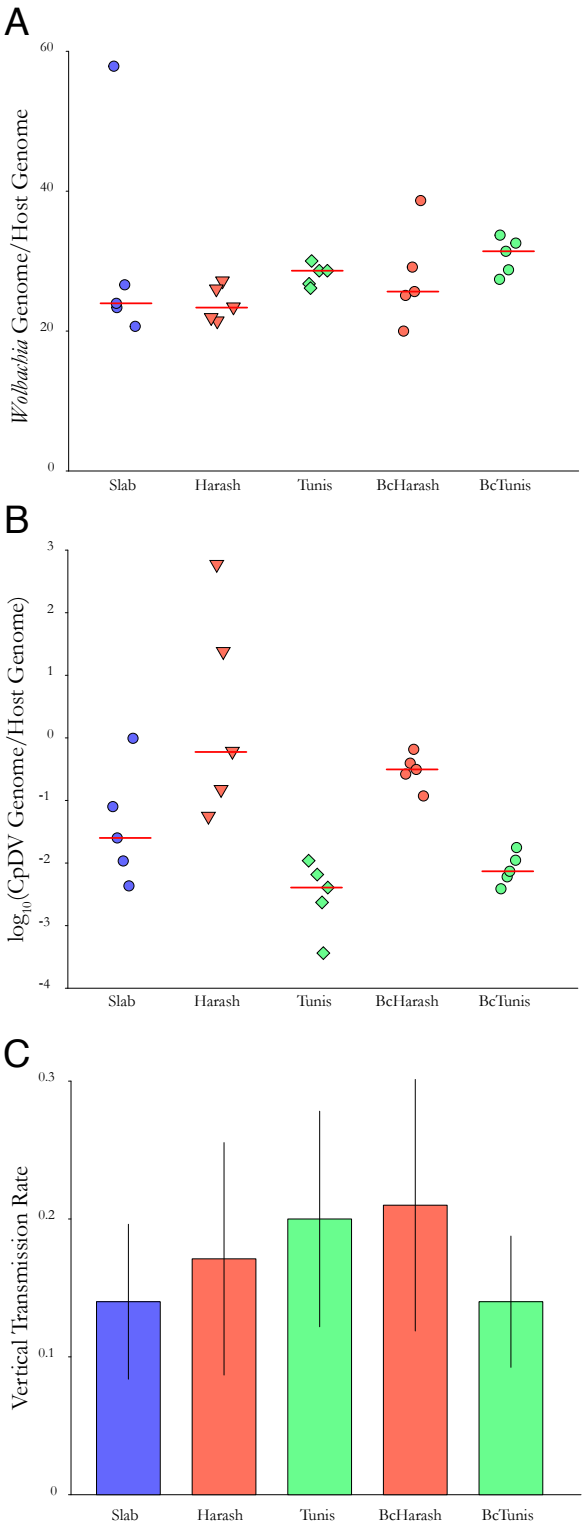

Figure 5 Slab line's genetic background was introgressed into Harash and Tunis lines, to establish BcHarash and BcTunis lines with the same genetic background as Slab (shown in circles) and same *Wolbachia* strain as Harash (shown in orange) or Tunis (shown in green) respectively. *Wolbachia* amount in ovaries (A). was not significantly different between the lines no matter what the *Wolbachia* strain (df=2, dev=17.703, p=0.87) and host genetic background were (df=1 dev=0.034, p=0.981). CpDV amount in ovaries (B) from three females were dissected and pooled. 5 pools per line have been tested by qPCR assays to quantify CpDV and *Wolbachia* amount. Mixed effect linear model showed no effect of *Wolbachia* amount or genetic background on the CpDV amount in the ovaries (*Wolbachia* amount dev=0.001, df=1, p=0.9, genetic background: dev=1.602, df=2, p= 0.449). Wolbachia strain, however significantly affected the CpDV amount (dev=12.236, df=1, p<0.001). CpDV vertical transmission rate (C) Females from each line were collected 5 days after their blood meal and isolated to lay eggs. Resulting L1 offspring was tested for CpDV prevalence and vertical transmission rate was calculated per female. A binomial generalized mixed effect model was fitted with host genetic background, *Wolbachia* strain as well as CpDV and *Wolbachia* amount in females' bodies as fixed effects and replicates as random effects. None of these variables had a significant effect on the vertical transmission rate.

significant part of the variance in the CpDV amount in the ovaries (lme, dev=12.236, df=1, p<0.001, Figure 5B) while the host genetic background had no significant effect on CpDV amount in the ovaries (lme, dev=1.602, df=2, p= 0.449; Figure 5B).

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

Using the same mosquito lines, we tested the vertical transmission of CpDV. For this, we let 10 females per line to lay eggs in isolation. To estimate a vertical transmission rate per female mosquito, both females and 10 L1 stage larvae per female were collected and tested for CpDV presence. All the mothers were CpDV positive. CpDV were transmitted to an average of 17% of the offspring (n=49, 0.172 ± 0.0317, Figure 5C). However, the vertical transmission rate highly diverged between individuals ranging from 0% (n=19 out of total 49 checked) to 90% (n=1, out of total 49 checked). This inter-individual variability was not significantly explained neither by the host genetic background (df=2, dev=0.278, p= 0.870, Figure 5C), *Wolbachia* strain (df=1, dev=0.152, p= 0.696, Fig3C), *Wolbachia* amount (df=1, dev= -0.135, p=0.713, Figure 5C) nor by CpDV amount in the whole body of the parental females (df=1, dev=-0.180, p=0.672, Figure 5C).

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

As all the *Cx. pipiens* isofemale lines were stably infected with *w*Pip (Wo+), to test the effect of the *Wolbachia* absence on CpDV presence and vertical transmission, we treated our Slab, BcHarash, and BcTunis lines, all having the same genetic background, with tetracycline (TC) for three generations to cure *Wolbachia*. We quantified *Wolbachia* and CpDV in dissected ovaries and carcasses of Wo+ (before TC treatment) and Wo- females (after TC treatment), as well as females during the 1st and 3rd generation of TC treatment (Figure 6). There was no significant change between 1st and 3rd generations in terms of CpDV amount (t = -0.643, df = 25.918, p-value = 0.525, Figure 6B). We thus grouped them together in a "TC treatment' group for further statistical analyses and figures.

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

In non-treated Wo+ females, higher amount of CpDV were measured in ovaries compared to carcasses (Wilcoxon rank sum test, W = 168, p = 0.021, Figure 6B). In the carcasses, TC treatment did not cause any significant effects on CpDV amount (Figure 6B, Wilcoxon rank sum test with Bonferroni correction; Wo+ *vs.* during TC treatment, W= 132.5, p=0.08; Wo+ *vs.* Wo-, W= 100 , p=0.138; during TC treatment *vs.* Wo-, W=308, p=1). On the contrary, in the ovaries CpDV amount significantly decreased during the TC treatment (Dunnett's test with Bonferroni Correction; Wo+ vs. during TC treatment, t=2.302, p=0.025; Figure 6B). However, in Wo- lines (three generations after the end of the tetracycline treatment) CpDV amount increased and reached a level not significantly different from non-treated Wo+ lines (Dunnett's test with Bonferroni Correction; Wo+ *vs.* Wo-, t= 0.118, p=1).

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

CpDV vertical transmission decreased significantly from an average of 25% to an average of 13% during tetracycline treatment (Wilcoxon rank sum test with Bonferroni correction; Wo+ *vs.* TC treatment, W= 488, p= 0.004, Figure 7). Three generations after the end of tetracycline treatment, vertical transmission in Wo- lines stayed marginally lower than non-treated Wo+ lines (Wilcoxon rank sum test with Bonferroni correction; Wo+ vs. Wo-, W= 576.5, p= 0.05, Figure 7).

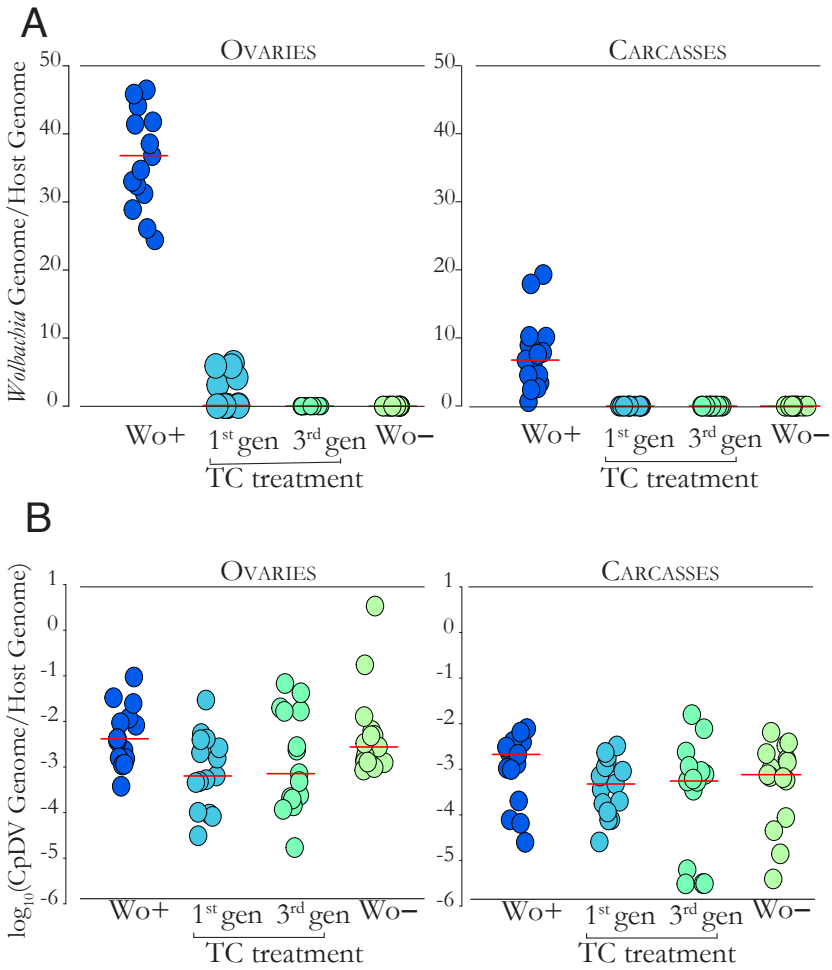

Figure 6 Effect of curing *Wolbachia* by tetracycline treatment on CpDV amount in *Cx. pipiens*. We have treated Slab, BcHarash and BcTunis lines with tetracycline (TC) for three generations and dissected the ovaries of 6 days old females. Dissections were performed on the non-treated mosquitoes (Wo+), first and third generation of mosquitoes during tetracycline treatment (during TC treatment) and three generations after the tetracycline treatment was finished (Wo-). Effect of tetracycline treatment on the amount of *Wolbachia* in ovaries and carcasses (A). *Wolbachia* amount decreased immediately starting from first generation during tetracycline treatment. There were no *Wolbachia* detected after first treatment generation neither in ovaries nor in carcasses. Effect of tetracycline treatment on CpDV amount (B). CpDV amount in the ovaries significantly decreased starting from the first round of tetracycline treatment but increased when tetracycline treatment stopped (Wo-)(Dunnett's test with Bonferroni Correction; Wo+ *vs.* during TC treatment, t=2.302, p=0.025; Wo+ *vs.* Wo-, t= 0.118, p=1). Carcasses had less CpDV compared to ovaries (Wilcoxon rank sum test, W = 168, p = 0.021). The effect of the treatment was not significant on the carcasses (Wilcoxon rank sum test with Bonferroni correction; Wo+ vs. during TC treatment, W= 132.5, p=0.08; Wo+ vs. Wo-, W= 100, p=0.138; during TC treatment vs. Wo-, W=308, p=1).

3.3.3 Discussion

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

The presence of CpDV capsids in the ovaries and eggs highlighted the possibility of vertical transovarial transmission. Transmission mechanisms of densoviruses are generally thought to be horizontal causing epizootics (Tijssen *et al.*, 2016). Nevertheless, vertical transmission has been shown both for some ambidensoviruses and brevidensoviruses. Interestingly, ambidensoviruses with different vertical transmission rates had different types of symbiotic interactions: while the 100% vertically transmitted ambidensovirus of *H. armigera* is mutualistic (Xu *et al.*, 2014), the *Myzus persicae* densovirus which is transmitted to 30-40% of the offspring has been shown to affect its hosts adversely (Van Munster *et al.*, 2003). In mosquitoes experimentally infected with brevidensoviruses, the

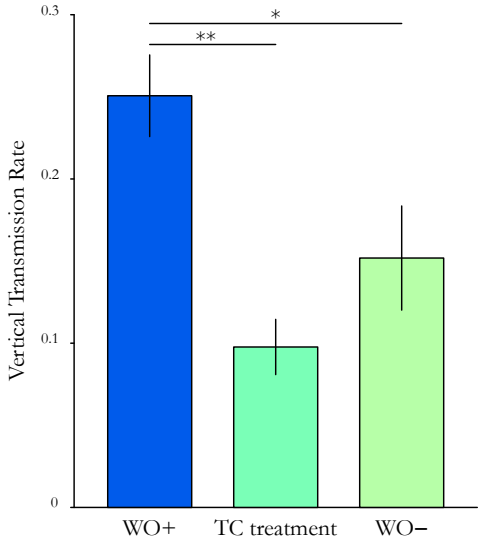

Figure 7 Effect of Wolbachia-curing tetracycline treatment on CpDV vertical transmission. In BcHarash, BcTunis and Slab lines, Females emerged from the larvae that underwent the first tetracycline treatment were isolated five days after bloodmeal to lay eggs. 10 females per line (total n=30), 10 L1 stage larvae per female were collected and tested for CpDV presence to estimate vertical transmission rate. This procedure was repeated for females emerged after the third generation of TC treatment and for the females Wo- (three generation after the end of tetracycline treatment). There was a significant decrease in vertical transmission during tetracycline treatment (Wilcoxon rank sum test with Bonferroni correction; Wo+ vs. during TC treatment, w= 488, p= 0.004). Three generations after end of the treatment the vertical transmission rate increased but still was marginally significant from the non-treated group (Wilcoxon rank sum test with Bonferroni correction; Wo+ vs. Wo-, w= 576.5, p= 0.05).

vertical transmission ranged between 28 to 62%: i) for *Ae. albopictus* densovirus, the vertical transmission rate ranged between 28-55% depending on the virus titer in parental females (Barreau *et al.*, 1997); ii) for *Ae. aegypti* densovirus vertical transmission ranged from 42% to 62% depending on the timing of the horizontal infection of the larvae (the larvae that were infected during L3 stage transmitted the virus less, as they became adults, than the larvae infected during L1 stage) (Suchman *et al.*, 2006), and iii) for *A. gambiae* densovirus vertical transmission was detected in 28% of larval offspring (Ren *et al.*, 2008). We showed that CpDV was transmitted to an average of 17% of the larval offspring in naturally infected *Cx. pipiens* lines. Even if the vertical transmission rate varied between individuals, this rate was quite stable (15%-21%) between the *Cx. pipiens*-*Wolbachia* lines tested through the different replicates. The importance of inter-individual variability in vertical transmission rate of CpDV revealed the complexity of the factors affecting vertical transmission. Individual differences in vertical transmission, for instance, could be due to when females have been infected by horizontal transmission (Suchman *et al.*, 2006), or whether they have been infected vertically or horizontally. Due to the lack of CpDV free lines, it was not possible to quantify the horizontal transmission or venereal transmission by experimental infections. Nevertheless, the infection rate changed from 17% at the L1 stage to 100% in tested mothers suggesting a high level of horizontal transmission.

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

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

To further investigate whether CpDV and *Wolbachia* had reciprocal interactions and whether CpDV could still be vertically transmitted in the absence of *Wolbachia*, we treated our lines with antibiotics. This treatment decreased both the amount of CpDV in the ovaries and the vertical transmission alike. However, following the end of the treatment CpDV amount in the ovaries increased in the *Wolbachia*-free lines. This suggested that this sudden decrease could be caused by either the direct influence of antibiotics on the host metabolism, or by the rest of the microbiota that putatively recovered in the generations following the end of the antibiotic treatment. Nevertheless, even though CpDV was able to be transmitted vertically in the absence of *Wolbachia*, the vertical transmission was lower in *Wolbachia*-free lines, suggesting their effect on CpDV transmission. Several endosymbiotic bacteria have been reported to modulate virus transmission. For instance Tomato yellow leaf curl virus' (a single stranded DNA plant virus) transmission is facilitated by GroEL protein secreted by its vector's endosymbiotic bacteria *Hamiltonella* (Gottlieb *et al.*, 2010). More recently, Jia et al. (2017) showed that Rice Dwarf virus (a plant double stranded RNA virus) binds to the envelopes of endosymbiotic bacteria *Sulcia* improving its vertical transmission in leafhoppers. Similarly, vertical transmission of *Wolbachia* could be hitchhiked by CpDV in *Cx. pipiens*. CpDV could potentially achieve this through the interactions of its capsids with bacterial surface glycans. Densovirus virions, as of many non-enveloped viruses, are constituted by a single major capsid protein with supersecondary structures which can act as interaction motifs (*e.g.* Single Jelly Roll) (Neu *et al.*, 2011; Huang *et al.*, 2014). These motifs can attach to bacteria's surface glycans which can serve as initial attachment factors for many viruses (Neu *et al.*, 2011; Huang *et al.*, 2014). Thus, CpDV capsids could potentially interact with a wide array of glycans, including *Wolbachia*'s surface glycans and such interactions could influence the infection outcome and lead to their co-vertical transmission (Kuss et al., 2011; Berger and Mainou, 2018).

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

3.3.4 Methods

3.3.4.1 Mosquito Lines

All the mosquito lines used in this study (Supplementary Table 1) have been maintained in insectary conditions (at 25 ± 2 °C and 75 ± 2% relative humidity and a 12:12 h photoperiod). During larval stage, they have been fed with a mixture of shrimp powder and rabbit pellets. Adults have been kept in 65 dm^3 cages and have been fed with a honey solution. Females were fed weekly with turkey blood using a Hemotek membrane feeding system (Discovery Workshops, United Kingdom).

Backcrosses

To test the effect of the *Wolbachia* genetic differences on CpDV amount and their vertical transmission, we introduced the cytoplasm of Tunis and Harash lines, along with their respective *w*Pip strains, into the Slab nuclear background as described by Duron et al (Duron *et al.*, 2012). With this backcrossing protocol we have established BcTunis and BcHarash lines with the same genetic background (over 97% of genome replacement of the original lines by Slab nuclear genome is estimated) and different *Wolbachia* types (*w*PipI and *w*PipIV respectively, Table 1). *w*Pip types were verified with a PCR/RFLP test on pool of larvae using pk1 primers as previously described (Altinli *et al.*, 2018).

Wolbachia free lines

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

3.3.4.2 Immunolabelling

Immunolabeling were performed on ovaries from 6 days old females and dechorionated eggs. The egg rafts were collected in 1-4 hours following egg laying. The egg- rafts were bleached (active ingredient, 9.6% of sodium hypochlorite) to separate the individual eggs and rinsed with distilled water. They were then fixed with 3.2% para-formaldehyde diluted in PBS-T (PBS 1X with Tween 0,02%) for 2 hours, and rinsed with PBS 1X. The chorion of each individual egg was removed manually with a needle under an optical microscope (Leica MZ 8).

All the samples were fixed for 1 h in 4% paraformaldehyde (PFA) and washed 3 times with PBS, then treated with 50 mM of NH_4Cl for 30 min to minimize autofluorescence. Ovaries and eggs were permeabilized with PBS containing 0.5% Triton and blocking was performed with 1% bovine serum albumin (BSA) in PBS for 1 h. Immunolabelling of CpDV were performed using a mouse primary JcDV anti-capsid antibody that co-reacts with CpDV capsids (Jousset et al., 2000), diluted at 1:300 in PBS with 0.2% Tween and incubated overnight à 4°C. Secondary antibodies (Alexa Fluor® 488 and 568; 1:500; Invitrogen) were used at 1:500 dilutions and incubated for 1 h (ovaries) or 6 h (eggs). Samples were incubated 30 min with Phalloidin-FITC (1:300; Sigma) to label actin cytoskeleton, and 5 min with DAPI (1 µg/ml; Invitrogen) to label nuclei and *Wolbachia*. Finally, labeled tissues and eggs were mounted in Dako (Sigma) on coverslips for observation with a Leica SPE confocal microscope equipped with suitable lasers and bandpass for the dyes (excitation 405 and emission 420-480 for DAPI; excitation 488 and emission 505-550 for Alexa Fluor® 488; emission 532 and emission 580-650 for TexasRed), using a 20X/0.55 HCX APO L W and a 40X/1.15 ACS APO oil objectives. We used the LAS AF (Leica) software to operate the microscope and all images were processed with ImageJ software.

3.3.4.3 Electron microscopy

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

Ultrathin sections were prepared from ovaries of 6 days old females and dechorionated eggs. Dissected ovaries and eggs were first fixed in 2.5% glutaraldehyde in PBS for 2 h at 4°C, and postfixed in 2% osmium tetroxide in the same buffer for 1 h at room temperature. Samples were then thoroughly rinced in distilled water, stained *en bloc* in 2% aqueous uranyl acetate solution for 2 h at room temperature, dehydrated in increasing-concentration ethanol baths, embedded overnight in Embed 812 resin (Electron Microscopy Sciences), and further polymerized for 2 days at 60°C. Then, ultrathin sections of approximately 100 nm, performed using an LKB Ultrotome III on copper grids, were counterstained with uranyless (Delta Instruments) and lead citrate and examined using a JEOL 1200 EX II transmission electron microscope operating at 100 kV and equipped with an Olympus camera (Quemesa model, 4008 x 2664 pixels) (MEA Platform, University of Montpellier, France).

3.3.4.4 Vertical transmission assays

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

Each L1 stage larva was individually collected to a 1.5 µl Eppendorf tube and crushed in 10 µl of STE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to obtain a crude DNA extraction. Two PCR reactions were conducted using these crude extractions, 1) to check the presence of CpDV using CpDVquantiF and CpDVquantiR primers and 2) to check the amplifiability of DNA using ace-2 gene primers, acequantidir and acequantirev (see above for sequences). The larvae crude extraction that did not allow any amplification in the latter were excluded from further analysis. PCR amplification conditions were as following: i) for CpDVquantiF and CpDVquantiR primers initial denaturation for 5 min at 94 °C, followed by 33 cycles of denaturation, annealing and elongation 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 min ii) for ace2quanti primers initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation, annealing and elongation respectively at 94 °C for 30 s, 52 °C for 30s, and 72 °C for 90 s, and a final elongation at 72 °C for 5 min.

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

3.3.4.5 Quantification of CpDV and *Wolbachia*

Ovaries were dissected from 6 days old female mosquitoes. Three ovaries were pooled together and 5 replicates were done per line. Qiagen extraction kit was used to extract DNA of ovary pools according to the manufacturer's instructions. DNA was eluted in 55 µl PCR gradient water. Negative controls were made to confirm that there was no contamination from the extraction kit and were always negative. Additionally to check the effect of *Wolbachia* and CpDV amount in the females on vertical transmission rate, DNA from the females

that laid eggs have been extracted using CTAB technique (Rogers and Bendich, 1989).

Real-time quantitative PCR (qPCR) was used to estimate the amount of *Wolbachia* and CpDV in each sample. qPCRs were performed using SYBR Green I Master (Roche) and analysed on a LightCycler 480 Instrument (Roche) using the LightCycler 480 software (version 1.5.1.62). On each DNA sample three PCR reactions were performed in triplicates using i) NS2 protein coding region specific primers to quantify CpDV (CpDVquantiF: 5'-CATTGGAGGAGAAAGTTGGAA-3', CpDV quantiR:5'-TCCCCATTATCTGCTGTCG-3'), ii) wsp specific primers to quantify *Wolbachia* (wolpipdir 5'-AGAATTGACGGCATTGAATA-3' and wolpiprev 5'-CGTCGTTTTTGTTTAGTT-GTG-3' (Berticat *et al.*, 2002)) and iii) *Culex* Ace-2 locus specific primers to quantify host genome copy as a reference (Acequantidir: 5'-GCAGCAC-CAGTCCAAGG-3', Acequantirev:5'-CTTCAGG-CCGTTCAAGTAG-3') (Weill *et al.*, 2000). Standard curves were created using dilutions of a pCR®4-TOPO- Vector (Invitrogen) containing a single copy of each *ace-2*, *wsp* and *ns2* gene fragments. Plates were designed to have all the compared groups of a biological replicate and prepared using Echo 525 Liquid Handler, Labcyte Inc. The amount of *Wolbachia* and CpDV were normalized with the amount of Ace-2 copies in order to obtain a number of virus or *Wolbachia* per cell.

3.3.4.6 Data Analysis

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

To analyse the effects of *Wolbachia* amount and diversity, on the amount of CpDV in the ovaries, one highly infected outlier from Harash line has been removed from the analysis and normality of the continuous response variable (log10 transformed amount of CpDV genome/ amount of host genome) has been checked with Shapiro–Wilk normality test. A linear mixed effect model is fitted with genetic background (Slab, Harash, Tunis), *Wolbachia* type (*w*Pip 1, *w*Pip 3 and *w*Pip 4) and *Wolbachia* amount as fixed effects and replicates as random effects using lme4 package (Bates, 2010). Homoscedasticity and normality of the residuals were checked using Breusch-Pagan test (ncvTest, in car package (Fox and Weisberg, 2011)) and Shapiro-Wilk test respectively. Likelihood ratio tests of the full model against the model without a given effect were used to obtain deviance and p-values.

To analyse the effects of *Wolbachia* diversity on vertical transmission of CpDV, a binomial generalized mixed effect model is fitted with genetic background (Slab, Harash, Tunis), *Wolbachia* type (*w*Pip 1, *w*Pip 3 and *w*Pip 4), *Wolbachia* amount and CpDV amount in the full body of the female as fixed effects and replicates as random effects.

CpDV in females were checked for the first and third generation of tetracycline treatment, they were grouped under during tetracycline treatment group given that these two groups didn't show any significant difference in terms of CpDV amount in the ovaries and represented the same treatment status. A Dunnett's test (glhrt function in multcomp package) (Hothorn *et al.*, 2008) have been performed for pairwise comparison of treatment stages to the non-treated control group.

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

3.4 Acknowledgments

We thank Sandra Unal and Patrick Makoundou for technical support. We thank Margareth Capurro for providing us with the "Brasil" *Culex* line. We also thank Dr Philippe Clair for his help in the Real Time Quantitative PCR experiments which were performed through the technical facility of the "qPCR Haut Debit (qPHD) Montpellier genomiX" platform. We acknowledge the imaging facility MRI, member of the national infrastructure France-BioImaging infrastructure supported by the French National Research Agency (ANR-10-INBS-04, «Investments for the future»). Mine Altinli received support from Ministère des affaires étrangères (Campus France) via the French

Embassy in Turkey and Project "ISEM Sud", 2016 for her PhD.

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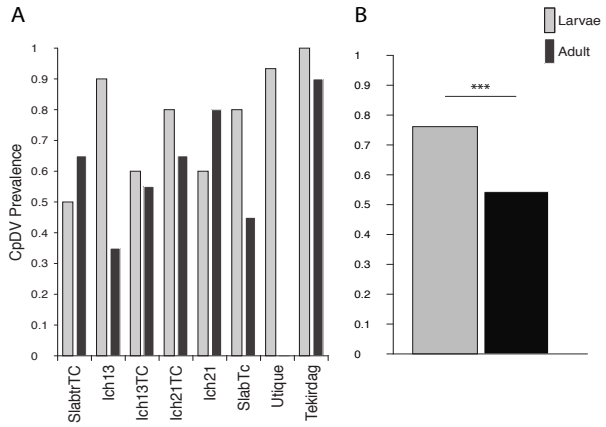
3.6 Appendix

3.6.1 Appendix A

3.6.1.1 CpDV prevalence in Laboratory lines

CpDV specific diagnostic PCR test, based on the amplification of partial NS2 gene revealed the presence



of CpDV in all the line that were kept in the insectary.

Figure 1. CpDV prevalence in insectary Lines (A). CpDV prevalence was higher in Larvae compared to adults (B)

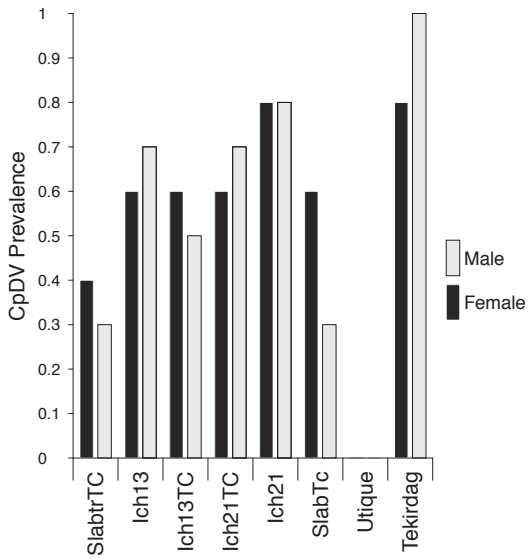


Figure 2. Prevalence in Males vs. Females.

3.6.1.2 Experimental Infections

Infection trials using homogenised and filtered larvae

SlabTR and Ich13 lines harbouring different *Wolbachia* groups have been used for the infections as well as their TC treated counterparts (SlabTRTC, Ich13TC).

About 500 larvae were collected from different lines in PBS and has been filtered with syringe filters 0.45 mm. 1/10 dilution of the homogenate has been tested using PCR. The ones that tested negative on PCR were used as negative control CpDV- while the ones tested positive used as CpDV+. Three different doses (25ul, 50ul, 70ul) have been tested.

For each group (line_ *Wolbachia* status_CpDV status_dose) 6 larvae were tested which added up to a total of 144 larvae. Data was analysed using R and survival curves estimated with Cox proportional hazards model implemented in Coxme package.

There was no significant difference between CpDV- positive and CpDV- negative treatment groups (Cox proportional hazards: $\chi 2$=1.765678 df=1 p>0.1, **Figure** A). On the other hand, survival decreased with the increase of the dose (Cox proportional hazards: $\chi 2$= 15.6863 df=2 p< 0.001**Figure** B), suggesting the toxicity of the filtered ground larvae in higher doses.

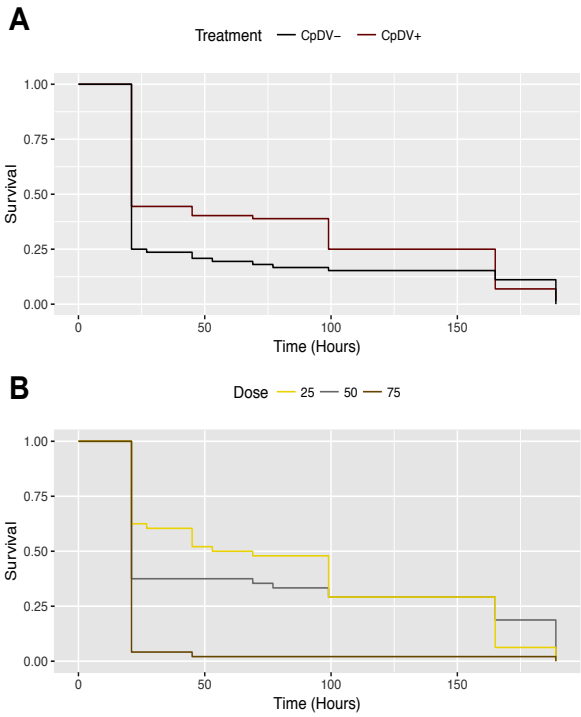


Figure 3 Experimental infections using crushed larvae

Infection trials with synthetic CpDV

C636 cells were transinfected with synthesized CpDV plasmid by our collaborators Anne-Sophie Gosselin-Grenet and Doriane Mutuel.

BcTU, BcHA, SlabTC, Ha total of 24 for each group CpDV- positive and CpDV negative tested. The amount of CpDV was quantified and 4e+6 geq/larvae was added to hemolysis tubes. Same amount of CpDV negative cell cultures have been added to hemolysis tubes for the negative control groups. 90ul of each added in 1 ml water-food mixture with 3rd instar larvae.

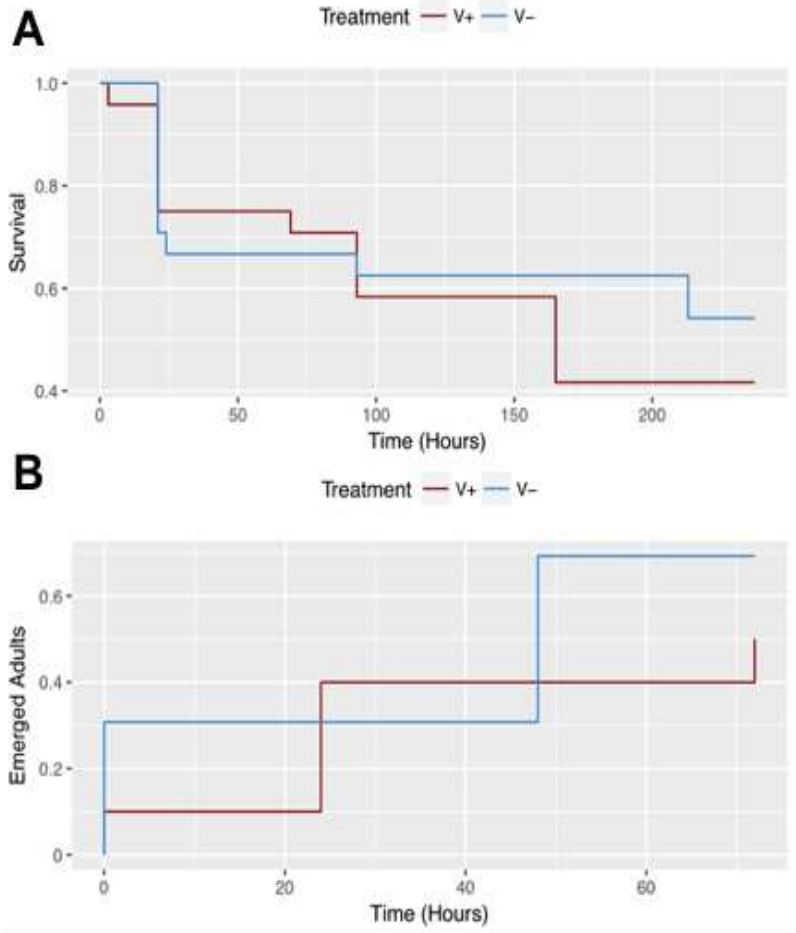


Figure 4. Experimental infections using CpDV transinfected C6/36 cells

3.6.2 Appendix B

Supplementary Table.1. Mosquito lines, their origin, genetic background and their Wolbachia group

Lines	***Origin***	***Host Genetic Background***	***Wolbachia group***
Slab	*USA* (Georghiou *et al.*, 1966)	*Slab*	*wPip III*
Harash	*Algeria* (Alout *et al.*, 2009)	*Harash*	*wPipIV*
Tunis	*Tunisia* (Duron *et al.*, 2005)	*Tunis*	*wPipI*
BcHarash	*SlabTC x Harash*	*Slab*	*wPipIV*
BcTunis	*SlabTC x Tunis*	*Slab*	*wPipI*
SlabTC	*USA*	*Slab*	*None*
BcHarashTC	*SlabTC x Harash*	*Slab*	*None*
BcTunisTC	*SlabTC x Tunis*	*Slab*	*None*
Brasil	*Brazil* (Capurro Margareth laboratory)	*Brasil*	*wPipI*
Ichkeul09	*Tunisia* (Bonneau *et al.*, 2018)	*Ichkeul09*	*wPipIV*
Ichkeul13	*Tunisia* (Bonneau *et al.*, 2018)	*Ichkeul13*	*wPipIV*
Istanbul	*Turkey* (Duron *et al.*, 2005)	*Istanbul*	*wPipIV*
Lavar	*France* (Duron *et al.*, 2005)	*Lavar*	*wPipII*
Utique	*Tunisia* (Atyame *et al.*, 2014)	*Utique*	*wPipI*

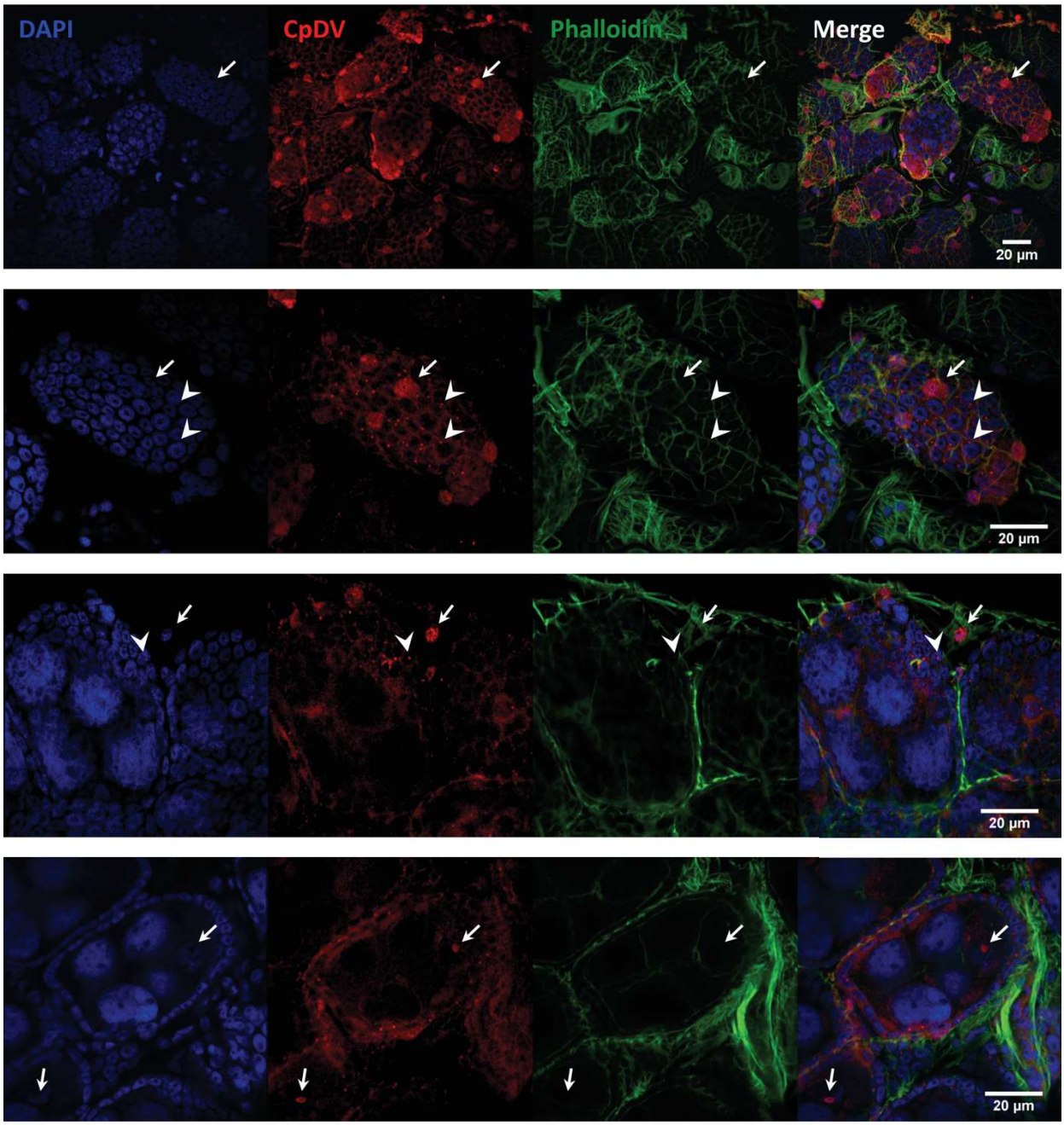


Figure 5. Confocal images of ovaries isolated from 6 days old *Cx. pipiens* females (Harash line). Immunolabeling of CpDV (in red) was performed with an anti-capsid antibody, actin was labelled with phalloidin-FITC (green) and nuclei with DAPI (blue). Arrows show CpDV-infected cells and arrowheads point cytoplasmic dots of CpDV labelling. Images shows split channels and merges of images from the figure 2.

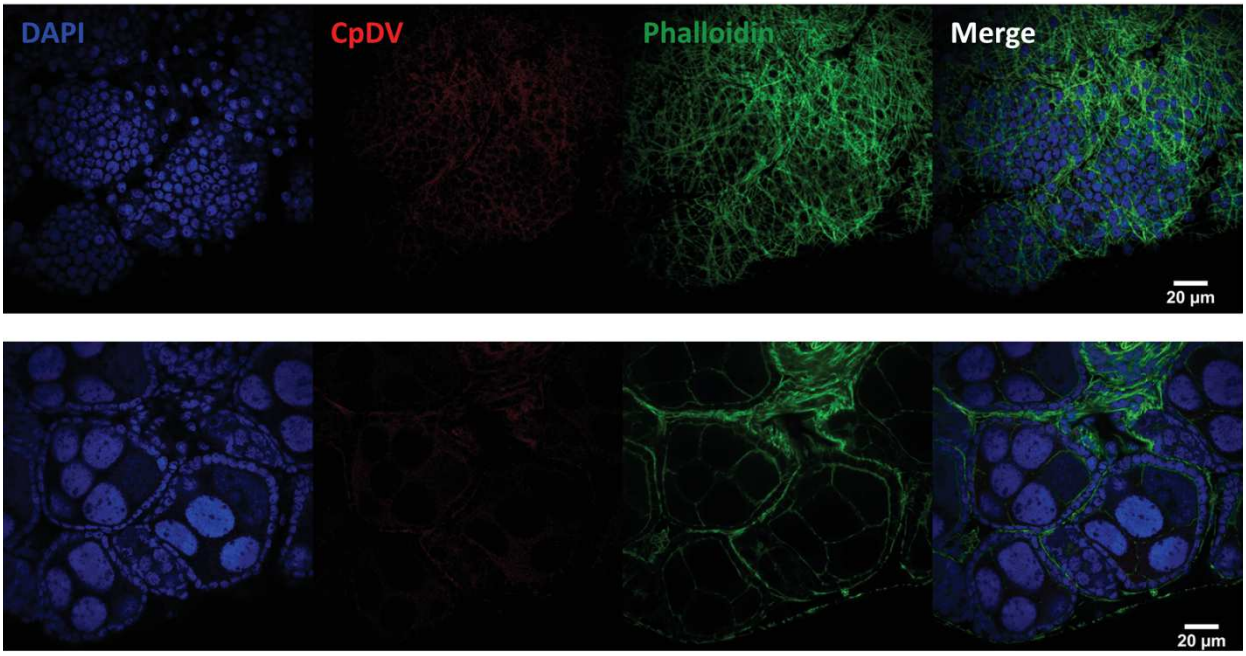


Figure 6. Confocal images of ovaries isolated from 6 days old *Ae. albopictus* females. Immunolabeling of CpDV (red) was performed with an anti-capsid antibody, actin was labeled with phalloidin-FITC (green) and nuclei with DAPI (blue) in the same conditions as in the supplementary figure 1. No red labeling was observed in *Ae. albopictus* ovaries.

3.6.3 References

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Chapter 4:
CpDV and *Wolbachia* Prevalence and Diversity in Turkey

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4.1 Introduction

Situated in Mediterranean basin between three continents -Asia, Europe and Africa- Turkey is a hotspot for biodiversity. Due to this unique geographical location and its suitable climate, Turkey can also provide ideal conditions for arthropod vectors and arthropod borne viruses. Hence it also represents a critical area for the control efforts of such arthropod borne viruses. Indeed a high diversity of mosquitoes have been revealed in the area, as well as the circulation of different strains of West-Nile virus and the diversity of insect-specific RNA viruses (Ergunay *et al.*, 2011, 2013, 2014, 2015; Erdem *et al.*, 2014; Ergünay *et al.*, 2016; Failloux *et al.*, 2017; Öncü *et al.*, 2018).

Important vectors of West Nile and St. Louis encephalitis viruses and avian malaria (Plasmodium spp.), mosquitoes of *Cx. pipiens* complex are always found infected with *Wolbachia* *w*Pip of the five genetically different groups (*w*Pip-I to V), which induce very complex cytoplasmic incompatibility patterns in their hosts including uni- and bi-directional incompatibility (Farajollahi *et al.*, 2011; Atyame *et al.*, 2014). The worldwide distribution of these groups have already been investigated, although samples from Turkey were restricted to one site in Istanbul (Dumas *et al.*, 2013).

Previous studies by our team in the ISEM, have showed that samples from Istanbul harbour *w*Pip from the group-IV which showed an interesting incompatibility pattern with other *w*Pip groups. Indeed *w*Pip group-IV carrying male mosquitoes were able to sterilize the females carrying other *w*Pip groups suggesting that this strain could be used in an 'Incompatible Insect Technique' (IIT) vector control strategy (Atyame, Pasteur, *et al.*, 2011; Atyame, Cattel, *et al.*, 2015). This species-specific approach consists of sterilization of field female mosquitoes by inundative releases of incompatible males which can lead to the reduction of mosquito population densities (Atyame, Pasteur, *et al.*, 2011; Atyame, Cattel, *et al.*, 2015).

Belonging to Densovirinae subfamily of Parvoviridae family, CpDV represents a unique mosquito densovirus: they differ from all the other mosquito densoviruses so far isolated in terms of their genome structure and sequence homology (Jousset *et al.*, 2000; Cotmore *et al.*, 2014). First isolated two decades ago following the observation of unusually high larval mortality in ISEM's insectary lines, CpDV has later been detected to persist in seemingly healthy laboratory colonies in all life stages of *Cx. pipiens* mosquitoes **(Chapter 3).** Their covert infection combined with vertical transmission could explain their long-term persistence in laboratory colonies and suggested that these viruses could also be really prevalent in natural mosquito populations **(Chapter3)**. Indeed we have previously reported a high prevalence of CpDV in worldwide distributed natural populations of *Cx. pipiens* **(Chapter 2)**, using a PCR diagnostic test.

Furthermore, CpDV-*w*Pip co-existence in the same host cells and their co- vertical transmission **(Chapter 2)** suggested that CpDV and *Wolbachia* could interact in their shared host environment. *Wolbachia* pathogen inference leading to a decrease in pathogen density and infection frequency has previously been shown in several arthropod species (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Mousson *et al.*, 2010; Hussain *et al.*, 2012; Braquart-Varnier *et al.*, 2015). In *Cx. pipiens s.l.* mosquitoes, *w*Pip can reduce the West Nile Virus infection depending on the *Wolbachia* density (Micieli and Glaser, 2014).

Wolbachia-CpDV interactions could, for instance, influence CpDV prevalence and diversification or *Wolbachia* *w*Pip group distribution in natural populations and eventually shape host populations. Especially where two different *w*Pip groups with different incompatibility patterns co-exist the impact of these interactions could be more important. In natural *Cx. pipiens* populations co-existing *w*Pip strains are more likely to be compatible with each other (Rousset *et al.*, 1991; Duron *et al.*, 2005, 2006, 2011; Engelstädter and Telschow, 2009; Atyame, Duron, *et al.*, 2011). In fact theory predicts that when the bi-directionally incompatible *w*Pip strains co-exist, the most prevalent strain should eventually invade the population (Rousset *et al.*, 1991; Engelstädter and Telschow, 2009). On the other hand when co-existing *w*Pip strains are unidirectionally incompatible, the CI-inducing strain is expected to invade the population once above a frequency threshold (Engelstädter and Telschow, 2009; Atyame, Labbé, *et al.*, 2015). On

the other hand, densoviruses could also influence these dynamics. If densoviruses are pathogenic for their host a more protective *w*Pip strain could invade the host population easier as it would benefit its host. In return probably, such a protective *w*Pip strain can make CpDV presence impossible in this *w*Pip-host combinations. Contrarily if CpDV is beneficial for the host the frequency of *w*Pip-host combination that favours the presence of CpDV would increase in the population.

In this context, natural populations of *Cx. pipiens* mosquitoes especially where two different *w*Pip groups with different incompatibility patterns co-exist could be interesting to study *Wolbachia*-CpDV interactions. Due to its unique geographical location, Turkey represents an area where the diversity from different continent meets and have a potential for investigation of *w*Pip and CpDV diversity and interactions. To this end, we explored first the *Wolbachia* diversity and incompatibility patterns (**Chapter 4.2.**, (Altinli *et al.*, 2018)). Secondly we focused on the prevalence of CpDV and diversity in natural populations of *Cx. pipiens* (s.l.) in Turkey.

4.2 *Wolbachia* diversity and incompatibility patterns in Turkey

Altinli, M., Gunay, F., Alten, B., Weill, M., and Sicard, M. (2018) *Wolbachia* diversity and cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey. *Parasites and Vectors* **11**: 198.

*Figure 1 colours have been modified to fit the rest of the thesis.

Altinli *et al. Parasites & Vectors* (2018) 11:198
https://doi.org/10.1186/s13071-018-2777-9

Parasites & Vectors

RESEARCH Open Access

Wolbachia diversity and cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey

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Abstract

Background: *Wolbachia* are maternally transmitted bacteria that can manipulate their hosts' reproduction causing cytoplasmic incompatibility (CI). CI is a sperm-egg incompatibility resulting in embryonic death. Due to this sterilising effect on mosquitoes, *Wolbachia* are considered for vector control strategies. Important vectors for arboviruses, filarial nematodes and avian malaria, mosquitoes of *Culex pipiens* complex are suitable for *Wolbachia*-based vector control. They are infected with *Wolbachia* *w*Pip strains belonging to five genetically distinct groups (*w*Pip-I to V) within the *Wolbachia* B supergroup. CI properties of *w*Pip strongly correlate with this genetic diversity: mosquitoes infected with *w*Pip strains from a different *w*Pip group are more likely to be incompatible with each other. Turkey is a critical spot for vector-borne diseases due to its unique geographical position as a natural bridge between Asia, Europe and Africa. However, general *w*Pip diversity, distribution and CI patterns in natural *Cx. pipiens* (*s.l.*) populations in the region are unknown. In this study, we first identified *w*Pip diversity in Turkish *Cx. pipiens* (*s.l.*) populations, by assigning them to one of the five groups within *w*Pip (*w*Pip-Ito V). We further investigated CI properties between different *w*Pip strains from this region.

Results: We showed a *w*Pip fixation in *Cx. pipiens* (*s.l.*) populations in Turkey by analysing 753 samples from 59 sampling sites. Three *w*Pip groups were detected in the region: *w*Pip-I, *w*Pip-II and *w*Pip-IV. The most dominant group was *w*Pip-II. While *w*Pip-IV was restricted to only two locations, *w*Pip-I and *w*Pip-II had wider distributions. Individuals infected with *w*Pip-II were found co-existing with individuals infected with *w*Pip-I or *w*Pip-IV in some sampling sites. Two mosquito isofemale lines harbouring either a *w*Pip-I or a *w*Pip-II strain were established from a population in northwestern Turkey. Reciprocal crosses between these lines showed that they were fully compatible with each other but bidirectionally incompatible with *w*Pip-IV Istanbul infected line.

Conclusion: Our findings reveal a high diversity of *w*Pip and CI properties in *Cx. pipiens* (*s.l.*) populations in Turkey. Knowledge on naturally occurring CI patterns caused by *w*Pip diversity in Turkey might be useful for *Cx. pipiens* (*s.l.*) control in the region.

Keywords: *Wolbachia*, *Culex pipiens*, Cytoplasmic incompatibility, Turkey, Vector control

Background

First discovered in *Culex pipiens* (*s.l.*) mosquitoes [1], the α-proteobacterium *Wolbachia pipientis* is one of the most common vertically transmitted cytoplasmic symbionts. Indeed, meta-analysis predicts *Wolbachia* infection in up to 50% of the arthropod species [2]. The success of their vertical transmission mainly relies on their host reproduction manipulation strategies; parthenogenesis induction, feminization, male killing and cytoplasmic incompatibility (CI) [3]. CI, the most common of these strategies, is modelled by a modification-rescue (*mod-resc*) system where *Wolbachia* modifies sperm of infected males (*mod* function), and only a compatible *Wolbachia* strain in the eggs can rescue (*resc* function) this modification [4]. Consequently, *Wolbachia* causes conditional sterility in crosses either between uninfected females and infected males [5]

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or like in the case of *Cx. pipiens* (*s.l.*), between females and males infected with incompatible *Wolbachia* strains [6, 7].

Consisting of several species, including important disease vectors with worldwide distribution (e.g. *Cx. pipiens* and *Cx. quinquefasciatus*) [8], mosquitoes of *Cx. pipiens* complex have a unique relationship with their endosymbiont *Wolbachia* (*w*Pip). *w*Pip is fixed in natural *Cx. pipiens* (*s.l.*) populations where they induce the most complex CI relationships yet described among arthropods, including uni and bidirectional incompatibility [7, 9–12]. So far, reciprocal crosses between many isofemale lines and four reference lines showed eight different *mod* and four different *resc* functions in *Cx. pipiens* (*s.l.*), resulting in the definition of 14 different cytotypes throughout the world [7].

In contrast to this observed diversity of CI patterns, *Cx. pipiens* *w*Pip strains are closely related, and all belong to a clade within *Wolbachia* B supergroup [12–14]. However, recent studies of fast evolving markers showed the presence of many genetically distinct *w*Pip strains in *Cx. pipiens* (*s.l.*) mosquitoes [10, 12, 15] distributed in five distinct phylogenetic groups (*w*Pip-I to V) [12]. Using a PCR/RFLP assay based on pk1 gene, encoding proteins with ankyrin motifs, a *w*Pip strain can be assigned to one of these five groups [12, 16]. A study of the *w*Pip worldwide distribution showed an important spatial structure of *w*Pip groups [16]. For instance, only *w*Pip-I was found in sub-Saharan Africa, South America and Southeast Asia, while *w*Pip-III was mainly observed in North America. Strains belonging to the *w*Pip-II group were mostly found in western Europe and *w*Pip-V in Asia. *w*Pip-IV group strains exhibit a patchy distribution in Europe, North Africa and Asia [16]. Also, *Wolbachia* genetic diversity and their CI patterns strongly correlate; most *w*Pip strains from the same group render their host compatible with each other (except few unidirectional incompatibilities) whereas those from different groups often lead to unidirectional or bidirectional incompatibilities [7]. Recently this huge diversity of CI patterns observed in *Cx. pipiens* has been explained by the amplification and the diversity of an operon in *w*Pip strains' genomes [17] composed of *cidA* and *cidB* genes involved in *Wolbachia* induced CI [18, 19]. No effect of host genetic background on the CI patterns [20] and no multiple infections by several strains have ever been shown [12, 16, 21].

Being a natural bridge between Africa, Asia and Europe, Turkey is a critical spot for many emerging and re-emerging vector-borne diseases [22, 23] and for the diversity of the vectors that transmit these diseases [23]. For instance, high diversity and abundance of *Cx. pipiens* (*s.l.*) species have been recorded in the area including *Cx. quinquefasciatus*, *Cx. pipiens* and its physiological variant *Cx. pipiens* f. *molestus* [24]. Arboviruses such as West Nile virus, mainly transmitted by these mosquitoes, have also been shown to circulate in Turkey [25–28]. Therefore, understanding *w*Pip diversity and their CI properties of *w*Pip to control *Cx. pipiens* (*s.l.*) populations in Turkey is a cornerstone for vector control in the region and prevention of putative epidemics extending through Europe, Asia and northern Africa. This knowledge can contribute to the biological vector control techniques using CI properties such as incompatible insect technique (IIT). IIT, the mass release of males harbouring incompatible *Wolbachia* into focal populations, has been shown to successfully decrease the female reproduction by sterilisation and reduce the pest/vector populations [29–34]. Nevertheless, our knowledge of the *w*Pip genetic diversity and the CI patterns in Turkey is yet limited to only one line established with samples collected in Istanbul in 2003 [35].

Here, we collected and analysed 753 *Cx. pipiens* (*s.l.*) individuals (larvae and adults) from natural populations across Turkey. We studied (i) the *w*Pip diversity in this geographically critical region in the crossroads of three continents, (ii) the CI relationships between *Cx. pipiens* lines from Turkey and (iii) the CI relationships between Turkish lines and reference lines to compare their CI properties to previously characterized *mod* (male crossing type) and *resc* (female crossing type) functions. Taken together, these results might be used in integrated vector control programs against *Cx. pipiens* (*s.l.*) in Turkey.

Methods

Sample collection and identification

A total of 753 samples from 59 different sampling sites in Turkey were tested for *Wolbachia* diversity. Most of these samples (n = 677) were collected during the larval stage, between July to September 2016 (Table 1). The rest of the samples has been collected as adults, using adult light traps, from May to September (2012–2015) (Table 1, Fig. 1). All of the sampling sites were situated outdoors with the only exception of sample site 16 (Table 1). Collected larvae and adults were morphologically identified as *Culex pipiens* (*s.l.*) / *Cx. torrentium* [36]. As the samples were only morphologically identified, we used *Cx. pipiens* (*s.l.*) to refer to *Cx pipiens* assemblage that includes both *Cx. pipiens* and *Cx. quinquefasciatus*, their hybrids and physiological forms [21, 35]. Samples were stored in 70% ethanol until DNA extraction before testing them for the *w*Pip presence and genetic characterisation.

Isofemale lines

For analysing the CI patterns induced by the *w*Pip strains belonging to different groups found in Turkey, egg rafts and larvae were collected from a population in Thrace region of Turkey, in Tekirdag (Table 1 sampling site 52, Fig. 1). Collected larvae were reared to adults in insectary conditions (at 25 ± 2 °C and 75 ± 2% relative

Table 1 Sampling sites, year, life stage and *w*Pip groups of *Culex pipiens* (*s.l.*) individuals collected from Turkey

Province	Sampling site	Latitude (°N)	Longitude (°E)	Breeding site type	Stage	Year	*w*Pip-I	*w*Pip-II	*w*Pip-IV
Adana	1	36.9475	35.485	Rural	A	2013	–	5	–
Ankara	2	39.8730	32.7370	Suburban	L	2016	–	27	–
	3	39.8716	32.7356	Suburban	A	2014	2	3	–
Artvin	4	41.3884	41.4335	Suburban	L	2016	1	35	–
	5	41.4919	41.5367	Suburban	L	2016	–	5	–
	6	41.3833	41.5716	Rural	L	2016	–	5	–
	7	41.3651	41.6835	Suburban	L	2016	1	20	–
	8	41.3911	41.6933	Rural	L	2016	7	17	–
	9	41.3646	41.6686	Suburban	L	2016	–	3	–
	10	41.3742	41.6235	Rural	L	2016	21	4	–
	11	41.3192	41.3534	Rural	L	2016	12	–	–
	12	41.3178	41.3412	Rural	L	2016	10	–	–
	13	41.3274	41.3022	Rural	L	2016	36	–	–
	14	40.7823	41.4991	Rural	A	2013	–	1	–
	15	41.3928	41.6937	Rural	A	2013	4	1	–
Aydin	16	37.4123	27.3612	Rural	A	2012	3	–	–
Bartin	17	41.8383	32.7115	Rural	L	2016	10	–	–
	18	41.7411	32.3827	Suburban	L	2016	5	–	–
Bursa	19	40.0948	29.4912	Urban	A	2013	1	9	–
Duzce	20	41.0708	30.9645	Rural	L	2016	7	3	–
Edirne	21	41.6134	26.9656	Rural	L	2016	–	16	11
	22	41.6731	26.9809	Rural	L	2016	11	–	–
	23	41.6635	26.5078	Suburban	L	2016	–	17	–
	24	40.8548	26.6897	Suburban	A	2012	–	4	–
	25	40.9404	26.4382	Rural	A	2012	–	1	–
Erzincan	26	39.2476	38.5050	Rural	A	2014	–	3	–
Eskisehir	27	39.7950	30.4972	Urban	L	2016	–	27	–
	28	39.2051	30.7145	Rural	A	2013	–	2	–
	29	39.7098	30.4035	Rural	L	2016	–	5	–
Hatay	30	36.2516	36.3166	Suburban	A	2015	1	–	–
Istanbul	31	40.9481	29.3050	Urban	L	2016	–	38	–
	32	40.9418	29.3016	Urban	L	2016	–	44	–
	33	40.9796	29.0557	Urban	L	2016	8	24	–
	34	41.0783	29.0136	Urban	A	2016	–	–	1
Kahramanmaras	35	37.5588	36.9737	Urban	A	2015	3	–	–
Karadeniz Ereglisi	36	41.2824	31.4241	Urban	L	2016	6	–	–
Kastamonu	37	41.8886	32.9995	Suburban	L	2016	5	–	–
Kirklareli	38	41.8458	27.8065	Rural	L	2016	–	10	–
	39	41.5239	27.0258	Rural	A	2015	–	6	–
	40	41.8300	27.0638	Rural	A	2015	–	2	–
Kocaeli	41	40.6882	30.2797	Urban	L	2016	–	15	–
Malatya	42	38.8180	37.9769	Rural	A	2014	–	1	–
Mardin	43	37.5607	40.8865	Rural	A	2013	5	–	–
	44	37.5477	40.9588	Rural	A	2013	5	–	–

Table 1 Sampling sites, year, life stage and *w*Pip groups of *Culex pipiens* (*s.l.*) individuals collected from Turkey *(Continued)*

Province	Sampling site	Latitude (°N)	Longitude (°E)	Breeding site type	Stage	Year	*w*Pip-I	*w*Pip-II	*w*Pip-IV
Osmaniye	45	37.1375	36.2010	Suburban	A	2015	1	1	–
Sakarya	46	41.0719	30.8454	Suburban	L	2016	–	5	–
Samsun	47	41.3689	36.2289	Urban	A	2013	3	–	–
Sinop	48	41.9915	35.0908	Suburban	L	2016	1	5	–
	49	41.9309	34.5819	Suburban	L	2016	6	–	–
Tekirdag	50	41.1503	27.8522	Urban	A	2012	–	3	–
	51	40.8886	27.4604	Urban	A	2015	–	6	–
	52[a]	41.0259	27.5805	Urban	L	2016	na	na	na
Tokat	53	40.1916	35.5139	Rural	A	2014	–	1	–
Trabzon	54	40.8938	39.7113	Suburban	L	2016	34	–	–
Yalova	55	40.6217	29.1770	Rural	L	2016	1	44	–
	56	40.6085	29.2080	Suburban	L	2016	–	42	–
	57	40.6428	29.0968	Suburban	L	2016	–	40	–
Zonguldak	58	41.4105	32.0890	Suburban	L	2016	10	–	–
	59	41.3537	32.0900	Suburban	L	2016	15	–	–
	60	41.4529	31.8203	Urban	L	2016	7	–	–

Abbreviations: A, adult; L, larva; na, not applicable
Note: Columns *w*Pip-I, *w*Pip -II and *w*Pip-IV indicate the amount of individuals infected with *w*Pip-I, *w*Pip -II and *w*Pip-IV, respectively, in a given sampling site
[a]Samples from this site were used to establish Tek *w*Pip-I and Tek *w*Pip-II lines

humidity and a 12:12 h photoperiod) and fed with a mixture of shrimp powder and rabbit pellets. To establish isofemale lines, females were fed with turkey blood using a Hemotek membrane feeding system (Discovery Workshops, Blackburn, United Kingdom) and were allowed to lay eggs five days later. Each egg raft (100–300 eggs) was isolated for hatching, and the isofemale line was established using resulting sibling larvae. A pool of first-instar larvae (L1) was tested to identify the *w*Pip group. Two isofemale lines each harbouring either a *w*Pip-I or *w*Pip-II strain were

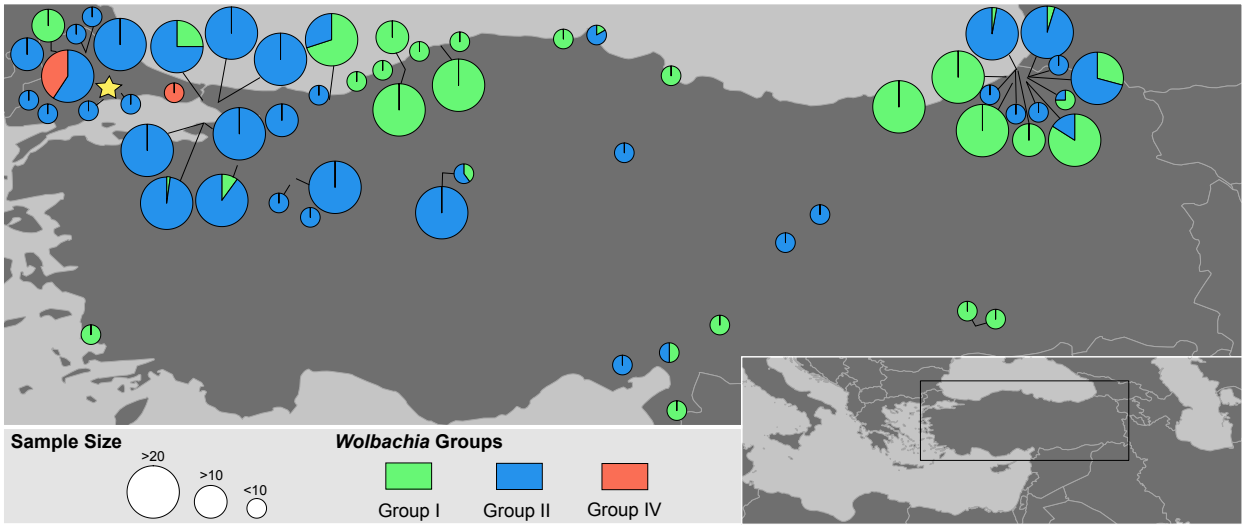

Fig. 1 Sampling sites and diversity of *w*Pip in *Cx. pipiens (s.l.)* populations in Turkey. A total of 753 samples were collected from 59 different sampling sites, tested with a PCR/RFLP assay on the pk1 gene (1.3 kb) and assigned to one of the five genetically distinct *w*Pip groups (*w*Pip-I to V). Results showed the occurrence of *w*Pip from three different groups in the area namely *w*Pip-I-II and IV. Size of the circle represents the sampling size. Percentage of a given *w*Pip group in a given population is shown in different colours; green: *w*Pip-I, blue: *w*Pip-II, orange: *w*Pip-IV. Star indicates the location of the samples (Tekirdag) that have been used to establish Tek *w*Pip-I and Tek *w*Pip-II lines. Reciprocal crosses between these lines and additional reference lines were performed to identify natural CI patterns caused by *w*Pip groups in this region.

reared for further crossing experiments in 65 dm^3 cages in insectary conditions and were fed with a honey solution and a weekly blood meal.

Crossing experiments between Turkish *Culex pipiens* lines

Tek *w*Pip-I and Tek *w*Pip-II isofemale mosquito lines were reared for at least four generations in insectary conditions to allow their acclimatisation before crossing experiments. Mosquitoes were isolated during pupal stage, and emerging adults were sexed. Then, 2–5 days old virgin males and females (n = 25–50) were used to carry out reciprocal crosses between them and with Istanbul *w*Pip-IV line. Females were fed with turkey blood using a Hemotek membrane feeding system (Discovery Workshops, United Kingdom) on the sixth day following caging and were allowed to lay eggs five days after the blood meal. Egg rafts were then isolated individually in 24 well plates filled with tap water until hatching. Embryonic development of all the unhatched egg rafts was verified to differentiate between non-fertilized egg rafts and CI induced embryonic death as previously described [37].

The crossing relationships were identified as following [7]: compatible (C) when > 90% of the rafts hatched in the two reciprocal crosses; and incompatible (IC), with two CI patterns: (i) unidirectionally incompatible crosses: when between 0–10% of the rafts hatched in one of the reciprocal crosses and > 90% in the other; and (ii) bidirectionally incompatible crosses: when less than 10% of the rafts hatched in both reciprocal crosses.

Crossing experiments to infer *mod* and *resc* functions

The *mod* (male crossing type) and *resc* (female crossing type) functions caused by many *w*Pip strains, which belong to different *w*Pip groups (I-V), have been identified by reciprocal crosses with 4 reference lines: LaVar (*w*Pip-II), MaClo, Slab (*w*Pip-III) and Istanbul (*w*Pip-IV) [7]. Here, we used same four reference lines to define the *mod-resc* functions of Tek *w*Pip-I and Tek *w*Pip-II isofemale lines and to compare them to previously defined ones [7].

Identification of *Wolbachia* diversity

DNA was extracted from the samples using CTAB method [38]. PCR assays were conducted using pk1 primers (PK1 Forward: 5'-CCA CTA CAT TGC GCT ATA GA-3' and PK1 Reverse: 5'-ACA GTA GAA CTA CAC TCC TCC A-3'-AM397079 [12]), which amplify a 1.3-kilobase (kb) fragment from ankyrin domain coding gene of *Wolbachia*. PCR amplifications were made in following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation, annealing and elongation respectively at 94 °C for 30 s, 52 °C for 30s, and 72 °C for 90 s, and a final elongation at 72 °C for 5mn. Resulting PCR products then used in RFLP assays first with *Taq*αI enzyme to discriminate specific *w*Pip alleles "a" or "e" (*w*Pip- I or *w*Pip-V; 991, 251, 107 bp), "b" (*w*Pip-III; 669, 665 bp), "c" (*w*Pip-II; 851, 498 bp) and "d" (*w*Pip-IV; 497, 251, 107 bp) [7, 16]. Secondly, since *Taq*αI digestion of "a" and "e" alleles show the same digestion pattern, pk1 PCR products of the samples showing this pattern were digested with *Pst*I enzyme to further discriminate "a" (*w*Pip- I; 903, 303, 141 bp) and "e" (*w*Pip-V; 903, 430 bp) alleles [7, 16]. Digested amplified fragments were separated by agarose gel electrophoresis (2%), stained with ethidium bromide (1 μg/ml) and visualized with UV light. Samples from laboratory mosquito lines with different *w*Pip groups and tetracycline-cured *Wolbachia* negative lines were included in every reaction as positive and negative controls, respectively, and always gave the expected result.

Statistical analyses

The occurrence of different *w*Pip groups was compared by a Chi-square test using R software (version 3.3.1).

Results

Diversity and distribution of *w*Pip groups

Wolbachia *w*Pip was present in all of the 753 *Cx. pipiens* (*s.l.*) individuals tested and they were further identifiable to one of the five previously described groups (*w*Pip-I to V). Co-infection of one individual by different *w*Pip groups was never observed. Out of five *w*Pip groups identified so far in the world, three of them (i.e. *w*Pip-I-II and IV) were represented in the studied area (Fig. 1). The abundance of these groups was significantly different from each other (χ^2 = 474.99, df = 2, P < 0.0001). While *w*Pip-II was the most dominant (n = 500, 66% of the samples, Table 1, Fig. 1) and widespread group (39 sampling sites out of 59 total) in Turkey; *w*Pip-IV was found only in two locations, both in Thrace Region (in Edirne, sampling site 21 and Istanbul sampling site 34, Table 1; Fig. 1), and was the least abundant group (n = 12, 1% of the samples, Table 1; Fig. 1). *w*Pip-I was found in 31 locations and a total of 241 individuals.

Co-existence of *w*Pip strains in different individuals from the same sampling sites

In 20 % of the sampling sites *w*Pip-I and *w*Pip-II co-existed (Table 1, Fig. 1). *w*Pip-IV was only found co-existing with *w*Pip-II in one sampling site but never found in the same sampling site with *w*Pip-I, even though they were sampled from nearby sites (~8 km) in north western Turkey (in Edirne, sampling site 21 and 23, Table 1; Fig. 1).

Naturally occurring CI patterns in Turkey

Two isofemale lines (Tek *w*Pip-I and Tek *w*Pip-II), harbouring two different *w*Pip strains from two different groups, were established from north western Turkey (Tekirdag Province, Table 1 sampling site 52, Fig. 1) to identify CI

patterns caused by different *w*Pip groups in the region. Reciprocal crosses between these lines showed that Tek *w*Pip-I and Tek *w*Pip-II were fully compatible with each other (Table 2). Both lines were bidirectionally incompatible with the line harbouring Istanbul strain (*w*Pip-IV, Table 2).

Mod and *resc* properties of Turkish *w*Pip strains

To compare *mod* and *resc* functions of Turkish *w*Pip strains with *mod* and *resc* functions of worldwide collected *w*Pip strains, we performed reciprocal crosses of Tek *w*Pip-I and Tek *w*Pip-II lines with the four reference lines [LaVar (*w*Pip-II), MaClo (*w*Pip-III), Slab (*w*Pip-III) and Istanbul (*w*Pip-IV)]. Tek *w*Pip-I males were compatible with LaVar (*w*Pip-II) and MaClo (*w*Pip-III) females while incompatible with Slab (*w*Pip-III) and Istanbul (*w*Pip-IV) females (Table 2). This type of *mod* property, inferred from similar crosses, has already been shown for the *w*Pip-I group from Tunisia; numbered "vi" [7]. Contrarily, Tek *w*Pip-II males demonstrated a new *mod* property, as they were incompatible with LaVar and Istanbul, and compatible with MaClo and Slab females (Table 2). We numbered this new *mod* as "ix" to continue the previously published numeration [7]. Both Tek *w*Pip-I and Tek *w*Pip-II lines showed the same *resc* type, which was characterised by the compatible crosses of females of these lines with all the males from the reference lines except Istanbul (Table 2). This *resc* type (*resc* "2") is the most common *resc* type found worldwide for *w*Pip-I and *w*Pip-II groups [7].

Discussion

In Turkey, all tested *Cx. pipiens* were infected with *Wolbachia* *w*Pip. Such fixation of *w*Pip has been demonstrated worldwide, including in the neighbouring country Iran [39], in *Cx. pipiens* and *Cx. quinquefasciatus* populations [35, 40–44]. A previous study in Turkey, however, showed a lower prevalence of *w*Pip [45]. This might be caused by the misidentification of a recently described cryptic species within *Cx. pipiens* complex that has been shown to lack *w*Pip infection and to be reproductively isolated from the other members of the complex [46, 47]. Similarly, *Cx. torrentium*, which is difficult to differentiate morphologically from *Cx. pipiens* (*s.l.*) mosquitoes is not infected with *Wolbachia* [41, 44, 48]. Therefore 100% *w*Pip infection rate of our samples confirmed that we only analysed *Cx. pipiens* complex members (excluding both previously mentioned cryptic species and *Cx. torrentium*) in the present study.

The identification of the *Cx. pipiens* taxa was left out of the scope of this study for several reasons. Previous studies on the diversification of *w*Pip in *Cx. pipiens* (*s.l.*) have proved that their diversity is not directly related to the nuclear genetic background of the mosquitoes, meaning that no *w*Pip group was specific for a *Cx. pipiens* sibling species [10, 21, 49]. It rather follows the same distribution as mitochondrial diversity (mtDNA) of mosquitoes, as *w*Pip are maternally transmitted to the next generation through the egg cytoplasm along with mitochondria [16, 46]. Moreover, CI properties are independent of the genetic background of *Cx. pipiens* (*s.l.*) and directly dictated by their *Wolbachia* [20]. Recent studies on *Cx. pipiens* (*s.l.*) in Turkey had shown that both *Cx. quinquefasciatus, Cx. pipiens* and its form *Cx. pipiens* f. *molestus*, were present in Turkey [24]. The co-existence of these sibling species in same sampling sites [24, 50] and the existence of hybrids [50–52] suggest that they can exchange *w*Pip strains easily in natural populations.

We have identified three different *w*Pip groups, i.e. *w*Pip-I-II and IV in *Cx. pipiens* (*s.l.*) mosquito populations in Turkey. The only previous sample from Turkey, which has been assigned to *w*Pip groups, was a *w*Pip-IV group strain collected in Istanbul in 2003 [16, 35]. Other than this single case, the *w*Pip diversity in Turkish *Cx.*

Table 2 Crossing relationships between lines from Turkey (Tek *w*Pip-I & Tek *w*Pip-II) and reference laboratory *Wolbachia* strains

	Males	Tek *w*Pip-I	Tek *w*Pip-II	Istanbul *w*Pip-IV	Slab *w*Pip-III	LaVar *w*Pip-II	MaClo *w*Pip-III
	Mod	vi	ix	viii			
Females	***Resc***						
Tek I	**2**		C (24)	**IC (24)**	C (9)	C (17)	C (34)
Tek II	**2**	C (26)		**IC (32)**	C (26)	C (20)	C (14)
Istanbul	**3**	**IC (58)**	**IC (36)**		IC (34)[a]	**IC (40)**[a]	C (31)[a]
Slab		**IC (32)**	C (27)	C (33)[a]		IC (30)[a]	IC (99)[a]
LaVar		C (15)	IC (33)	**IC (26)**[a]	C (8)[a]		C (10) [a]
MaClo		C (18)	C (20)	IC (53)[a]	C (43)[a]	C(36)[a]	

[a]Data taken from Duron et al. [10]

Note: Reciprocal crosses between Tek *w*Pip-I, Tek *w*Pip-II, Istanbul *w*Pip-IV lines have been performed to identify natural CI patterns induced by these strains in the region. Additional reciprocal crosses between Turkish lines and 4 reference laboratory lines [LaVar (*w*Pip-II), MaClo and Slab (*w*Pip-III) and Istanbul (*w*Pip-IV)] have been performed to define the *mod-resc* functions of Tek *w*Pip-I and Tek *w*Pip-II isofemale lines and to compare them to previously defined ones by Atyame et al. [7]. Crosses were classified as either compatible (C, raft hatching > 90%) or incompatible (IC, raft hatching = 0–10%). Bidirectionally incompatible crosses are shown in bold. The number of egg-rafts collected for each cross is indicated in parentheses

pipiens populations was to date completely unknown. Although *w*Pip diversity was investigated in regions around Turkey [16]. Dumas et al. [16] have found *w*Pip-I strains in Middle East (Lebanon, Israel, Jordan) and in northern Africa (Tunisia), *w*Pip-II strains widely distributed in eastern Europe and Cyprus, and *w*Pip-IV strains in a patchy distribution in Europe -in areas dominated by other *w*Pip groups. We demonstrated that *w*Pip strains belonging to *w*Pip-I, *w*Pip-II and *w*Pip-IV, previously identified near Turkey, are all present in the *Cx. pipiens* (*s.l.*) populations within this country, suggesting that Turkey is a crossroads for *w*Pip strains from eastern Europe, Africa and Middle East as for their vector hosts.

The most widespread groups in Turkey, *w*Pip-I and *w*Pip-II, induce reciprocal compatibility between their hosts and co-exist in many populations. Indeed, different *w*Pip strains can co-exist in a single natural *Cx. pipiens* (*s.l.*) population [12, 35, 43, 53] and these coexisting strains are usually compatible with each other [43]. Mathematical models confirm that only compatible strains can stably coexist in unstructured and panmictic host populations when the fitness costs related to infection by different *w*Pip strains are the same [54, 55]. When bidirectional incompatibility inducing *w*Pip strains co-exist in one population the most prevalent strain is expected to eventually invade the population [54, 55] and when unidirectional incompatibility inducing *w*Pip strains co-exist, CI-inducing strain is expected to invade the population once above a frequency threshold [49, 55]. Therefore, a stable co-existence of incompatible strains is predicted to be rare. However, an example of the co-existence of unidirectional incompatibility inducing strains, belonging to a *w*Pip-I group and *w*Pip-IV group, has been shown in Tunisia while mathematical models predicted that *w*Pip-I should have invaded this area in only 4 generations [5, 49]. Atyame et al. [49] hypothesized that low dispersal and extinction-recolonization events could explain this stable co-existence. In one site in Turkey, we observed the co-existence of bidirectional incompatibility inducing strains (*w*Pip-IV and *w*Pip-II). The low prevalence of *w*Pip-IV and its incompatibilities suggest that it should disappear from the population. However, we have evidence that *w*Pip-IV strains were already present at least 13 years ago at Istanbul since it has been sampled in 2003 [35]. This persistence of *w*Pip-IV, at low frequencies, could be explained by higher fitness costs associated with *w*Pip-I and *w*Pip-II infections or by extinction-recolonization events of *w*Pip-IV-infected individuals as it has been suspected in Tunisia [43, 49, 55]. Fitness difference could be, for instance, linked to differences in fecundity [56, 57] or to a possible ability of the different *w*Pip strains to protect their hosts against other microbial infections [58–62]. Further studies on the differences between *w*Pip strains in terms of infection costs and pathogen protection might help to understand stable co-existence of bidirectionally incompatible *w*Pip-IV strains observed in Turkey.

To study the phenotypical diversity of crossing types in Turkey, we crossed Turkish isofemale lines harbouring *w*Pip-I and *w*Pip-II strains with four reference lines defined by Atyame et al. [7]. We inferred both their *mod* and *resc* functions and compared them to the eight *mod* and four *resc* functions already described worldwide. The Tek *w*Pip-I line showed the most common *resc* functions for a *w*Pip-I infected line (i.e. *resc* 2) but a rare *mod* function previously defined in few lines harbouring *w*Pip-I or *w*Pip-II strains (i.e. *mod* "vi"). The Tek *w*Pip-II line showed the same *resc* function as *w*Pip-I (i.e. *resc* 2) but a totally new *mod* function (i.e. *mod* "ix"). Our findings are consistent with theoretical predictions and empirical data suggesting new *mod* functions can more easily evolve and spread in the population than new *resc* functions [7, 63].

Natural CI properties induced by *Wolbachia* can be used to control the vector populations: the mass release of males harbouring incompatible *Wolbachia* into the natural populations can decrease the female reproduction and eradicate the pest/ vector populations (IIT) [29, 30]. Indeed, *w*Pip induced CI has been used against *Cx. quinquefasciatus* (formerly named *Cx. pipiens fatigans*) for the first time in 1967 to control filariasis in Southeast Asia [31]. More recently, natural CI properties caused by *w*Pip infection have been found promising to control *Cx. pallens* (no longer considered as a valid species) in China [32] and *Cx. pipiens* populations in La Réunion Island [33, 34]. In the latter study, a *w*Pip-IV strain from Istanbul has been successfully used to sterilise *w*Pip-I females in semi-field conditions. We demonstrated that this Istanbul strain also induces bidirectional incompatibility with mosquitoes harbouring *w*Pip-I or *w*Pip-II in Turkey. This means that most *Cx. pipiens* females in Turkey, except in few sites in the Thrace region, can be sterilised by the release of males infected with Istanbul strain. Although further studies on intrapopulation CI variability, mating choice, hatching rate and population dynamics in semi field populations are needed for *w*Pip-IV Istanbul to be used in future vector control programs in Turkey, a critical region for vector-borne diseases, our results suggest that it could constitute a good candidate.

Conclusions

We identified *w*Pip diversity in natural *Cx. pipiens* (*s.l.*) populations in Turkey. The previously described *w*Pip-IV group was in fact restricted to only two populations while *w*Pip-I and *w*Pip-II group are widely distributed and coexist in many populations all over the country. The *w*Pip-IV strain Istanbul was found bidirectionally incompatible with individuals harbouring *w*Pip-I or *w*Pip-II from Turkey. This highlights the potential of *w*Pip-IV

harbouring males as a vector control to sterilise local *Cx. pipiens* populations, particularly where only *w*Pip-I or *w*Pip-II harbouring females were found.

Abbreviations
C: Compatible; CI: Cytoplasmic incompatibility; IC: Incompatible; *Mod*: Modification ability; *Resc*: Rescue ability; *w*Pip: *Wolbachia pipientis*

Acknowledgements
We are thankful to Dr Hilal Bedir (Kafkas University, Kars, Turkey), Dr Berna Demirci (Kafkas University, Kars, Turkey) and Dr Mustafa Akiner (RTE University, Rize, Turkey) for their guidance during fieldwork, to Dr Sırrı Kar (Namık Kemal University, Tekirdağ, Turkey) for the collection of *Cx. pipiens* from Tekirdağ (Turkey), to Patrick Makoundou, Sandra Unal and Marco Perriat-Sanguinet from team EVAS (ISEM) for technical support.

Funding
This work was funded by the French ANR program (project ANR-16-CE02-0006-01 "CIAWOL", 2017-2020). Mine Altinli received support from Ministère des affaires étrangères (Campus France) *via* the French Embassy in Turkey and Project "ISEM Sud", 2016 for her PhD.

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Authors' contributions
MA, FG, MW and MS contributed to the conception of the study. MA, BA, FG did the sample collection. MA and FG identified samples, MA did the *Wolbachia* genotyping. MA and MS carried out crossing experiments. MA analysed the data and interpreted the results. MA and MS drafted the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 11 January 2018 Accepted: 6 March 2018
Published online: 20 March 2018

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4.3 CpDV Prevalence and Diversity in Turkey

4.3.1 Methods

4.3.1.1 Sample Collection.

Same larvae samples used in **Chapter 4.2** were also investigated for the prevalence and diversity of CpDV. The samples collected as adults from May to September in 2012-2015 using adult light traps, have been tested before the second collection and no CpDV was detected in these samples (n=76). To investigate whether the life stage of the mosquito can have an effect on the prevalence of CpDV, I collected new larvae samples from July to September 2016 some of which I let to become adults in insectary conditions in the water from which have been collected and stored as emerged adults in 70% ETOH.

4.3.1.2 PCR diagnostic test

CpDV genome contains three non-structural (NS) genes; NS3 ORF which is on the 5' side of NS1, and NS2 ORF overlapping the 5' half of the NS1 ORFs (Baquerizo-Audiot *et al.*, 2009)((Figure 1). In CpDV genome, NS1 and NS2 coding sequences split into four ORFs, instead of two ORFs like in other ambidensoviruses (Kerr *et al.*, 2005; Baquerizo-Audiot *et al.*, 2009) (Figure 1). NS1 gene includes highly conserved endonuclease called SF3 helicase which is generally used for densovirus taxonomy (Kerr *et al.*, 2005)(Figure 1).

We designed a diagnostic PCR using CpDV specific primers which amplify a 238 bp fragment **(Chapter 2)** within NS1-NS2 region (Figure 1). A total of 236 adults and 430 larvae were tested.

To investigate the diversity of CpDV in natural populations of *Cx. pipiens* mosquitoes in Turkey, specific primers to amplify fragments from both NS1 and VP coding region of CpDV genome have been used (Figure 1). These primers amplified most of the NS1' ORF of NS1 gene (Figure 1) that encompasses the SF3 helicase domain in the 5'end of NS1 and as well as other less conserved sites (1681-2648; 967 bp) and a part of VP region of CpDV that includes both conserved PLA2 and stretches for 1389 bp (3570-4959; 1389 bp, Figure 1). To increase the chance of amplifying these fragments, semi-nested PCRs were conducted with previously described protocols **(Chapter 2, see Supplementary Table 2 for protocols and primers).**

We have further designed specific primers within NS3 area to be able to have access to much more polymorphic region to discriminate CpDV between geographically close samples (Figure 1). For this a 1566 nt long fragment, including NS3 coding region, has been amplified using CpDV_355_F (5'-GGTCTGAATTGGCTGATGC-3') and CpDV_1902_R (5'- GTGTCCCAG-CAACTTCTC GA- 3') primers. Resulting PCR products have been used as a template to amplify a 1072 nt long fragment using CpDV_355_F and CpDV_1387_R(5'-CGAAATTAGCATTT-GACTCTCC-3').

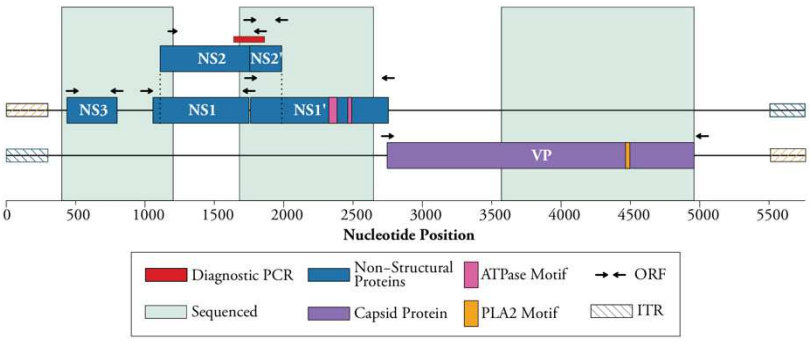


Figure 1.CpDV genome. CpDV has an ambisense genome: its non-structural genes (NS) are on one strand while its structural gene (VP) are on the complementary strand. CpDV has 3 different NS genes. NS1 and NS2 are transcribed from the same strand. Their ORFs overlap on the same strand and both are split into two ORF. ORFs shown with arrows. NS1 and VP genes have very conserved motifs such as PLA2 and endonuclease motifs. In red fragments used for diagnostic PCR test shown, and in green sequenced NS3, NS1 and VP fragments.

For both PCR reactions protocol was as following: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30s, and 72 °C for 90 s, and a final elongation at 72 °C for 5 min.

With three of these fragments amplified for most of the samples, we covered about 54% of the CpDV genome (3256 over 6000 nt, Figure 1).

All the PCR products were purified using AMPure (Agencourt) with Biomek 4000. Following the purification, DNA was quantified by nanodrop and sequencing reaction has been performed using 100ng of DNA (50ng/kb). After the completion of sequencing reaction (96°C for 5min, 30 cycles of 96°C 15 sec, 50°C 10sec and 60°C 4mn) products have been purified using CleanSEQ (Agencourt) with Pipeteur Biomek 4000, before the capillary electrophoresis of the sequences.

4.3.1.3 Data Analysis

For probabilistic phylogenetic analyses we have used concatenated matrix of all the NS3, NS1 and VP amplicons, from the Turkish populations. We have also added samples from three phylogenetic groups found in the worldwide phylogeny of CpDV (**Chapter 2**)(Slab, Armenia, Montpellier) and Reference genome, to be able to compare the diversity in Turkey to the worldwide diversity. MAFFT v7.273 (Katoh and Standley, 2013) was used to align the concatenated sequences.

In order to avoid misleading phylogenetic signal due to recombinant parts of sequences, signature of recombination we used the RDP, GENECONV, Bootscan, MaxChi, and LARD methods with default parameters, as implemented in the RDP4 software (Martin *et al.*, 2015). Recombinant regions of the sequences have been excluded while the rest of the sequence was kept. Modelfinder (Kalyaanamoorthy *et al.*, 2017) was then used to find the best fitting model (Lemmon and Moriarty, 2004). Phylogeny of CpDV in Turkey was then inferred under the HKY85 + \Gamma model of sequence evolution using PhyML. Statistical support of the phylogenetic nodes was computed using a bootstrap procedure (Felsenstein, 1985).

4.3.2 Results

4.3.2.1 Prevalence of CpDV.

Excluding all the adults that were collected between 2012-2015 with adult traps and were negative, 60% of the samples tested showed a presence of CpDV (Figure 2). The proportion of infected larvae was lower compared to infected adults, 58% and 64% respectively. The effect of the life stage was significant however it only explained a low amount of deviance (binomial glm, df=1, dev=18.896,p<0.001, Figure 3A). There was also a significant effect of collection location on the CpDV prevalence (binomial glm, df=36, dev=210.74, p<0.001). There was no effect of *w*Pip group on the prevalence of CpDV (binomial glm, df=2, dev=0.745, p>0.5, Figure 3B).

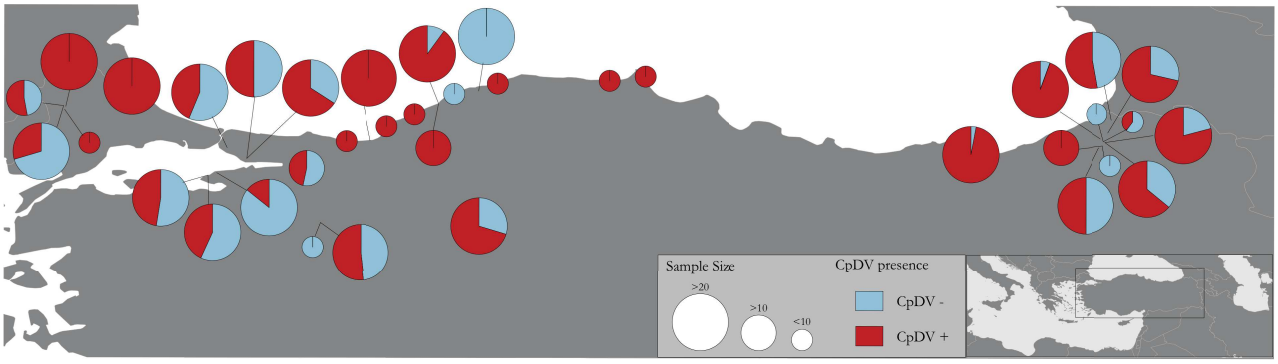


Figure 2. Prevalence of CpDV in natural populations of *Cx. pipiens* (s.l.) in Turkey. A PCR test based on the amplification of partial NS2 region is used to detect CpDV. Only samples that have been collected in 2016 are shown. All samples collected between 2012-2015 with adult traps were negative.

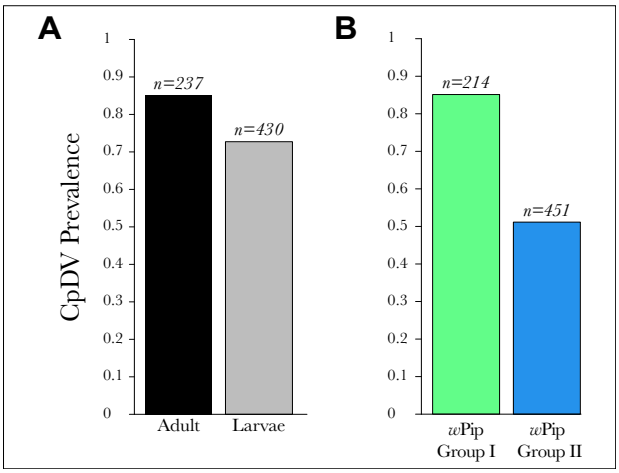


Figure 3 Life stage & CpDV prevalence (A). CpDV presence was detected using a PCR diagnostic test amplifying NS2 region. Adults were more likely to be CpDV positive (binomial glm, df=1, dev=18.896, p<0.001). ***w*Pip group & CpDV prevalence (B).** Mosquitoes that have been tested for their *w*Pip group also tested with PCR diagnostic test and prevalence of CpDV was recorded. Generalized linear model did not show any effect of *w*Pip diversity on (df=2, dev=0.745, p>0.05)

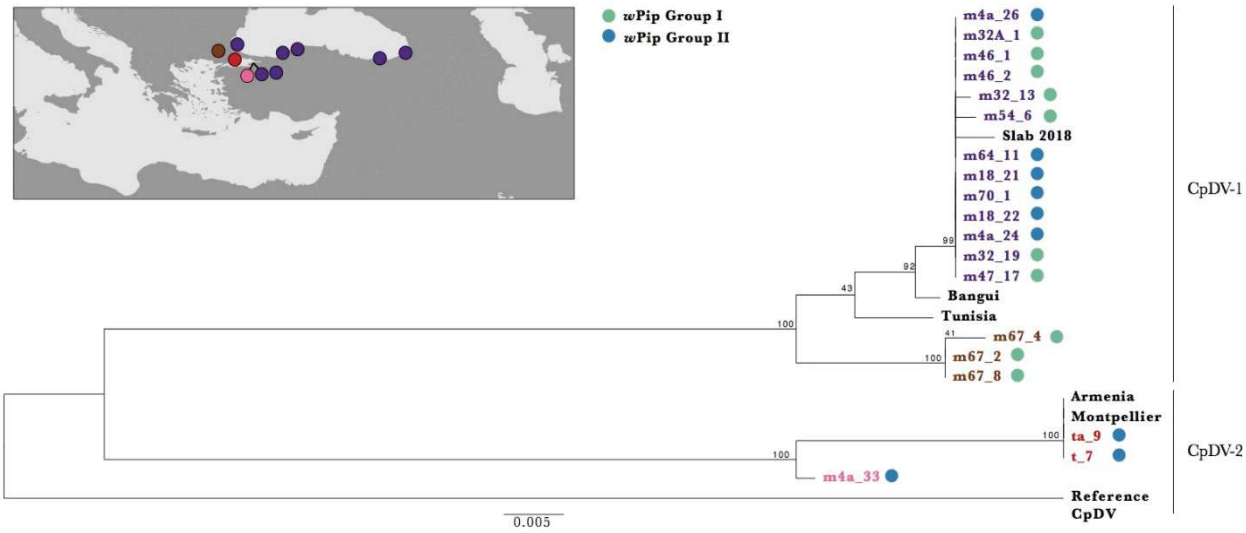
As discussed in **Chapter 4.2** mosquitoes infected with *w*Pip-IV were limited to two locations and there were only 11 of them collected. Therefore, we excluded these samples and analysis of the subset of the data only including the dominant *w*Pip groups (*w*Pip-I and *w*Pip-II) gave similar results (binomial glm, df=1, dev=0.047, p>0.5, Figure 4)

4.3.2.2 Diversity of CpDV

Maximum likelihood tree based on the partial NS3-NS1 and VP sequences showed that there were four distinct CpDV variants in Turkey (depicted with different colours on the map and sample names in Figure 4). Reference CpDV genome, along with other samples from around the world (Slab 2018, Bangui, Tunisia, Armenia, Montpellier) have been used to understand the diversity in Turkey compared to worldwide phylogeny of CpDV (**Chapter 2**). General CpDV diversity in Turkey grouped in two clades (Figure 4). Most of the sequenced samples belong to CpDV-1 (as defined in **Chapter 2**, Figure 4). These samples were collected mostly from Northern Turkey and they were really closely related to each other (purple points on the map, Figure 4) and the current laboratory variant Slab 2018. Interestingly all the four variants were present in North-Western Turkey. One variant (shown in red, Figure 4) was really closely related to the samples from Armenia and Montpellier.

Both *w*Pip-I and *w*Pip-II harbouring mosquitoes were found infected with variants from CpDV-1. On the contrary, CpDV-2 was only found in the mosquitoes harbouring *w*Pip-II *Wolbachia*.

Figure 4 CpDV diversity in Turkey. Maximum likelihood phylogenetic tree constructed using partial NS3, NS1 and VP sequences. Bootstrap values shows the statistical support of the nodes. Closely related variants were given a colour (purple and brown within CpDV-1; Pink and red within CpDV-2) and the location from where these variants were collected are shown on the map. *w*Pip groups associated with the same samples were given in green (*w*Pip-I) or blue (*w*Pip II). Reference CpDV genome, along with other samples from around the world (Slab 2018, Bangui, Tunisia, Armenia, Montpellier) have been used to understand the diversity in Turkey compared to worldwide phylogeny of CpDV.



4.4 Conclusions

A high prevalence of CpDV has been shown worldwide (**Chapter 2**). Focusing on Turkey we investigated the possible effects of life stage and *w*Pip genetic diversity. Samples that have been collected as adults did not show any CpDV presence, although when larvae have been collected, emergent adults were more likely to be infected. The longer storage time of the samples collected as adults (2012-2015) could have made impossible to detect CpDV but one should keep in mind that we were able to amplify and identify *w*Pip strains using the same DNA extractions. Alternatively, CpDV could be emerging in Turkey only recently, reaching higher frequency in 2016. Another possibility could be that CpDV infected adults are less likely to be collected with adult traps and there could be several reasons for that: i) high mortality in larvae resulting in very low prevalence in adults, ii) reduced adult lifespan, iii) reduced dispersal. A similar pattern has been observed in AeDV Thai strain prevalence in natural populations of *Ae. aegypti* and *Ae. albopictus* (Kittayapong *et al.*, 1999). In *Ae. aegypti* adults collected from Thailand (n=97), AeDV Thai strain prevalence was 44.3% but there were no AeDV in *Ae. albopictus* adults tested (n=79) although AeDV Thai strain was able to infect both species in experimental infections (Kittayapong *et al.*, 1999). Interestingly, in our laboratory lines we have observed the opposite: randomly sampled adults were less likely to be infected by CpDV compared to larvae **(Chapter 3).** Given that samples from natural populations were collected as soon as they emerged, a reduced lifespan of the adults could explain the difference observed between natural and insectary populations. Other mosquito densoviruses have been shown to reduce the lifespan of their *Ae. aegypti* adults (Suchman *et al.*, 2006) although the effect of CpDV on their host is still not clear.

After identifying *w*Pip diversity in Turkey we have also investigated whether there is an effect of *w*Pip diversity on CpDV prevalence. Although the percentage of mosquitoes harboring *w*Pip-I that were also CpDV positive was higher than the other *w*Pip groups, this effect was not significant. In fact most of the variance in the data was explained by the location where the mosquitoes have been collected. The fact that populations where different *w*Pip groups co-existed was rare made further analysis of *w*Pip diversity-CpDV prevalence correlation impossible, especially since we observe such an high impact of the collection location. On the other hand if mosquitoes harboring different *w*Pip groups were collected from the same environmental conditions, both of the groups would have the same probability to be infected, in terms of the effect of the environmental conditions, hence we could compare their CpDV infection status directly.

We have identified two clades of CpDV in Turkey, from three that has been found worldwide **(Chapter 2)**. Similar to what we have observed in worldwide populations, most of the CpDV variants belonged to the CpDV-1 clade. There was no clear association between CpDV and *w*Pip diversity. All of the samples collected from the populations around the Blacksea coast showed the same variant of CpDV suggesting the efficient dispersal of this variant between the populations of this area. It is known that adult mosquitoes can contaminate new oviposition sites with mosquito densoviruses (Wise de Valdez *et al.*, 2010), they can also be transmitted by human activity. It is also important to note that most of these samples collected around Blacksea coast were collected from old tyres filled with rain water. On the other hand the lack of the two other variants (shown in red and brown Figure 5) in this area might suggest a barrier between the populations harbouring the red, brown and purple variants or a more recent introduction of red and brown variants to Turkey.

Our results demonstrated the diversity of both *w*Pip groups and CpDV in Turkey, especially in North-Western part (Thrace region). Both the diversity of insect-specific flaviviruses and the circulation of West-Nile virus vectored mainly by *Cx. pipiens* mosquitoes have previously been demonstrated in the same region (Ergunay *et al.*, 2013, 2014, 2015; Öncü *et al.*, 2018). Indeed Turkey with its unique geographical location, climate and arthropod diversity, could represent a suitable habitat for circulating viruses to amplify even more and for emergence of new viruses. Overall our results were also in accordance with the previously reported Cx. pipiens diversity for the area (Gunay *et al.*, 2015) and suggested Turkey could be a place where different populations of *Cx. pipiens* mix.

This highlights the importance of this area, for surveillance efforts against arboviruses to prevent putative epidemics and for investigation of the ecological interactions between *w*Pip groups, arboviruses and insect-specific viruses in natural mosquito populations.

4.5 References

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Chapter 5:
Towards Understanding *Wolbachia*-CpDV interactions in natural populations

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5.1 Influence of *Wolbachia* on prevalence, virus load and diversity of *Culex pipiens* densoviruses

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Influence of *Wolbachia* on prevalence, viral load and diversity of *Culex pipiens* densoviruses

Abstract: *Culex pipiens* densovirus (CpDV), belong to *Densovirinae* subfamily and have been initially isolated from laboratory *Cx. pipiens* colonies. Recently their wide prevalence and their ability to vertically transmit were reported, highlighting their long-term associations with their hosts. In nature *Cx. pipiens* are always found infected with *Wolbachia* *w*Pip from one of the five phylogenetic groups (*w*Pip-I to V). *Wolbachia* are endosymbiotic bacteria which deeply modify their host phenotypes including their reproduction, for instance *via* cytoplasmic incompatibility, and their survival for instance *via* viral interference. Given the importance and co-occurrence of these two *Cx. pipiens* endosymbionts, we have investigated the putative reciprocal influence between CpDV and *Wolbachia* in natural populations of *Cx. pipiens*. For this we have chosen a specific zone in Northern Tunisia where two genetically distinct incompatible *w*Pip groups co-exist in same environmental conditions and where the prevalence of CpDV is high. Our results showed an effect of *Wolbachia* groups and their titers, on the prevalence, viral load and diversity of CpDV. Overall our results represented a good evidence for *Wolbachia* and CpDV interactions in natural populations and highlighted the potential importance of bacterial ensodymbiont on insect specific virus ecology.

5.1.1 Introduction

Densoviruses (*Densovirinae*) are small single stranded DNA viruses of arthropods (Cotmore *et al.*, 2014; Tijssen *et al.*, 2016). They belong to *Parvoviridae* family, a virus family that consists of animal viruses, including vertebrate (*Parvovirinae*) viruses (Cotmore *et al.*, 2014; Tijssen *et al.*, 2016). Their existence in a broad range of invertebrate hosts, sometimes even as endogenous viral elements, suggests that they might have an important influence on their hosts' phenotypes and evolution (Liu *et al.*, 2011; Thézé *et al.*, 2014; Metegnier *et al.*, 2015; François *et al.*, 2016). Mosquito densoviruses (MDVs) have long attracted scientific attention as vector control tools due to the pathogenicity and host specificity reported for some of them (Buchatskiĭ *et al.*, 1987; Carlson *et al.*, 2006; Johnson and Rasgon, 2018). Most of MDVs are closely related (82-99% sequence homology) belonging to the genus *Brevidensovirus* (Cotmore *et al.*, 2014). However one MDV, namely *Culex pipiens* densovirus (CpDV), differs extensively from other MDVs and belongs to *Ambidensovirus* genus (Jousset *et al.*, 2000; Baquerizo-Audiot *et al.*, 2009). Indeed CpDV with their larger genome (6kb compared to 4kb for brevidensoviruses), ambisense genome organization and sequence homology, is closely related to lepidopteran and blattodean Ambidensoviruses but not to other MDVs (Jousset *et al.*, 2000; Baquerizo-Audiot *et al.*, 2009; Cotmore *et al.*, 2014). First discovered during high mortality episodes of laboratory *Cx. pipiens* larvae (Jousset *et al.*, 2000), CpDV have since been shown to persist at low densities in seemingly healthy *Cx. pipiens* lines and found widespread in natural populations worldwide (**Chapter 2**, **Chapter 3**). Moreover, their hosts *Cx. pipiens* can harbour both *Wolbachia* -widespread endosymbiotic bacteria of arthropods- and CpDV in their germinal cells resulting in the vertical co-transmission of both endosymbionts (**Chapter 3**).

Such co-existence of *Wolbachia* and CpDV within the same cells in *Cx. pipiens*, especially during transmission, (**Chapter 3**) suggests that these endosymbionts may interact and influence their respective prevalence, load and diversity. Both antagonistic and beneficial interactions between *Wolbachia* and viruses have previously been reported. However, antagonistic interactions have

been by far more studied showing that *Wolbachia* can decrease virus multiplication by indirectly modulating their host resistance or tolerance, or directly by competing with the virus for host resources (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Moreira *et al.*, 2009; Mousson *et al.*, 2010; Hussain *et al.*, 2012). The strength of this antagonism seems to mostly depend on variations in *Wolbachia* titers between different strains (Lu *et al.*, 2012; Osborne *et al.*, 2012; Bian *et al.*, 2013; Martinez *et al.*, 2017). For instance, *Wolbachia* (*w*Pip), when exhibiting a higher titer in some *Cx. pipiens s.l.* lines, reduced the West Nile Virus load (Micieli and Glaser, 2014). To date, such antagonistic interactions of *Wolbachia* have only been revealed against RNA viruses but never against DNA viruses (Teixeira *et al.*, 2008; Graham *et al.*, 2012). On the contrary, *Wolbachia* seem to increase *Spodoptera exempta* nucleopolyhedrovirus (SpexNPV) load, a double stranded DNA virus, and decrease the host survival in African armyworm. Furthermore in Drosophila *Wolbachia* did not have an effect on Insect Iridescent virus (double stranded DNA virus) infection in Drosophila (Teixeira *et al.*, 2008; Graham *et al.*, 2012).

As many medically important mosquito species are infected with *Wolbachia* (Rasgon and Scott, 2003; Dumas *et al.*, 2013; Baldini *et al.*, 2014; Gomes *et al.*, 2017), either naturally or stably transinfected, *Wolbachia* could be a major determinant of MDVs dynamics in natural mosquito populations. Because all the mosquitoes of *Cx. pipiens* complex are naturally infected with *w*Pip strains belonging to five genetically distinct phylogenetic groups *Wolbachia*-CpDV interactions might be frequent both in laboratory colonies and natural populations (**Chapter 3**). The *w*Pip strains strongly manipulate their host's reproduction by causing cytoplasmic incompatibility (CI), a sperm-egg incompatibility resulting in embryonic death (Werren, 1997; Atyame, Delsuc, *et al.*, 2011). The diversity of *w*Pip strains leads to a huge diversity in CI patterns in *Cx. pipiens*, including bidirectional (where the incompatibility is reciprocal between different strains) and unidirectional incompatibilities (where only one of the strains leads to an incompatible cross when carried by the male mosquitoes) (Atyame 2014). This diversity has recently been explained by the amplification and the diversification of an operon within WO phage region in *w*Pip genomes (Bonneau *et al.*, 2018). In natural *Cx. pipiens* populations, different *w*Pip strains can coexist when they are compatible with each other (Duron *et al.*, 2006, 2011; Atyame, Duron, *et al.*, 2011; Altinli *et al.*, 2018) while a stable co-existence of incompatible strains is predicted to be rare in unstructured and panmictic host populations (Rousset *et al.*, 1991; Engelstädter and Telschow, 2009). A rare example of stable co-existence between incompatible *w*Pip strains, belonging to *w*Pip-I and *w*Pip-IV groups, over at least a 7-years period has been shown in Northern Africa. By mapping the distribution of *w*Pip strains in Algeria and Tunisia and describing their CI patterns, Atyame et al. (2015) predicted that the *w*Pip-I should outcompete the *w*Pip-IV in only several generations (Atyame *et al.*, 2015). While the authors further hypothesized that low dispersal and extinction-recolonization events may contribute to explain this stable co-existence, *Wolbachia's* virus interference could also influence this stability: if *w*Pip groups interact differently with viruses either in antagonistic or beneficial ways, it can also influence their co-existence in addition to their CI properties (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Brownlie and Johnson, 2009; Fenton *et al.*, 2011; Hughes *et al.*, 2011).

The presence of CpDV has recently been revealed in this contact zone between these two *w*Pip groups (**Chapter 2**). Here, we tested the hypothesis that mosquitoes harbouring *w*Pip-I and *w*Pip-IV groups might differ in terms of their interactions with CpDV may be affecting its dynamics and distribution. To this end, we have studied CpDV prevalence and analysed them together with previously published results of *w*Pip groups distribution in the same samples (Atyame *et al.*, 2015). We additionally quantified both CpDV and *w*Pip loads to study their potential reciprocal influence. Lastly, given their co-vertical transmission (**Chapter 3**), we investigated putative link between CpDV diversification and *w*Pip diversity in natural populations of *Cx. pipiens* from this area.

5.1.2 Methods

5.1.2.1 Sample collection

All the samples have been collected between 2010 and 2011 from natural populations of *Cx. pipiens s.l.* in Tunisia (Atyame *et al.*, 2015) during larval stage (Supplementary Table 1). DNA has been extracted as described in Atyame et al. (2015) and stored in -80 °C after its utilization for identification of *w*Pip groups (Atyame *et al.*, 2015).

5.1.2.2 Prevalence of CpDV inferred by diagnostic PCR test on NS2 region

A CpDV specific PCR based diagnostic test was used to infer CpDV prevalence in the area. This test consists of an amplification of a 238 bp fragment using CpDV-For (5'- ACCACCACGGACTGGTATGA- 3') and CpDV-Rev (5'-TCCCCATTATCTTGCTGTCG-3') primers within conserved NS2 coding region. PCR was performed with the following conditions: initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation, annealing and elongation 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 min (**Chapter 1**).

5.1.2.3 Diversity of CpDV by sequencing variable NS3 region

To detect the diversity in geographically close populations, a 1566 nt long fragment including NS3 coding region, has been amplified using CpDV_355_F (5'-GGTCTGAATTGGCTGATGC-3') and CpDV_1902_R (5'- GTGTCCCAGCAACTTCTCGA -3') primers. Resulting PCR products have been used as a template to amplify a 1072 nt long fragment using CpDV_355_F and CpDV_1387_R (5'-CGAAATTAGCATTTGACTCTCC-3'). These fragments than sequenced using only CpDV_1387_R primer resulting in 800 nt long sequences.

For both PCR reactions protocol was as following: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 56 °C for 30s, and 72 °C for 90 s, and a final elongation at 72 °C for 5 min. PCR products were purified using AMPure (Agencourt) with Biomek 4000. Following the purification, DNA was quantified by nanodrop and sequencing reaction has been performed using 100ng of DNA (50ng/kb). After the completion of sequencing reaction (96°C for 5min, 30 cycles of 96°C 15sec, 50°C 10sec and 60°C 4mn) products have been purified using CleanSEQ (Agencourt) with Pipeteur Biomek 4000, before the capillary electrophoresis of the sequences.

5.1.2.4 Assessing CpDV and *Wolbachia* loads in a single qPCR assay

To test whether the *Wolbachia* load differed between mosquitoes harbouring different *Wolbachia* groups (*w*Pip-I and *w*Pip-IV) and whether it affected the CpDV load in the area, we quantified CpDV and *Wolbachia* amount for the same individuals using Real-time quantitative PCR (qPCR). SYBR Green I Master (Roche) and LightCycler 480 Instrument (Roche), LightCycler 480 software (version 1.5.1.62) were used to collect and analyse the results. Three PCR reactions were performed in triplicates using for each DNA sample i) NS2 protein coding region specific primers to quantify CpDV (CpDVquantiF: 5'-CATTGGAGGAGAAAGTTGGAA-3' , CpDVquantiR: 5'-TCCCCATTATCTTGCTGTCG-3'), ii) *wsp* specific primers to quantify *Wolbachia* (wolpipdir 5'-AGAATTGACGGCATTGAATA-3' and wolpiprev 5'-CGTCGTTTTTGTTTAGTTGTG-3' (Berticat *et al.*, 2002)) and iii) *Culex* Ace-2 locus specific primers to quantify host genome copy as a reference (acequantidir: 5'-GCAGCACCAGTCCAAGG-3', acequantirev: 5'-CTTCACGGCCGTTCAAGTAG-3') (Weill *et al.*, 2000). Dilutions of a pCR®4-TOPO- Vector (Invitrogen) containing a single copy of each *ace-2*, *wsp* and *ns2* gene fragments were used as standard curves. As such the amount of *Wolbachia* and CpDV were normalized with the amount of ace-2 copies in order to obtain a number of virus or *Wolbachia* per cell. We have tested 114 samples in total and samples were chosen to balance the years and *Wolbachia* types (n=114; *w*Pip-I n= 52, *w*Pip-IV n=62; Col-

lected in 2010 n=69, Collected in 2011 n=45). Samples that showed CP>33 for any of the PCR reactions have been excluded because of the high uncertainty of the quantification at such low titter levels.

5.1.2.5 Phylogenetic Analyses of CpDV diversification

Sequences were aligned using MAFFT v7.273 (Katoh and Standley, 2013). In order to avoid misleading phylogenetic signal due to recombinant parts of sequences, recombinant region was identified and removed using RDP4 software (Martin *et al.*, 2015). Modelfinder (Kalyaanamoorthy *et al.*, 2017) has been used to find the best fitting model (Lemmon and Moriarty, 2004). Phylogenetic tree has been constructed under the HKY85 + \Gamma model of sequence evolution using RAxML v8.2 (Stamatakis, 2014). Statistical support of the phylogenetic nodes was computed using a bootstrap procedure (Felsenstein, 1985). Reference genome of CpDV (Baquerizo-Audiot *et al.*, 2009) has been used as an outgroup to root the phylogenetic tree.

5.1.2.6 Statistical Analyses

All data analyses have been performed in R version 3.3.1.

To analyse the effects of *population, collection year* (2010-2011) and *Wolbachia groups (w*Pip-I, *w*Pip-IV) on CpDV prevalence, we first fitted a generalized linear model from binomial family. Likelihood ratio tests of the full model against the model without a given effect were used to obtain deviance and p-values. A subset of the data then created to include only the populations where both *Wolbachia* strains co-existed and where at least one sample was CpDV positive. Hence in each population tested, the probability of a given sample to be infected by CpDV assumed to be independent from the location where samples were collected from. A binomial generalised linear model was used to test the effect of the *collection year* (2010 or 2011) and *Wolbachia groups (w*Pip-I, *w*Pip-IV) on CpDV prevalence. To investigate the effects of *Wolbachia* groups, *Wolbachia* load and location on CpDV load a generalised linear model was fitted.

Spatial correlation between the distribution of CpDV clades and geographical location of collection sites as well as *w*Pip group distribution have been tested using Mantel test included in ecodist package (Goslee and Urban, 2007).

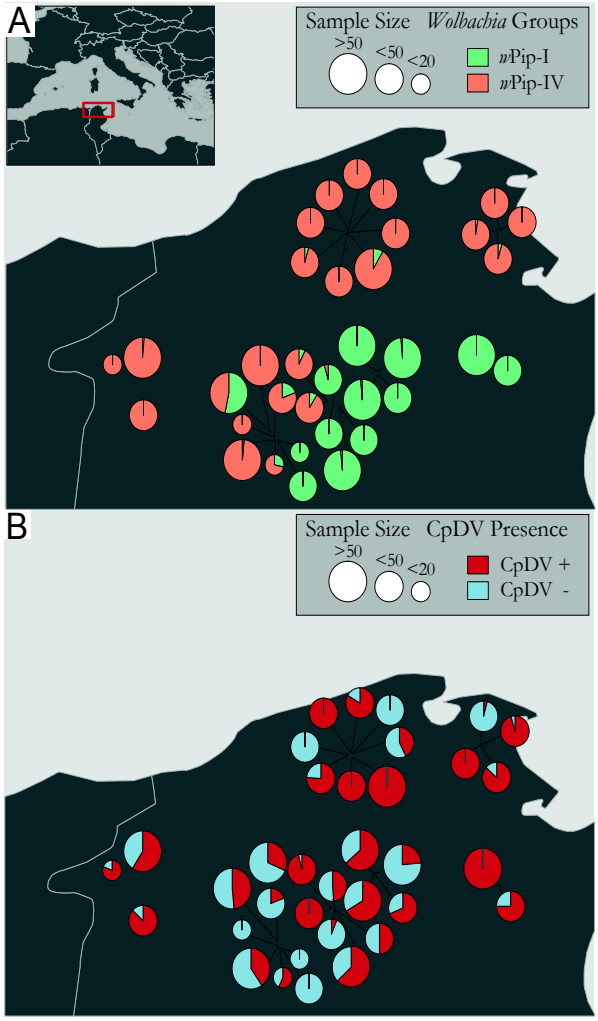

Figure 1 *Wolbachia* groups and CpDV prevalence in the contact zone. A total of 1588 samples collected from 35 different populations between 2008-2011, have been tested. **A. *w*Pip strain distribution in the area.** (Adapted based on the results of Atyame et al. 2015) There are two distinct *w*Pip strains in the area; *w*Pip IV in south-eastern populations and *w*Pip I north-western populations with a narrow contact zone between them. In the samples tested, the distribution of *Wolbachia* strains was significantly affected by year and population (glm, year: df=3, dev=17.128, p<0.001; population: df=34, dev=1587.3, p<0.001). **B. CpDV prevalence and distribution in the area.** A total of 933 was infected. In this global analysis of all sampled individual there was no apparent significant link between CpDV prevalence and *w*Pip groups distribution (glm, df=1, dev=0.109, p>0.05).

5.1.3 Results

5.1.3.1 Analysis of CpDV prevalence in the all sampling area

We used 1230 samples, that have been collected between 2010-2011 by Atyame et al. (2015) and *w*Pip groups identified (Figure 1A) and further tested them to infer CpDV prevalence in and around the *w*Pip-I/*w*Pip-IV contact zone. In such global analysis of all these samples from different locations (*i.e.* populations), *Wolbachia* groups (*i.e.* *w*Pip-I versus *w*Pip-IV) had no significant effect on CpDV prevalence (glm, df=1, dev=0.108, p=0.741, Figure 1). However, CpDV prevalence significantly differed between locations (Figure1B, Population: glm, df=34, dev=638.04, p<0.001). CpDV prevalence was quite stable between years 72 % in 2010 (n=676) and 76% in 2011 (n=554).

5.1.3.2 Analysis of CpDV prevalence in host populations where different *Wolbachia* strains co-existed in similar ecological conditions

The absence of CpDV in some locations could reflect that these populations never had encountered CpDV because the environmental conditions where not favourable for infection. As this could bias the results, we performed the analysis on a subset of the data only considering the populations where at least one individual was CpDV positive 1 (per year/per population) and both *Wolbachia* groups co-existed. This way, we were able to assess *w*Pip I and *w*Pip IV groups effects on CpDV prevalence in same ecological conditions (*i.e.* the same probability of getting infected with CpDV for *Cx. pipiens* infected with *w*PipI and *w*PipIV).

CpDV prevalence in relation to Wolbachia groups

Main factor that affected the CpDV prevalence, specifically in the contact zone, were the *Wolbachia* groups (i.e. *w*Pip-I *versus* *w*Pip-IV) (glm, df=1, dev= 16.492, p< 0.001, Figure 2). Indeed, mosquitoes harbouring the *w*Pip-IV were 34 % (RR=1.338; OR=2.791, 95% CI: 1.709-4.542; p<0.001) more likely to be CpDV positive. This difference was constant between the two years of sampling that were not significantly differing from one another (glm, df=1, dev=2.739, p= 0.097, Figure 2).

5.1.3.3 Relation between CpDV and *Wolbachia* loads

CpDV load got as high as 82900 CpDV/host cell in only one sample from El Manar population (Figure 3A). This sample probably representing a peak of an acute infection was an outlier that we excluded from further analyses. Overall, the average load of CpDV per host cell of *Cx. pipiens* larvae was 3

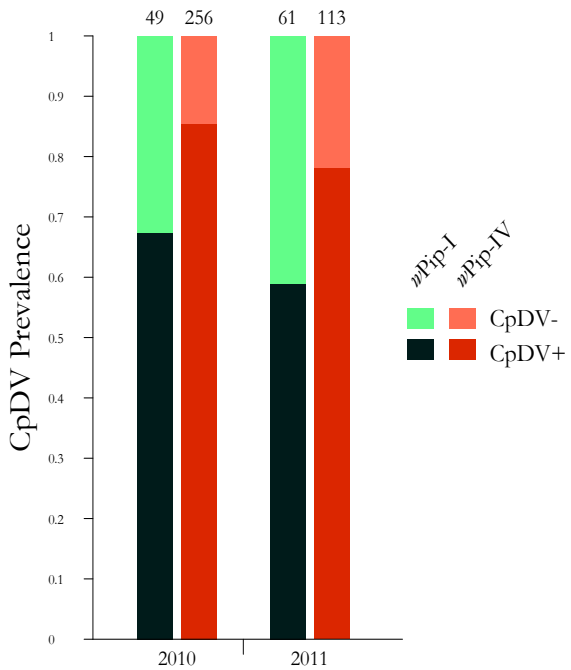

Figure 2. Effect of *Wolbachia* group on CpDV prevalence in the *Cx. pipiens* populations where both *w*Pip-I, *w*Pip-IV and CpDV co-exist in the same environmental conditions. The data set was reduced only to locations where both *Wolbachia* strains and CpDV present. The proportions of both CpDV positive (in darker colours) and CpDV negative (in lighter colours) mosquitoes were indicated relative to the groups of *Wolbachia* *w*Pip-I versus *w*Pip-IV they carry. Sample size for each *w*Pip group is shown on the top of the bars. A total of 378 samples over 479 samples were found CpDV positive. CpDV distribution was significantly affected by the *Wolbachia* group (glm, df=1, dev= 16.492, p<0.001). Mosquitoes harbouring the *w*Pip-IV were 34 % (RR=1.338; OR=2.791, 95% CI: 1.709-4.542; p< 0.001) more likely to be CpDV positive. Year of collection did not have a significant effect in the complete model (glm, df=1, dev=2.739, p>0.05).

(±0.911). However, CpDV load significantly differed between populations where larvae were sampled from (df=8, dev=127.27, p< 0.001, Figure 3A). This was mainly based on the samples from the El Manar population that showed the highest mean of CpDV/host cells (El Manar n=21, mean=14.881±3.925) while samples from Fontaine (n=61, mean=0.055±0.0180) populations showed really low amount.

Average *Wolbachia* amount per host cell was 0.356 (±0.052). The *Wolbachia* load was not different between *Wolbachia* groups or collection years (df=1, dev=0.162, p=0.359 and df=1, dev=0.571, p=0.085, respectively). However, *Wolbachia* load differed between populations (df=8, dev=3.147, p=0.038).

CpDV load differed with respect to *Wolbachia* load (df=1, dev=9.839, p< 0.001, Figure 3B), although there was no significant effect of *w*Pip groups on the CpDV load (df=1, dev=0.256, p>0.05). More specifically, CpDV load was positively correlated with *Wolbachia* load (r^2=0.11 p< 0.001, Figure 3C)

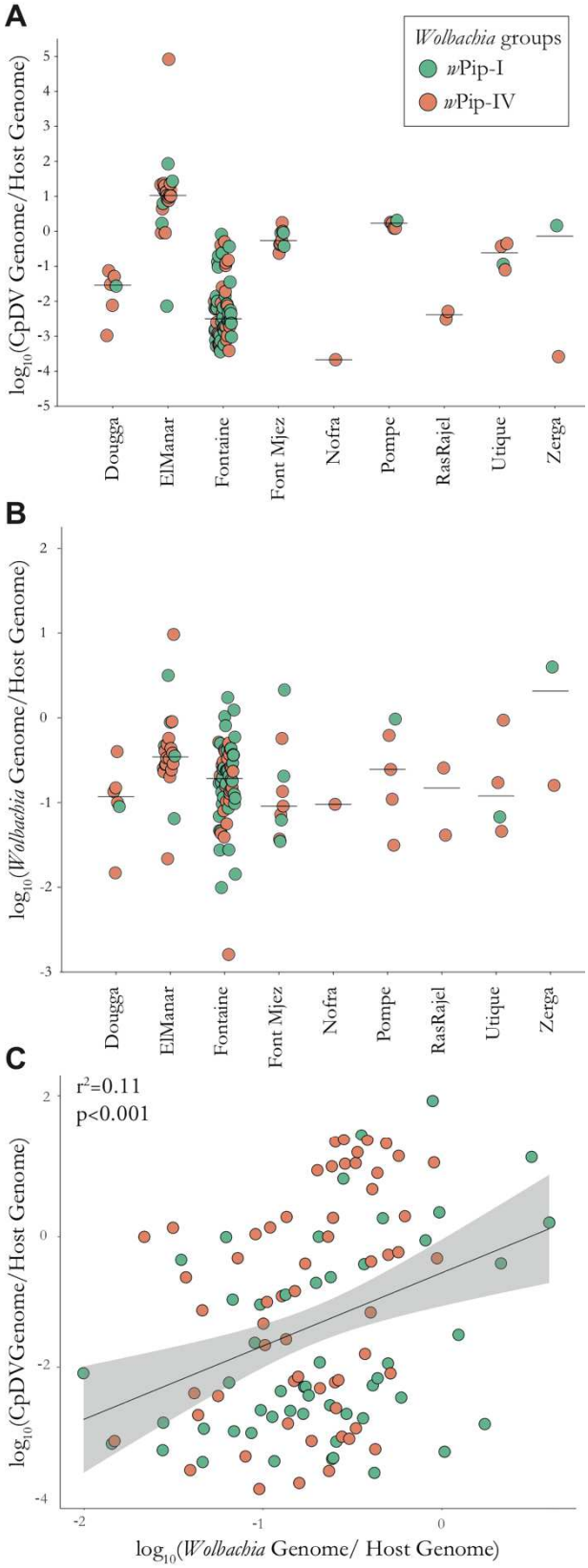

Figure 3. Relation between *Wolbachia* and CpDV amounts in the *Cx. pipiens* populations where both *w*Pip-I, *w*Pip-IV and CpDV co-exist in the same environmental conditions. Both *Wolbachia* and CpDV densities were quantified by qPCR assays using *wsp* and NS2 gene copy number, respectively, relative to *ace2* mosquito gene in a total of 114 samples. Samples with Cq>33 for any of the qPCR reactions have been excluded from the analysis and the graph because of the high uncertainty of the quantification. **A. CpDV load.** CpDV load reached 82900 CpDV/host cell in one sample from El Manar population and this was excluded from further analysis as an outlier. CpDV load was affected mainly *Wolbachia* loads (df=1, dev=9.840, p< 0.001) but also by population (df=8, dev=127.27, p<0.001). Samples from the El Manar population showed the highest mean of CpDV / host cells (El Manar n=21, mean=14.881±3.925) while samples from Fontaine (n=61, mean=0.055±0.0180) showed really low amount. **B. *Wolbachia* load**. Outliers from Fontaine population were excluded from analyses. Average *Wolbachia* amount per host cell was 0.356 (±0.052). *Wolbachia* load also seemed to be influenced by CpDV load (glm, df=1, dev=3.050, p<0.001) and the population (df=8, dev=3.1472, p<0.05) but not by the collection year (df=1, dev=0.571, p>0.05) and the *Wolbachia* group (df=1, dev=0.162, p>0.05). **C. Correlation between *Wolbachia* and CpDV loads.** CpDV load significantly increased with the increased *Wolbachia* load (r^2=0.11, p<0.001).

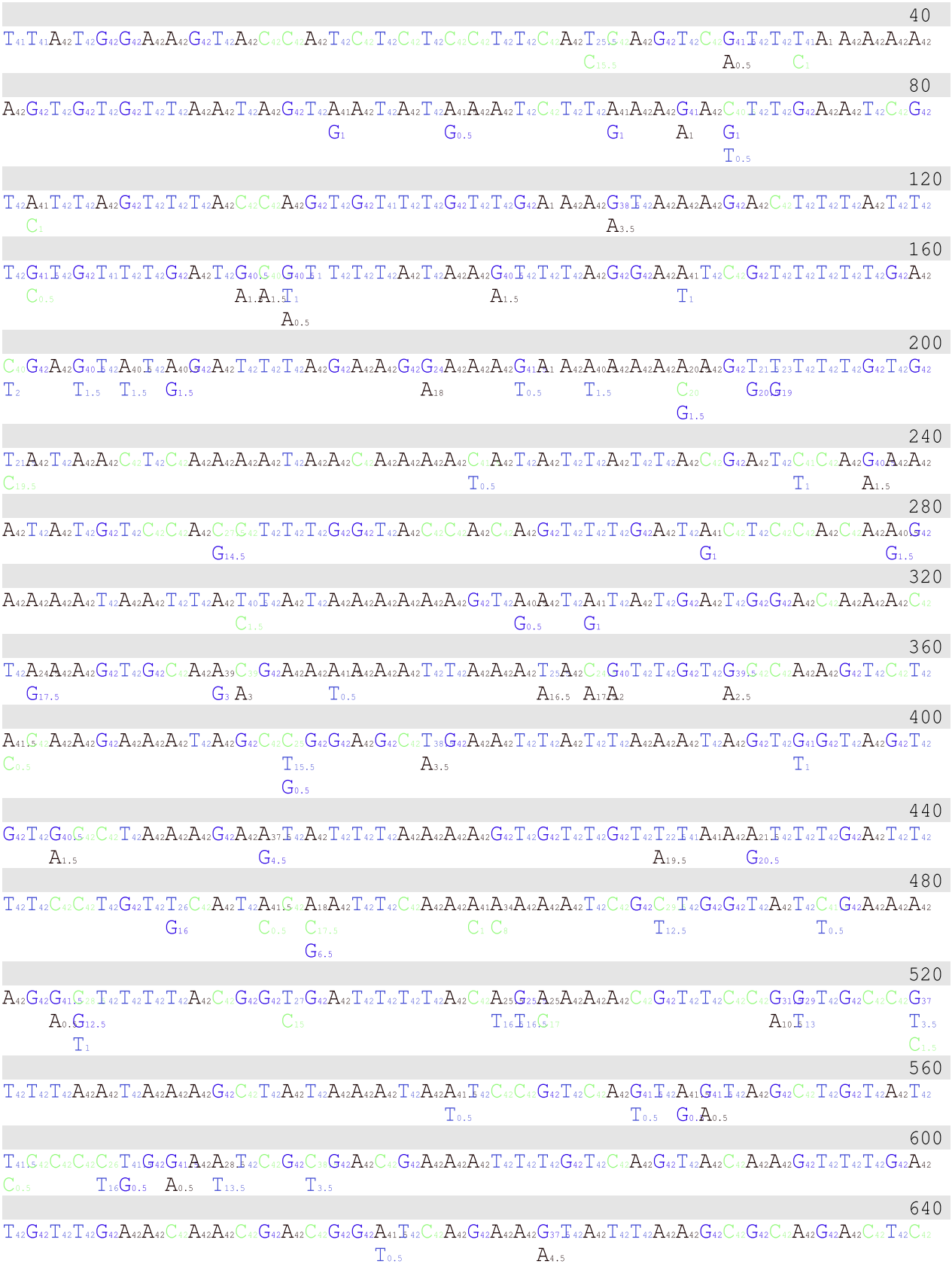

Figure 4. Sequence Alignment of CpDV genomes. To infer the diversity of CpDV from geographically close locations we have sequenced a divergent part of the CpDV genome including NS3 coding region. Subscript under each nucleotide represents how many variant had a given nucleotide. $N_{0.5}$ indicates double peaks. A total of 42 sequences are shown after clonal sequences were removed. Numbers on the right side represents the nucleotide position within the sequenced fragment. Figure generated using classic.datamonkey.org

5.1.3.4 Diversification of CpDV in relation with *Wolbachia* groups

CpDV samples are genetically divers on the sequenced 800 nt long fragment including NS3 (Figure 4). Phylogenetic analyses based on these sequences showed that CpDV variants are grouped in two well-supported clades namely CpDV-Ia and CpDV-Ib (Figure 5). CpDV variants collected from the same populations, while showing some variabilities, generally clustered together (Figure 5). Especially the samples from Oued Melah, El Battan and Tabarka formed clades which were well-supported by bootstrap test (Figure 5). Samples from some other populations also grouped together while their bootstrap values were not as strong (*e.g.* Utique, Pompe, Figure 5).

Both CpDV-Ia and CpDV-Ib were found in mosquitoes infected with either one of the *w*Pip groups. However, their distribution correlated with the distribution of *w*Pip groups in the region (Mantel, r= 0.34, p=0.02, Figure 6A-Figure 6B) while was independent from geographical distances between the different populations they have been collected from (Mantel test, r=0.17, p=0.13). These statistical results mean that *w*Pip group variation might be the major ecological variable influencing CpDV-Ia and CpDV-Ib distributions in *Cx. pipiens* populations.

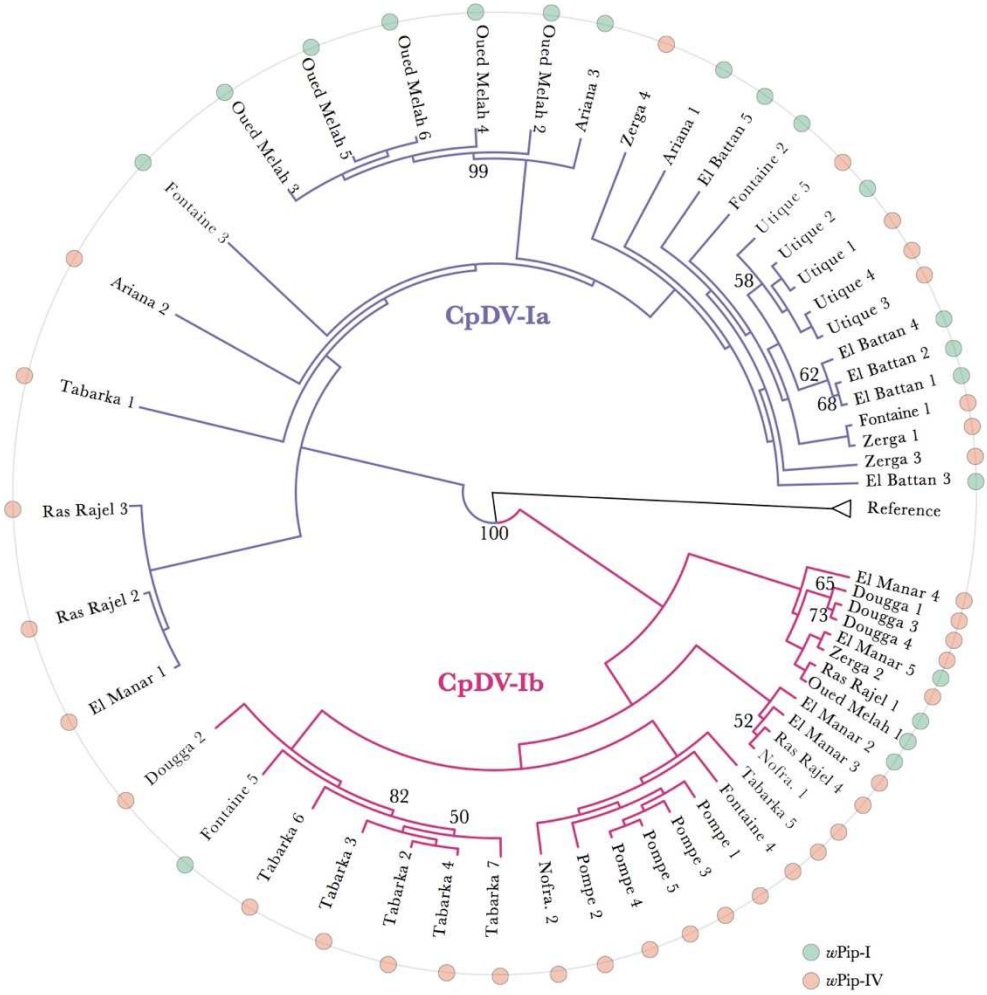

Figure 5 CpDV diversification in northern Tunisia. Maximum likelihood tree based on the sequences of CpDVs NS3 coding region. CpDV samples found in the area grouped in two well-supported clades, CpDV-Ia and CpDV-Ib (highlighted in purple and pink). CpDV variants from same populations generally clustered together with high bootstrap values (*e.g.* samples from Oued Melah, El Battan and Tabarka). Some other populations also grouped together while their bootstrap values (100 iterations) were not as strong (*e.g.* Utique, Pompe). While both CpDV-Ia and CpDV-Ib clades could be found in both *w*Pip-I and *w*Pip-IV infected mosquitoes, CpDV-Ib found more in wPip-IV infected mosquitoes.

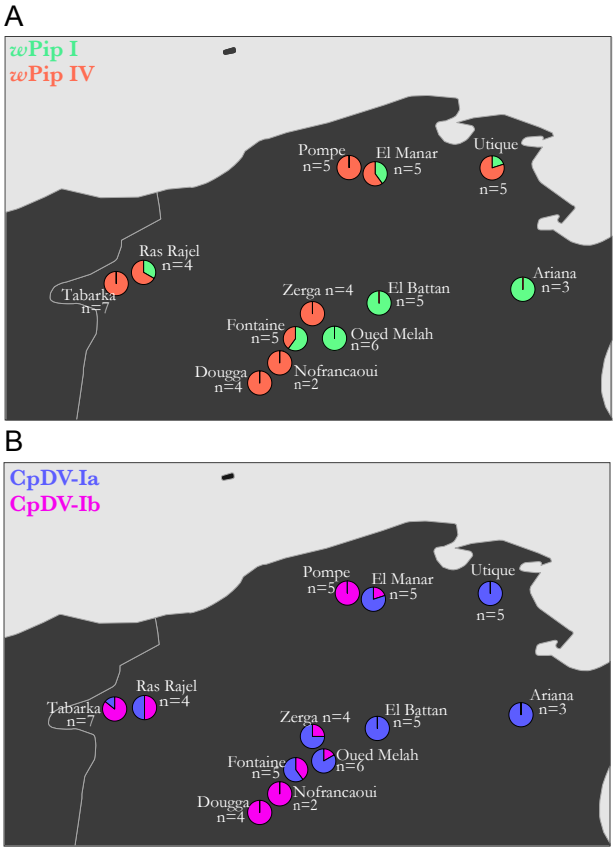

Figure 6 Similar spatial distribution of *Wolbachia* groups (A) and CpDV clades (B) (Mantel, r= 0.34, p<0.05). There was no spatial correlation between the distribution of the CpDV clades and geographical distance between their collection location (Mantel test, r= 0.17 ,p>0.05).

5.1.4 Discussion

We studied CpDV load, prevalence and diversification in a specific area, Northern Tunisia, where a rare stable contact zone between two incompatible *Wolbachia* strains have previously been revealed (Atyame *et al.*, 2015). All the studied parameters suggested reciprocal interactions between the two endosymbionts in natural populations.

By studying the viral load in relation to *Wolbachia* load, we observed a positive correlation between them independently from *Wolbachia* group infecting the host. Such positive correlation between symbionts' loads suggests a beneficial interaction between CpDV and *Wolbachia*. In most of *Wolbachia*-virus interactions studied so far, especially in Drosophila and mosquitoes, the load of the bacterial symbiont had generally been reported to negatively correlate with the virus load (Hussain *et al.*, 2012; Lu *et al.*, 2012; Frentiu *et al.*, 2014; Martinez *et al.*, 2017). However, a positive interaction between CpDV and *Wolbachia* have already been revealed in laboratory mosquito lines where mosquitoes infected with *Wolbachia* tended to exhibit higher CpDV loads than Tetracycline treated ones (**Chapter 3**). In addition to their effect on CpDV load, another type of *Wolbachia* and CpDV was also highlighted by differential CpDV prevalence in *w*Pip-I carrying mosquitoes versus *w*Pip-IV carrying ones. While in the whole study area prevalence of CpDV did not appear to be influenced by *w*Pip groups, this influence was observed in a subset of this area, where both *w*Pip groups (*w*Pip-I and *w*Pip-IV) and CpDV co-existed in same sampling sites. Indeed, by analyzing only the individuals living in the similar ecological conditions and certainly having the same probability to get infected by CpDV, we showed that *w*Pip-IV was associated with higher prevalence of CpDV compared to *w*Pip-I. This "beneficial" effect of one *Wolbachia* genotype on a virus prevalence can be seen, at first, as counterintuitive because of the growing evidence of *Wolbachia* antagonistic interactions with many RNA viruses such as DENV (Walker *et al.*, 2011; Lu *et al.*, 2012; Bian *et al.*, 2013; Frentiu *et al.*, 2014), Zika (Dutra *et al.*, 2016) and WNV (Glaser and Meola, 2010; Micieli and Glaser, 2014). However, antagonistic *Wolbachia*-DNA virus (such as Densoviruses) interactions have never been shown. Contrarily, *Wolbachia* has been reported to enhance the DNA (*Baculovirus*) infection in lepidopteran hosts (Graham *et al.*, 2012). While these differences of *Wolbachia* interference could be due to the differences between their interactions with either DNA or RNA viruses, they could also result from differences in the interactions between insect hosts and viruses. For instance, insects can be the principal host for viral multiplication for some viruses specialized on insects, such as the abovementioned DNA viruses baculoviruses and densoviruses, while they can act only as vectors for some others, such as abovementioned arboviruses DENV, WNV, Zika which are RNA viruses. Interestingly, contrary to the antagonistic interactions reported between *Wolbachia* and arboviruses, insect specific RNA viruses infec-

tion rate and loads increased in *Aedes aegypti* stably transinfected with *w*Mel compared to their *Wolbachia* free counterparts in natural populations (Amuzu *et al.*, 2018).

Viruses generally studied for their pathogenicity although they can also be seen as endosymbionts of their hosts with interactions that might range from mutualism to antagonism (Roossinck, 2015; Roossinck and Bazán, 2017). To date CpDV's true nature with their hosts is unknown. Initially they have been isolated during high mortality periods in laboratory *Cx. pipiens* larvae (Jousset *et al.*, 2000). However, it was so far impossible to replicate this 'deadly' infection in laboratory conditions (personal communication) and densonucleosis have not been firmly observed in infected adults in laboratory lines (**Chapter 3**). Studies on CpDV transmission in laboratory lines demonstrated a persistent infection by CpDV in apparently healthy laboratory *Cx. pipiens* lines (**Chapter 3**). Although the mechanisms of this persistence and the putative mutualistic side of CpDV have to be investigated in further experiments, other densoviruses have been shown to have mutualistic interactions with their hosts. For instance, an ambidensovirus genetically close to CpDV, namely *Helicoverpa armigera* densovirus-1 is beneficial for their hosts as it increases their developmental rate, female fecundity, lifespan and the resistance of their host against microbiological agents of control, such as *Bacillus thuringiensis* and a pathogenic baculovirus (Xu *et al.*, 2014). MDVs can also have negligible effects on survival of their hosts (Ren *et al.*, 2014) even if many MDVs have been shown to affect their mosquito host adversely (Barreau *et al.*, 1996; Ledermann *et al.*, 2004; Rwegoshora and Kittayapong, 2004; Carlson *et al.*, 2006; Becnel *et al.*, 2007). Moreover, the virulence of the host-virus interactions can evolve through time. Indeed, repetitive challenges by a densovirus from generation to generation can result in a decrease of the pathogenicity of the virus due to a higher resistance of the host or to the emergence of a persistent infection. The latter has been described in *Aedes aegypti* mosquitoes in which survival increased progressively when they were challenged with AThDV infections for successive generations while the offspring were always as infected (Roekring *et al.*, 2006). While it is difficult to interpret the beneficial interactions between CpDV and *Wolbachia* endosymbionts with limited knowledge on CpDV-host interactions, the higher prevalence of CpDV in mosquitoes harbouring *w*Pip-IV could be a result of its improved efficient vertical transmission due to the bacterial symbiont (**Chapter 3**). Indeed, Altinli et al. (submitted, **Chapter 3**) demonstrated that *w*Pip-IV infected mosquitoes had higher loads of CpDV in their ovaries compared to *w*Pip-I. If CpDV is beneficial for the hosts, the fact that CpDV hitchhikes this *w*Pip-IV strain could be favourable for both symbionts.

By sequencing a polymorphic NS part of CpDV genome, we investigated their microevolution in geographically close locations. Three distinct CpDV clades (CpDV-I, CpDV-II, CpDV-III) had previously been described in a worldwide study of CpDV diversity (**Chapter 2**). Samples from Tunisia belonged to CpDV-I clade (**Chapter 2**). The different CpDV variants found in the area all seem to derive from a common ancestor that further diversified in two closely related but distinct clades as named CpDV-Ia and CpDV-Ib. Furthermore, most of the samples collected from same locations clustered together indicating population structuration and that CpDV diversification occurs at local scales. Within species diversity of MDVs had never been studied to such an extent, although several variants have been discovered from the two recognized MDV species *Brevidensovirus*, Dipteran brevidensovirus-I and Dipteran brevidensovirus-II (Cotmore *et al.*, 2014). Similarly, the diversity of *Ambidensovirus* species was poorly studied. In fact it is limited to one study on *Myzus persicae nicotinae* densovirus (MpnDV) which revealed that the MpnDV were structured geographically with low level gene flow between locations, suggesting their high vertical transmission and putative co-evolution with their hosts (Song *et al.*, 2016). The distribution of CpDV-Ia and CpDV-Ib clades, which diversified in Tunisian populations, was independent from geographical distances between the different populations but correlated with the distribution of *w*Pip groups in the region (Figure 5). Assuming CpDV transmission is mainly vertical from

mother to offspring, *w*Pip could drive the distribution and evolution of CpDV in natural populations especially due to their CI properties. Indeed, CI can theoretically prevent gene flow between incompatible hosts which can result in the divergence of the CpDV. Given their co-vertical transmission it is possible that *w*Pip and CpDV co-diverge in the natural *Cx. pipiens* populations. On the other hand, we have reported CpDV horizontal transmission at high rate in laboratory conditions (**Chapter 3**), if this is similar in natural conditions co-divergence of these endosymbionts together should be rare in natural populations. The fact that we observe this co-divergence could be related to the ability of CpDV to disperse to new populations. Mosquito densoviruses have been shown to be carried to new oviposition sites by adult mosquitoes but it is usually in really low levels and might not always be enough to infect other larvae horizontally (Wise de Valdez *et al.*, 2010). Hence a CpDV is more likely to infect a new oviposition site via vertical transmission to the offspring of the infected female. Horizontal transmission then might play the major role for the infection of the larvae from uninfected females or females carrying a different CpDV. Therefore, it is expected that whichever variant infects the most females in a given area would also be found more in the larvae in the same location despite the occasional horizontal transmission of CpDV between larvae from different mothers. This could also explain why we do not observe a strict association between *w*Pip-IV or *w*Pip-I with two CpDV variants, but a strong association between their distribution despite the expected high horizontal transmission of CpDV.

Strong variations between different sampling locations in CpDV load and prevalence also suggested that there might be different variants circulating with different infectivity and that co-infections could occur. Although these questions were not in the scope of our study, we did not observe any general patterns between CpDV clades and CpDV load. For instance, CpDV load was lowest in Fontaine and highest in El Manar while both populations exhibited similar diversity of CpDV hosting variants from both clades. Similarly, co-infections by different CpDV variants could also result in high CpDV load. Although we did not quantify these co-infections in our study, we observed many double and triple peaks in CpDV sequences of the samples from El Manar population, where we also observed high viral loads.

Both MDVs (Buchatskiĭ *et al.*, 1987; Buchatsky, 1989; Afanasiev *et al.*, 1994; Carlson *et al.*, 2006; Ren and Rasgon, 2010; Johnson and Rasgon, 2018) and *Wolbachia* (Laven, 1967; Atyame, Pasteur, *et al.*, 2011; Hoffmann *et al.*, 2011; McGraw and O'Neill, 2013; Frentiu *et al.*, 2014) are considered as promising vector control tools. Although more studies have been carried on the effects of *Wolbachia* on viral infections in the natural populations, these are generally focused on RNA viruses, especially arboviruses, and our knowledge on insect specific viruses is very limited (Amuzu *et al.*, 2018). The prevalence of DNA viruses, especially densoviruses, highlights their importance on their hosts and possibly on their host' microbiota. Our study pointed out the interactions between *Wolbachia* and CpDV in natural *Cx. pipiens* populations, future studies are warranted to investigate these complex tripartite interactions between insect-specific viruses and *Wolbachia* in other mosquitoes. Indeed, especially in field releases of *Wolbachia* trans-infected mosquitoes, these tripartite interactions could be crucial for the dynamics of important vectors, their endosymbionts and the viruses they transmit, in natural populations.

Overall our results suggested that CpDV-host interactions could be diverse in nature and CpDV-*Wolbachia* interactions can influence the outcomes of CpDV infection for the host. The study of the arthropod viruses in the context of symbiosis - rather than focusing only on their pathogenicity- both in natural populations and experimental settings could lead to a better understanding of the evolution of mutualism and pathogenicity of viruses, and their interactions with the rest of the microbiota.

5.1.5 Acknowledgements

We thank Sandra Unal and Patrick Makoundou for technical support, Dr Philippe Clair for his help in the Real Time Quantitative PCR experiments which were performed at the “qPCR Haut Debit (qPHD) Montpellier génomiX” platform. Sequencing data were produced at the LabEX "Centre Méditerranéen de l'Environnement et de la Biodiversité" in the GENSEQ platform. We also thank Emmanuel Douzery for helpful discussions.

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Chapter 6:
Discussion

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6.1 Summary of Results

During my PhD project we have shown:

- A high prevalence and high diversity of a mosquito densovirus, CpDV in natural host populations (**Chapter 2**)
- That the CpDV evolution was shaped by recombination and purifying selection (**Chapter 2**)
- Highly significant migration routes and the lack of a geographical structure in CpDV diversity which suggested their efficient dispersal worldwide (**Chapter 2**)
- Persistent CpDV infection in apparently healthy laboratory lines (**Chapter 3**)
- CpDV vertical transmission and their presence in oocytes along with *Wolbachia* (**Chapter 3**)
- Effect of host microbiota including *Wolbachia* on the amount of CpDV in the ovaries and on CpDV vertical transmission (**Chapter 3**)
- High prevalence and diversity of both *w*Pip and CpDV in Turkey, a critical region for vector control efforts (**Chapter 4**)
- *Wolbachia*-CpDV interactions in Tunisian natural populations of *Cx. pipiens* (**Chapter 5**):
    - An influence of *w*Pip groups on CpDV prevalence
    - A positive correlation between *w*Pip density and CpDV density in infected mosquitoes suggesting their mutualistic interactions
- Microevolution of CpDV in geographically close populations and putative influence of *w*Pip group variations on CpDV diversification in Tunisia (**Chapter 5**)

6.2 Discussion

The aim of this PhD thesis was first to study the prevalence and diversity of a unique mosquito densovirus, CpDV, in natural populations of *Cx. pipiens* mosquitoes. Once their relevance for *Cx. pipiens* ecology was established, we have further investigated interactions with their hosts and host's endosymbiont *Wolbachia* both in laboratory lines and natural populations.

Mosquito densoviruses (MDV), relatively more studied than many other arthropod viruses, are still rarely studied in an ecological and evolutionary context to date. Indeed, the research around MDVs mainly focused on their pathogenicity and/or their application as a vector control tool (Buchatskiĭ *et al.*, 1987; Carlson *et al.*, 2006; Johnson and Rasgon, 2018). They were nevertheless thought to be widespread in natural populations as their presence in mosquito larvae and adults as well as in insectivorous bat faeces has been reported (Kittayapong *et al.*, 1999a; Rwegoshora *et al.*, 2000; Ma *et al.*, 2011; Ng *et al.*, 2011; Ge *et al.*, 2012). Only for one MDV general high prevalence in natural populations and the effect of abiotic factors on this prevalence has been shown (AaeDV Thai strain)(Kittayapong *et al.*, 1999a; Rwegoshora *et al.*, 2000). CpDV, given their fundamental differences from the rest of MDVs, could be expected to have different life cycles, host interactions and transmission modes. Their genomic differences from other MDVs could also suggest that they have been acquired from another species. Using samples that have been collected at different times worldwide, we have revealed for the first time that a MDV can be prevalent in such a large scale and persistent through the years in *Cx. pipiens* populations (**Chapter 2**). While this prevalence was not as high as it could be observed for some endosymbionts, such as *Wolbachia w*Pip, it highlighted their importance for *Cx. pipiens* ecology.

By sequencing some of the CpDV variants found in natural populations, we sought to understand their diversity, evolution, dispersal and population dynamics (**Chapter 2**). Knowledge of within species diversity of densoviruses was limited at best to couple of variants that are sequenced

(Cotmore *et al.*, 2014), except one study of MpnDV in China (Song *et al.*, 2016). Authors indeed reported a high diversity of MpnDV which often clustered accordingly to geographically defined aphid host populations (Song *et al.*, 2016). Using methods that are now widely used for fast evolving human viruses such as Zika and influenza (Baillie *et al.*, 2012; Thézé *et al.*, 2018), we have also observed a high diversity of CpDV clustered in three clades and their efficient dispersal worldwide. Parvoviruses usually have been reported to have a high substitution rate making their evolution fast and allowing the use of similar methods to estimate the Most Recent Common Ancestor (tMRCA) and population dynamics (Shackelton *et al.*, 2005; Firth *et al.*, 2009; Streck *et al.*, 2011; Lin *et al.*, 2017; Grecco *et al.*, 2018). We demonstrated a prevailing role of recombination and purifying selection on the evolution of CpDV as expected in ssDNA viruses in general (**Chapter 2**). General incongruence of different genes (NS and VP) in CpDV possibly caused by recombination made it difficult to infer their real tMRCA with certainty (**Chapter 2**). The current phylodynamics methods cannot take recombination into account hence we had to exclude the recombinants from the datasets (**Chapter 2**). Such exclusions of the recombination seemed to change tMRCA estimates a lot (**Chapter 2**). Being one of the major forces of virus evolution recombination constitutes one of the challenges that phylodynamics methods are facing today, new phylogenetic methods might improve this current situation (Frost *et al.*, 2014). As our study was limited to already extracted DNA samples (**Chapter 2**) **new sampling of fresh material could allow to sequence more samples, and/or the whole genome sequencing could be a way to circumvent this problem and possibly can give better estimates.** Additionally, while our approach using Sanger sequencing allowed us to use older samples, we were not able quantify within host diversity even though we have observed double and sometimes triple peaks in some electropherograms (**Chapter 2, Chapter 3**). **Future studies employing next generation sequencing methods could also be useful to understand the within host diversity, especially the effects of this diversity on the host-virus interactions.**

Discovery of the worldwide prevalence and persistence of CpDV brought an important question in mind: how do they transmit from host to host? To answer this question we have tried first to test their horizontal transmission by using infected crushed larvae and cell cultures transinfected with synthetic CpDV plasmid **(Chapter 3)**. First approach was failed as these filtered homogenates were toxic in higher volumes where we reached an adequate amount of CpDV to experimentally infect other larvae (**Chapter 3**). Trials using second approach were also impeded as we faced difficulties to culture cells highly infected with synthetic CpDV and most importantly as our mosquito lines were already persistently infected with CpDV (**Chapter 3**). This persistence suggested they might be vertically transmitted along with their hosts' endosymbiont *Wolbachia*. MDVs have been known to be transmitted vertically but their interactions with the rest of the microbiota are not yet investigated (O'Neill *et al.*, 1995; Barreau *et al.*, 1997; Kittayapong *et al.*, 1999b; Rwegoshora and Kittayapong, 2004; Suchman *et al.*, 2006; Barik *et al.*, 2016). Moreover, as CpDV is genetically closer to ambidensoviruses than brevidensoviruses (other MDVs), whether their biology would be similar to ambidensoviruses or MDVs was not clear. Investigating the vertical transmission in laboratory lines naturally infected with CpDV, we have shown that i) CpDV was vertically transmitted in low amounts, ii) they were inside the oocytes (where also *Wolbachia* is) and not just on the surface of the eggs, iii) microbiota and *Wolbachia* in particular seem to have a role on the amount and transmission of CpDV (**Chapter 3**). Although we were not able to quantify properly due to the lack of CpDV free mosquito lines, horizontal transmission seems to be high given the low amount of vertical transmission to the first instar larvae and high amount of prevalence in the emerged adult females (**Chapter 3**).

Transmission modes of endosymbionts could give insights about their evolution as to whether they would evolve towards mutualism or antagonism. In our lines, CpDV was observed to cause

densonucleosis and was possibly the reason of high larval mortality when it was first isolated in around 1994 (Jousset *et al.*, 2000). Almost two decades later, CpDV seems to persist in low amounts and be vertically transmitted in our seemingly healthy laboratory lines. This shift in virulence suggests a change in CpDV over the years which made the new variant less pathogenic to their hosts. Another result that might be in support of this hypothesis, came with the improvement of the transinfection and CpDV isolation methods which allowed us to use a synthetic CpDV and a natural isolate from our lines, Ich13 isolate, to perform experimental infections (**Chapter 3**). While the results are preliminary (only one replicate), they suggested that synthetic CpDV (that is designed based on reference genome) was pathogenic while Ich13 isolate (current variant in the laboratory lines) seemed to improve host survival. Moreover, CpDV sequences from the laboratory lines collected between 1990-2018 indicated a shift from CpDV 2 clade to CpDV 1 in a short period of time between 1994 (reference genome) to 2001 (Slab 2001)(**Chapter 2**). This change in CpDV could be caused by their evolution with the host, as it is possible that a variant with lower pathogenicity, or even beneficial for the host, emerged and became widespread in the insectary populations. On the other hand it is also possible that another CpDV variant arrived to the insectary with the establishment of a new *Cx. pipiens* line collected from natural populations and outcompeted the pathogenic CpDV (reference genome). The results of recombination analysis suggest that the truth could be lying somewhere in between these two hypotheses (**Chapter 2**). Indeed, a new variant could have arrived to the insectary around 1997, and the co-infection by reference variant (CpDV-2 clade) and the new variant (CpDV-1 clade) might have resulted in the recombination event we have detected in Slab 1997 sample (**Chapter 2**). After this, either the arriving variant might have out competed the reference variant or newly emerged recombinant variant could have evolved further with their hosts. **To gain more insights on how this change occurred in the insectary populations, we now are sequencing samples that have been collected from our insectary lines established from different mosquito populations between 1990-2018. By analysing these sequences, we will be able to follow the arrival of new variants, their evolution and recombination events in the insectary lines, especially between 1997 to 2001.**

Another way to understand the evolution of the CpDV in the insectary lines would be to continue comparing a natural isolate from the insectary with a synthetic CpDV having the same genome, in addition to synthetic CpDV with the reference genome. This would help us to compare the infectivity of both genomes and synthetic vs. natural isolates. However, it is crucial to first establish CpDV free lines to separate the effects of the virus from the effects of a co-infection by different variants. **Once established this model system could also be used to test both the evolution of CpDV, their interactions with their hosts and *Wolbachia* more directly. For instance, How the mosquito is affected by CpDV infection? What is the role of the immune system? If immune system is activated by a CpDV infection does this affect the rest of the microbiota? How the changes in microbiota affect the *Culex* and hence CpDV? What is the route for the CpDV horizontal infection of *Cx. pipiens* (ingestion, anal papillae)? Easy manipulation of the CpDV genome by simple cloning techniques could also make functional studies possible**.

Last part of this PhD thesis focused on *Wolbachia* and CpDV interactions in natural populations (**Chapter 4- Chapter 5**). We have first identified both *Wolbachia* and CpDV presence and diversity in natural populations in Turkey (**Chapter 4**). Turkey can provide ideal conditions for both the amplification of already circulating viruses and the emergence of new viruses due to its geographical location, its climate and the biodiversity of arthropods vectors. Previous studies have shown the diversity of mosquitoes and the RNA viruses they carry (arboviruses and insect-specific viruses), although *Wolbachia* diversity in *Cx. pipiens* mosquitoes was unknown (Ergunay *et al.*, 2011, 2013;

Erdem *et al.*, 2014; Ergunay *et al.*, 2015; Gunay *et al.*, 2015; Failloux *et al.*, 2017; Öncü *et al.*, 2018). We have revealed the diversity of *w*Pip groups and their cytoplasmic incompatibility patterns, and highlighted the potential of *w*Pip-IV harbouring males as a vector control to sterilise local *Cx. pipiens* populations, particularly where only *w*Pip-I or *w*Pip-II harbouring females were present. In this context, **local studies of *w*Pip diversity and CI patterns in finer scale could be useful for *Wolbachia* based vector control in the region.** We have also revealed CpDV diversity especially in the Thrace region (North-western Turkey). This proved that Turkey could be a great place to study virus-virus, virus-*Wolbachia* interactions in nature, while our study so far stayed more in explorative nature due to the lack of previous studies on *w*Pip in the region. **Indeed, adding the investigation of diversity and prevalence of insect-specific viruses to the existing arbovirus surveillance efforts in Thrace region- where we have detected both *w*Pip diversity and CpDV diversity- could shed light on *w*Pip, arboviruses and insect-specific virus dynamics in natural populations.**

In Northern Tunisia *w*Pip diversity and their CI patterns have already been studied in detail by our team EVAS at ISEM (Atyame *et al.*, 2015). Authors discovered a contact zone where mosquitoes harbouring different *w*Pip groups, *w*Pip group I and IV, co-existed. Their CI patterns predicted the invasion of the contact zone by the strain inducing uni-directional CI, namely *w*Pip-I, in only couple of generations although the contact zone was stable over seven years. Using a part of these samples, we have investigated the prevalence of CpDV in the same area and the effect of different *w*Pip groups on this prevalence (**Chapter 5**). Our initial hypothesis was that a pathogen protection conferred by one of the *w*Pip strains, in this case *w*Pip-IV, could possibly explain why *w*Pip-I could not invade the whole host populations in the contact zone. Conversely, we demonstrated that CpDV was more likely to infect mosquitoes harbouring *w*Pip-IV. While it is difficult to interpret these interactions without knowing the nature of the CpDV-host interactions, this higher prevalence of CpDV in mosquitoes harbouring *w*Pip-IV could be a result of efficient vertical transmission, rather than a more efficient protection by *w*Pip-I. In support of this hypothesis we have observed, in the laboratory (**Chapter 3**), that *w*Pip-IV infected mosquitoes had higher amount of CpDV in their ovaries compared to *w*Pip-I infected mosquitoes. Indeed, the density of CpDV seemed to be controlled by a cytoplasmic factor, *e.g. Wolbachia*, rather than host genetic background (**Chapter 3**). However due to the high variance in vertical transmission rate at individual females' level, we were not able to show such a difference in vertical transmission between mosquitoes harbouring different *w*Pip groups (**Chapter 3**).

In the field, while the prevalence was affected by the *w*Pip group, there was no effect of this group on the CpDV density (**Chapter 5**). However, the density of CpDV was positively correlated with *w*Pip density (**Chapter 5**). This suggested that *w*Pip could enhance CpDV infections in natural populations. *w*Pip protection against West Nile Virus (RNA virus) by reducing the virus density has previously been reported (Glaser and Meola, 2010; Micieli and Glaser, 2014). On the other hand *Wolbachia* appear to enhance DNA virus infections so far which was consistent with our results (Teixeira *et al.*, 2008; Graham *et al.*, 2012). The interactions between *Wolbachia* and DNA viruses could be different as these viruses are likely to affect their hosts differently. It is possible that arboviruses vectored by mosquitoes have less of an impact on mosquito biology compared to mosquito-specific viruses. Indeed recently, *Wolbachia* has been reported to enhance an insect specific RNA virus (Anphe virus) in *Ae. aegypti* cells (Parry and Asgari, 2018). Furthermore, natural populations of *Ae. aegypti* stably transinfected with *Wolbachia* displayed higher insect-specific RNA virus infection rates and loads compared to their *Wolbachia*-free counterparts (Amuzu *et al.*, 2018). **These CpDV-*Wolbachia* interactions and possibly the interactions between CpDV and arboviruses could also be tested in future experimental infection studies to understand their interactions in natural populations better.**

By sequencing NS3 coding region in CpDV genome, we revealed the microevolution of geographically close populations in Northern Tunisia. All

belonging to CpDV1 clade (**Chapter 1**), variants from the region were closely related as expected. Nevertheless, they grouped in two statistically well supported distinct groups (**Chapter 5**). Mapping these two distinct groups revealed their similar distribution with *w*Pip groups (**Chapter 5**). Given their co-vertical transmission it is possible that these two endosymbionts co-diverge and as a result their distributions would be similar in natural populations. On the other hand, in the insectary conditions, we have observed a high rate of CpDV horizontal transmission (**Chapter 3**), if this is similar in natural conditions it would be difficult to observe the co-divergence of these endosymbionts together. Similar distribution of *w*Pip and CpDV could be explained in this case by the way CpDV disperses between different water bodies. Indeed, we know that CpDV can be vertically transmitted to the larvae in a new location where infected females lay eggs (**Chapter 3**). Although adult mosquitoes have also been shown to contaminate a water body with other MDVs, it is usually in really low levels and often not enough to infect the larvae (Wise de Valdez *et al.*, 2010). Hence it is possible that whichever variant infects the most females in that area would also be found more in the larvae there despite the occasional horizontal transmission of CpDV between larvae from different mothers. This could also explain why we do not observe a strict association between *w*Pip group IV and *w*Pip I with the two CpDV variants while the association is clearer between geographical distribution of both. **Studying the effects of CpDV on their hosts, and their horizontal transmission by experimental infections can also help to understand how they might diverge together with their hosts and hosts vertically transmitted endosymbiont *Wolbachia* in natural populations.**

Although my PhD focused mainly on CpDV and CpDV-*Wolbachia* interactions, endosymbiont interactions could be much more intricate in nature than only tripartite interactions. It is possible that the rest of the microbiota also affects CpDV-*Wolbachia* interactions. For instance, diversity and presence of insect-specific RNA viruses in natural populations of mosquitoes have been shown in the recent years. Nevertheless, their effects on their hosts and host microbiota are still poorly studied. Such a *Cx. pipiens* associated RNA virus have recently been discovered in Northern Tunisia (Bigot *et al.*, 2018) offering the possibility to study a DNA virus-RNA virus-*Wolbachia*-*Culex pipiens* interactions. We could not investigate this question in the **Chapter 5** as it was not possible to take RNA viruses into account in our study due to the use of readily extracted DNA samples. **Future studies could possibly take these multi-partner species interactions in environments into account using their co-occurrence and their correlation patterns as it is now being used for bacterial species in microbiota studies**.

MDVs have long been investigated due to their pathogenicity and their narrow host range as a vector control strategy. There are two methods i) their direct use to kill mosquito larvae ii) their use as transducing agents (Buchatskiĭ *et al.*, 1987; Afanasiev *et al.*, 1994; Ward *et al.*, 2001; Carlson *et al.*, 2006; Ren and Rasgon, 2010; Suzuki *et al.*, 2014). First method consists of the application of highly pathogenic MDV directly to the larval habitats (Carlson *et al.*, 2006; Johnson and Rasgon, 2018). High mortality during larval stages could indeed reduce mosquito populations but could also lead to rapid resistant evolution due to high selection pressure caused by MDV (Johnson and Rasgon, 2018). It is argued that the second method could be evolution proof, as the real target would be older mosquitoes which are more likely to carry arthropod borne viruses (Ren and Rasgon, 2010). Sublethal MDVs would not cause as high selective pressure as a pathogenic MDV as they would allow larvae to survive and young adults to reproduce (Ren and Rasgon, 2010). While both methods are promising in terms of mosquito-borne disease control efforts, they are still hindered by the lack of knowledge on the basic biology and ecology of MDVs, and the lack of MDVs specific to all medically important mosquitoes (Johnson and Rasgon, 2018). **In future, CpDV, or the knowledge on their effects on host ecology and evolution, could also prove useful for these control strategies. Especially since they might be**

used as transducing agent due to their persistence in mosquito populations and low pathogenicity against *Cx. pipiens* (s.l.).

In this context, but also to understand CpDV biology better, it would be useful to study their host specificity. Being similar to the other MDVs in terms of hosts they infect while being genetically closer to lepidopteran ambidensoviruses (Baquerizo-Audiot *et al.*, 2009; Cotmore *et al.*, 2014), it might be possible that they infect both of these hosts. Furthermore a genetically similar densovirus to CpDV has been recently discovered in *Drosophila* (Kapun *et al.*, 2018). Transinfection of cell cultures with CpDV have previously shown that they were able to amplify in *Anopheles* and *Culex* cells while in the *Aedes*, *Drosophila* and *Spodoptera* cells they did not amplify (Jousset *et al.*, 2000). Our preliminary results also showed that CpDV did not cause any mortality in *Ae. albopictus* larvae (**Chapter 3**). **Future studies on *Anopheles* mosquitoes, lepidopterans as well as *Drosophila* could shed more light to host specificity of CpDV.**

Virus discovery have long depended on the observation of outbreaks and the research to understand their pathogenicity depended on their culture. The advances of sequencing technology and molecular biology techniques allowed researchers to discover the ubiquity and the diversity of viruses. Despite these advances, virus discovery mainly focused on vertebrate viruses. Arthropods with their ancient evolutionary history represent 80% of animal diversity on our planet. Assuming a correlation between host and virus diversity, this might mean that we are still oblivious to most of the existing virus diversity. Furthermore, even the known diversity of these viruses is poorly studied in term of virus ecology. The ubiquity of endosymbiosis in arthropods and the ability of arthropods to support (sometimes multiple) symbionts suggest that their interactions with their viruses might be of symbiotic nature, intimate long-term associations with outcomes ranging from mutualistic to antagonistic.

In this context, this new approach to study viruses, while still presents many challenges could answer broad range of questions about the ecology and evolution of host-microbiota interactions. Densoviruses, as ancient and diverse as their arthropod hosts, are ubiquitous in nature and have small genomes that are easy to manipulate with basic cloning techniques. Hence, they could be good models to study host-virus-bacteria interactions. In the intersection of these many novel areas, our results, while may have raised more questions than they answered, suggested that **CpDV-*Cx.pipiens*-*Wolbachia* system could be a good model system to study the ecological interactions between these partners and their evolution together both in the nature and in laboratory conditions.**

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Chapter 7:

Résumé détaillé de la thèse

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7.1 Introduction

Près de trois décennies après la révolution conceptuelle autour des interactions hôtes-bactéries montrant que ces dernières ne représentaient pas seulement une menace mais sont impliquées dans des interactions très variables avec leurs hôtes, la métagénomique virale a récemment bouleversé notre façon de voir les virus. Tout a commencé par l'étude des communautés virales marines au début des années 2000. Depuis, le nombre de métaviromes publiés a augmenté de façon significative et, avec eux, notre appréhension de la diversité des virus, de leur écologie et de l'étendue des conditions environnementales dans lesquelles ils interagissent avec leurs hôtes (Breitbart et Rohwer, 2005 ; Edwards et Rohwer, 2005 ; Rosario et Breitbart, 2011). Des études récentes ont permis de mettre en évidence de façon claire que la plupart des virus ne causent pas de dommages détectables à leurs hôtes contrairement à ce que l'on pensait (Roossinck et Bazán, 2017). Ainsi, la meilleure façon de décrire la grande majorité des virus, étant données leurs relations intimes et parfois bénéfiques avec leurs hôtes, serait de les qualifier d'endosymbiotes. En effet, tout comme les endosymbiotes bactériens, leurs interactions avec leurs hôtes peuvent se situer n'importe où - du mutualisme à l'antagonisme dans le continuum des interactions durables (Roossinck, 2015a). Si les relations antagonistes, de type parasitaire, sont les plus étudiées, des virus mutualistes ont également été caractérisés notamment chez les plantes (Roossinck, 2015b). Par exemple, il a été démontré que plusieurs virus végétaux confèrent à leurs hôtes une tolérance à la sécheresse et au froid (Xu et al., 2008), soulignant que l'impact des virus sur l'écologie et l'évolution de leurs hôtes n'est pas seulement celui d'une pression parasitaire. Il est donc à présent nécessaire de conduire des études intégratives considérant les virus comme étant engagés dans des interactions durables symbiotiques le long d'un gradient allant du mutualisme au parasitisme (Roossinck et Bazán, 2017).

Un autre changement de paradigme, également lié au progrès des techniques de séquençage, a été de montrer que la virulence d'un micro-organisme n'étaient pas la seule résultante d'une interaction entre un micro-organisme (" le " pathogène) et un hôte mais plutôt la conséquence d'interactions multiples entre micro-organismes au sein de l'organisme hôte (Vayssier-Taussat et al., 2014). Pourtant, et c'est le cas même dans le contexte de la symbiose mutualiste, les interactions entre les symbiotes au sein d'un hôte n'ont été que peu étudiées et les recherches se sont surtout concentrées sur les interactions binaires hôte-symbiote (Douglas, 2016). Il est cependant clair que les endosymbiotes peuvent interagir entre eux directement ou indirectement par le biais de leur phénotype étendu (Almand et al., 2017 ; Douglas, 2016). Par exemple, les souris ayant un microbiote normal meurent deux fois moins que celles qui n'ont pas de microbiote lorsqu'elles sont infectées par un poliovirus par voie orale. D'autres types d'interactions directes semblent affecter la transmission horizontale et verticale des virus des végétaux par leurs vecteurs-insectes. Ainsi, il a été démontré qu'un symbiote bactérien obligatoire commun chez les cicadelles, nommé *Sulcia*, permettait au « virus nain

du riz » de se transmettre verticalement à la progéniture de leur vecteur commun. Le virus nain du riz fait de l'autostop grâce à une interaction spécifique entre une protéine de sa capside virale et une protéine de la membrane externe de *Sulcia* (Jia et al., 2017).

Dans ce contexte théorique, au cours de mon doctorat, j'ai étudié ces interactions multiples dans un système naturel tripartite hôte-bactéries-virus, entre les moustiques *Culex pipiens*- les endosymbiotes bactériens *Wolbachi*a- et le virus CpDV, à la fois dans les populations naturelles et les élevages.

- Les symbiotes bactériens, dont *Wolbachia*, ont été caractérisés depuis de nombreuses années comme étant des partenaires intimes des organismes multicellulaires. Le fait que les micro-organismes soient fondamentaux dans l'édification du phénotype des animaux et des végétaux, a révolutionné de nombreux aspects de la science, y compris les sciences appliquées et médicales.
- Les insectes représentent des modèles d'étude pertinents dans l'endosymbioses car ils sont très diversifiés et capables d'établir des relations symbiotiques avec des organismes de toutes origines évolutives.
- Les densovirus, aussi anciens et divers que leurs hôtes arthropodes, semblent également être des virus pertinents pour étudier l'écologie évolutive des interactions hôte-virus-bactéries. Les connaissances sur ce virus CpDV étaient avant cette thèse limitées à leur caractérisation génétique et structurelle à partir de moustique issus des élevages de l'institut des sciences de l'évolution de l'Université de Montpellier.

7.1.1 Les objectives de la thèse

Dans ce document de thèse, je présente comment nous avons étudié la prévalence et la diversification du CpDV à travers le monde (**Chapitre 2**). Puis comment, nous avons décrypté leur cycle de vie et leur dynamique dans nos colonies de laboratoire en particulier en étudiant leur transmission verticale (**Chapitre 3**). Enfin, comment nous avons exploré les interactions *Wolbachia*-CpDV dans les populations naturelles de *Cx. pipiens* en étudiant leur prévalence et leur évolution à l'échelle locale (**Chapitre 4 et 5**).

7.2 Les résultats

Au cours de mon travail de doctorat, nous avons montré :

- Une prévalence élevée et une grande diversité d'un densovirus de moustique, le CpDV, à l'échelle mondiale dans les populations hôtes naturelles (**Chapitre 2**)
- Que l'évolution du CpDV a été façonnée par la recombinaison et la sélection purificatrice (**Chapitre 2**)
- L'absence d'une structure géographique dans la diversité du CpDV qui suggère leur dispersion efficace dans le monde entier (**Chapitre 2**)
- Infection persistante par le CpDV dans des lignées de laboratoire apparemment en « bonne santé » (**Chapitre 3**)
- La transmission verticale du CpDV en utilisant les ovocytes où ils peuvent être en interaction intime avec *Wolbachia* (**Chapitre 3**)
- L'effet du microbiote-hôte, y compris *Wolbachia*, sur la charge de CpDV dans les ovaires et sur la transmission verticale du CpDV (**Chapitre 3**)
- La forte prévalence et diversité des *Wolbachia* *w*Pip ainsi que des CpDV en Turquie, une région critique dans la lutte antivectorielle (**Chapitre 4**)
- Une influence des *Wolbachia* sur la prévalence, la charge virale, et la diversité des CpDV dans les populations naturelles tunisiennes de *Cx. pipiens* :

  -du fait de la corrélation positive entre les densités des *w*Pip et celle des CpDV chez les moustiques infectés suggérant des interactions synergétiques entre les endosymbiote bactérien et viral (**Chapitre 5**)

  - car la prévalence des CpDV est influencée par les différents groupes de *w*Pip dans la région (**Chapitre 5**)

  - car la diversification des CpDV est peut-être influencée par les différents groupes de *w*Pip (**Chapitre 5**)

7.3 Discussion

L'objectif de ma thèse de doctorat était tout d'abord d'étudier la prévalence et la diversité d'un densovirus original de moustique, le CpDV, dans les populations naturelles du moustique *Cx. pipiens*. Une fois sa répartition et les relations phylogénétiques étables, nous avons étudié plus en avant les interactions avec leurs hôtes et l'endosymbionte *Wolbachia* dans les lignées de laboratoire et les populations naturelles.

Les densovirus de moustiques (MDVs) qui ont été comparativement plus étudiés que de nombreux autres virus arthropodes, le sont encore rarement dans un contexte écologique et évolutif. En effet, les recherches conduites sur les MDVs se sont principalement concentrées sur leur pathogénicité et/ou leur application comme outil de lutte antivectorielle (Buchatskiĭ et al., 1987 ; Carlson et al., 2006 ; Johnson et Rasgon, 2018). On pense néanmoins qu'ils sont largement répandus dans les populations naturelles car leur présence dans les larves de moustiques et les adultes ainsi que dans les fèces de chauves-souris insectivores a été signalée (Kittayapong et al., 1999a ; Rwegoshora et al, 2000 ; Ma et al, 2011 ; Ng et al, 2011 ; Ge et al., 2012). Cependant, seule une étude avait jusqu'à lors décrit la prévalence élevée d'un MDV (la souche AaeDV Thai) dans les populations naturelles et l'effet de facteurs abiotiques sur cette prévalence (Kittayapong et al., 1999a ; Rwegoshora et al., 2000). Étant données les caractéristiques propres des CpDV, on peut s'attendre que ces derniers aient des cycles de vie, des interactions avec leur(s) hôte(s) et des modes de transmission différents. Leurs différences phylogénétiques et génomiques par rapport aux autres MDVs pourraient également suggérer qu'ils ont été acquis par un transfert horizontal ou qu'ils ont évolué récemment à partir d'un MDV ancestral. En utilisant des échantillons prélevés dans le monde entier, nous avons révélé pour la première fois qu'un MDV, le CpDV, est distribué à l'échelle mondiale et persiste au fil des générations dans les populations de *Cx. pipiens*. Bien que la prévalence des CpDV ne soit pas aussi élevée que celle observée pour d'autres endosymbiontes comme l'endosymbiote *Wolbachia* *w*Pip, qui lui est fixé, elle souligne leur potentielle importance dans l'écologie du moustique *Cx. pipiens*.

En séquençant de nombreux variants de CpDV échantillonnés dans les populations naturelles, nous avons étudié leur diversité, leur évolution, leur dispersion et leur dynamique démographique (**Chapitre 2**). La connaissance de la diversité des densovirus à l'intérieur d'une même espèce hôte était jusqu'à lors réduite à une étude de densovirus chez les pucerons en Chine (Song et al., 2016). Les auteurs avaient mis en évidence une grande diversité de virus qui se regroupaient en fonction des populations de pucerons géographiquement structurées (Song et al., 2016). En utilisant des méthodes qui sont maintenant largement utilisées pour les virus humains à évolution rapide tels que l'agent du Zika our de la grippe (Baillie et al., 2012 ; Thézé et al., 2018), nous avons révélé une grande diversité de CpDV regroupés en trois clades et leur dispersion efficace à travers le monde, fait peu courant pour les virus arthropodes (**Chapitre 2**). Nous avons par ailleurs montré un taux de substitution élevé, ce qui accélère l'évolution des CpDV et permet méthodologiquement l'utilisation de méthodes pour estimer l'ancêtre commun le plus récent (tMRCA) ainsi que d'inférer la dynamique des

populations virales (Shackelton et al., 2005 ; Firth et al., 2009 ; Streck et al., 2011 ; Lin et al., 2017 ; Grecco et al., 2018). Nous avons démontré un rôle prédominant de la recombinaison et de la sélection purifiante sur l'évolution des CpDV comme déjà observé chez d'autres virus à ADN simple brin (**Chapitre 2**). L'incongruence phylogénétique entre les différents gènes (NS et VP), du fait d'événements de recombinaisons, ont rendu difficile d'inférer avec certitude le tMRCA (**Chapitre 2**). En effet, les méthodes de phylodynamiques actuelles ne peuvent pas prendre en compte les recombinaisons et nous avons donc dû exclure les recombinants de nos données. Etant l'une des forces majeures de l'évolution virale, la recombinaison constitue l'un des défis auxquels les méthodes phylodynamiques sont confrontées aujourd'hui et de nouvelles méthodes phylogénétiques permettant de les prendre en compte verront sûrement le jour prochainement (Frost et al., 2014). Notre étude se limitait à des échantillons d'ADN déjà extraits, un nouvel échantillonnage de matériel frais pourrait permettre de séquencer plus d'échantillons, en ayant accès aux génomes entiers des CpDV ce qui nous permettrait d'avoir des analyses plus robustes. Des études futures utilisant des méthodes de séquençage NGS nous permettrait par ailleurs d'avoir accès au virome des *Cx. pipiens*.

L'étude de la prévalence mondiale et la mise en évidence de la persistance des CpDV à l'échelle mondiale soulève une question importante : comment se transmettent-ils d'un hôte à l'autre ? Pour répondre à cette question, nous avons d'abord essayé de tester leur transmission horizontale en utilisant des filtrats de broyats de larves infectées mais aussi des cultures cellulaires transinfectées par un plasmide portant un génome synthétique du CpDV. La première approche a échoué car ces homogénats filtrés étaient toxiques (même dans les contrôles) (**Chapitre 3**). Les essais utilisant la deuxième approche ont également été entravés car nous avons eu des difficultés à obtenir des doses importantes de CpDV synthétiques à partir de nos cultures cellulaires. Un problème majeur est surtout que nos lignées au laboratoire étaient déjà toutes infectées de façon persistante par le CpDV (**Chapitre 3**). Cette persistance suggérait que ces virus pourraient être transmis verticalement en même temps que l'endosymbiote *Wolbachia* présent naturellement dans toutes nos lignées. Nous savions que certains MDVs étaient capables de transmission verticale, cependant l'influence du microbiote sur cette transmission n'avait jamais été abordée (O'Neill et al., 1995 ; Barreau et al., 1997 ; Kittayapong et al, 1999b ; Rwegoshora et Kittayapong, 2004 ; Suchman et al, 2006 ; Barik et al, 2016). En étudiant pour la première fois la transmission verticale dans des lignées de laboratoire naturellement infectées par le CpDV et *Wolbachia*, nous avons montré que i) la transmission verticale avec des faibles charges virales, ii) la présence des CpDV dans les ovocytes (où se trouve aussi le *Wolbachia*) et pas seulement à la surface des œufs, iii) par des traitements antibiotiques que le microbiote, en particulier *Wolbachia* semble influencer positivement la transmission verticale du CpDV (**Chapitre 3**). Bien que nous n'ayons pas pu la quantifier de manière robuste la transmission horizontale en raison de l'absence de lignées de moustiques sans CpDV, celle-ci semble être élevée étant donnée la transmission verticale aux larves du premier stade

larvaire (~20%) et la prévalence élevée chez les femelles adultes émergées (~100%) (**Chapitre 3**).

Les modes de transmission des endosymbiontes sont fondamentaux dans l'évolution de leur virulence à savoir leur trajectoire vers le mutualisme ou l'antagonisme. Dans les lignées de laboratoire, il a été observé que le CpDV pouvait causer de la densonucléose et qu'il était probablement la cause d'une mortalité larvaire élevée lorsqu'il a été isolé pour la première fois dans la fin des années 1990 (Jousset et al., 2000). Deux décennies plus tard, le virus CpDV semble persister en faibles quantités et se transmettre verticalement dans nos lignées de laboratoire dont les individus ne semblent pas malades. Ce changement de virulence suggère un changement dans le virus au fil des années. Le séquençage des variants de CpDV présents au laboratoire pendant les 30 dernières années a en effet indiqué un changement dans les variants présents. Dans les années 90, les variants appartenaient au clade CpDV 2 alors qu'à l'heure actuelle ils appartiennent au clade CpDV1. Ce « shift » a eu lieu avant les années 2000 (**Chapitre 2**). Nous pensons que ce remplacement d'un variant par un autre est dû à l'importation de nouveaux échantillons dans nos élevages dont les nouveaux variants de CpDV ont exclu les variants résidants. En effet, un échantillon de l'année 1997, zone de transition entre les deux clades, montre une séquence composite, issue de recombinaison, entre un CpDV 1 et un CpDV 2 suggérant qu'ils ont coinfectés les mêmes hôtes pendant une période restreinte. Nous devrons dans les années à venir tester la différence de virulence entre les virus actuels et passés (CpDV 2 versus CpDV 1). Pour cela, il nous faudra améliorer les méthodes de transfection des virus en cellule et l'isolement des CpDV à partir de larves afin de comparer de nombreux CpDV synthétiques ou provenant d'isolat naturel en réalisant des infections expérimentales comparatives. Pour cela, nous sommes en train de mettre en place une lignée cellulaire de *Cx. pipiens* qui puisse optimiser le succès de la transfection. Bien que les résultats déjà acquis en termes de virulence des CpDV soient préliminaires (un seul réplicat), ils suggèrent que le CpDV synthétique (qui est conçu à partir du génome de référence qui est un CpDV 2) était pathogène tandis que l'isolat actuellement présent dans le laboratoire (CpDV 1) est au contraire plutôt mutualiste. C'est résultats sont de première importance et représentent le chainon manquant de cette thèse. Cependant, pour mener ces expériences de manière rigoureuse, il est crucial d'établir d'abord des lignées exemptes de CpDV pour séparer les effets confondant d'un variant testé avec les effets du variant déjà présent dans la lignée. Une fois établi, un système d'infection expérimental pourrait être utilisé pour réaliser des manipulations d'évolution expérimentales des CpDV et mieux étudier ses interactions avec ses hôtes et *Wolbachia*. Nous pourrions, par exemple, répondre aux questions suivantes, comment les traits de vie du moustique sont-ils modifiés (positivement ou négativement) par l'infection par le CpDV ? Quel est le rôle du système immunitaire dans cette interaction ? Si le système immunitaire est activé par une infection par le VCDPC, est-ce que cela affecte le reste du microbiote ? Quelle est la voie d'infection horizontale des *Cx. pipiens* par les CpDV (ingestion, papilles anales) ? Les manipulations qui semblent aisée du génome du CpDV par de simples techniques de clonage pourraient également permettre des approches fonctionnelles.

La dernière partie de cette thèse portait sur les interactions entre *Wolbachia* et CpDV dans les populations naturelles (**Chapitre 4- Chapitre 5**). Nous avons d'abord révélé la présence et la diversité des *Wolbachia* et des CpDV dans les populations naturelles de *Cx. pipiens* en Turquie (**Chapitre 4**). La Turquie offre des conditions propices tant pour l'amplification des virus déjà en circulation que pour l'émergence de nouveaux virus en raison de sa situation géographique, de son climat et de la biodiversité des arthropodes vecteurs. Des études antérieures ont montré la diversité des moustiques et des virus à ARN qui circulent dans cette région (arbovirus et virus spécifiques aux insectes) (Ergunay et al., 2011, 2013, 2015 ; Erdem et al, 2014 ; Gunay et al, 2015 ; Failloux et al, 2017 ; Öncü et al, 2018). Cependant, la diversité des *Wolbachia* et des CpDV chez les moustiques *Cx. pipiens* était jusqu'à lors inconnue. J'ai pu montrer que la diversité des *Wolbachia* (et de leur patrons d'incompatibilité cytoplasmique) mais aussi des CpDV, en particulier dans la région de Thrace (nord-ouest de la Turquie), était très importante. Cela démontre une nouvelle fois que la Turquie, une zone au carrefour entre l'Europe, l'Asie et l'Afrique est une région pertinente pour étudier les interactions virus-virus et les interactions virus-*Wolbachia* dans les populations naturelles de vecteurs.

D'autre part, dans le nord de la Tunisie, la diversité des *w*Pip et de leurs patrons d'IC avaient déjà été étudiés de manière approfondie par notre équipe EVAS à l'ISEM (Atyame et al., 2015). Ainsi, les auteurs avaient découvert une zone de contact où des moustiques hébergeant deux groupes différents de *w*Pip, les groupes *w*Pip I et IV, coexistaient. Leurs patrons d'IC respectifs permettaient de prédire l'invasion de la zone de contact par la souche induisant un IC unidirectionnel, à savoir *w*PipI, en quelques générations seulement. Cependant, la zone de contact a été montrée comme stable pendant plus de sept ans. En utilisant une partie des échantillons, nous avons étudié la prévalence du CpDV dans la même région et l'effet des différents groupes de *w*Pip sur sa prévalence (**Chapitre 5**). Notre hypothèse initiale était qu'une protection contre les pathogènes (ici CpDV) conférée par l'un des groupes de *w*Pip, en l'occurrence *w*Pip IV, pourrait expliquer pourquoi le groupe *w*Pip I ne pourrait pas envahir l'ensemble des populations hôtes dans la zone de contact. De manière surprenante, nous avons démontré que ce sont les individus infectés par le groupe *w*Pip IV qui étaient plus susceptibles d'héberger le CpDV. Bien qu'il soit difficile d'interpréter ces résultats sans avoir pu étudier la nature des interactions CpDV-hôte, la prévalence plus élevée de CpDV chez les moustiques porteurs du *w*Pip-IV pourrait résulter d'une transmission verticale plus efficace du CpDV en interaction avec ce groupe de *Wolbachia*. En appui de cette hypothèse, nous avons observé, en laboratoire (**Chapitre 3**), que les moustiques porteurs de *w*Pip IV avaient dans leurs ovaires une quantité de CpDV supérieure aux moustiques porteurs de *w*Pip I. Dans cette même zone, bien que la prévalence soit affectée par les groupes de *w*Pip, il n'y a aucun effet des différents groupes sur les variations de charges virales du CpDV. Cependant, nous avons montré que la charge virale de CpDV est positivement corrélée aux densités des *w*Pip. Cela suggère que les interactions entre *Wolbachia* et les virus pourraient ne

pas toujours être en accord avec l'emblématique « protection » en général accordée à *Wolbachia*. S'il est vrai que dans le cas des arbovirus (virus à ARN) des interactions de nature antagoniste ont été principalement démontrées ce n'est pas le cas pour les virus à ADN (Teixeira *et al.*, 2008; Graham *et al.*, 2012). Il possible que ce ne soit pas le fait que les virus soit à ADN ou à ARN qui soit la cause des différences mais plutôt la nature de leur interaction avec l'hôte (hôte principal versus vecteur) qui change la relation entre *Wolbachia* et virus. En effet, les arbovirus vectorisés par les moustiques ont *a priori* moins d'impact sur la biologie des moustiques, donc sur leur valeur sélective, que les virus spécifiques aux moustiques. Il a été récemment signalé que la présence de *Wolbachia* renforçait la multiplication d'un virus à ARN spécifique d'insecte dans des cellules d'*Aedes aegypti*. De plus, les populations naturelles d'*Ae. aegypti* transinfectées de façon stable par *Wolbachia* présentaient des taux d'infection et des charges d'ARN spécifiques aux insectes plus élevés que leurs homologues sans *Wolbachia* (Amuzu *et al.*, 2018). Ces résultats démontrent que la relation entre *Wolbachia* et les virus n'est pas toujours antagoniste et que nous pourrions bien démontrer dans les années à venir que CpDV est un mutualiste de *Cx. pipiens*. Par ailleurs, en séquençant la région codante NS3, très variable dans le génome du CpDV, nous avons pu étudier la microévolution des CpDV au sein de populations de *Cx. pipiens* géographiquement proches dans le Nord de la Tunisie. Tous les variants de Tunisie appartiennent au clade CpDV1 (**Chapitre 1**), mais se regroupent en deux clades distincts et bien soutenus statistiquement (**Chapitre 6**). Nous montrons une forte structuration spatiale des variants de CpDV. De plus, l'étude de la distribution de ces deux groupes distincts a révélé un lien potentiel avec la distribution des deux groupes de *w*Pip. Compte tenu de leur co-transmission verticale, il est possible que ces deux endosymbiontes co-divergent et que leurs distributions résultent de cette co-transmission.

Bien que mon doctorat ait principalement porté sur les interactions CpDV-*Wolbachia*, les interactions entre endosymbiontes sont par essence de nature beaucoup plus complexe que de « simples » interactions tripartites. Il est probable que le reste du microbiote affecte également les interactions CpDV-*Wolbachia* et réciproquement. Ainsi, la diversité et la forte prévalence de virus à ARN spécifiques d'insectes dans les populations naturelles de moustiques ont été démontrées au cours des dernières années. Néanmoins, les recherches sur leurs effets sur leurs hôtes et le microbiote hôte n'ont pas encore étaient menées. Un tel virus à ARN associé à *Cx. pipiens* a récemment été découvert dans le nord de la Tunisie (Bigot *et al.*, 2018) offrant la possibilité d'étudier les interactions entre virus à ADN et à ARN spécifiques des insectes, l'endosymbiote *Wolbachia* et *Cx. pipiens* dans cette zone géographique. Les études futures devront tenir compte de ces interactions virales multipartenaires comme c'est le cas actuellement dans l'étude des endosymbiotes bactériens en regard du reste du microbiote.

Les MDV ont longtemps été étudiés en raison de leur pathogénicité et de leur spectre d'hôtes étroit en tant qu'agents potentiellement stratégiques en lutte antivectorielle. Deux méthodes ont été envisagées pour leur utilisation : i) leur utilisation directe pour tuer les larves de moustiques en association ou non avec d'autres traitements; ii)

leur utilisation comme agents transducteurs (Buchatskiĭ et al., 1987 ; Afanasiev et al., 1994 ; Ward et al., 2001 ; Carlson et al, 2006 ; Ren et Rasgon, 2010 ; Suzuki et al., 2014). La première méthode consiste à appliquer le MDV pathogène directement dans les habitats larvaires (Carlson et al., 2006 ; Johnson et Rasgon, 2018). Une mortalité élevée au stade larvaire pourrait en effet réduire les populations de moustiques, mais pourrait aussi entraîner une évolution rapide de la résistance en raison des fortes pressions de sélection exercées par des MDV épandus à hautes doses (Johnson et Rasgon, 2018). Certains auteurs soutiennent, qu'au contraire, la deuxième méthode pourrait « résister à l'épreuve de l'évolution », car la véritable cible serait les moustiques plus âgés, qui sont plus susceptibles de transporter des arbovirus et de les transmettre (Ren et Rasgon, 2010). Les MDV faiblement pathogènes, utilisés dans cette technique, ne causeraient pas une pression sélective aussi élevée que les MDV très pathogènes et permettraient ainsi aux larves de survivre et aux jeunes adultes de se reproduire mais empêcheraient la transmission des arbovirus (Ren et Rasgon, 2010). Bien que les deux méthodes soient prometteuses en termes de lutte contre les maladies vectorielles, elles sont encore entravées par le manque de connaissances sur la biologie et de l'écologie de base des MDV et par le manque de MDV spécifiques aux moustiques d'importance médicale (Johnson et Rasgon, 2018). Dans ce contexte, nos études sur le CpDV pourraient trouver un sens appliqué, en particulier comme agent transducteur en raison de sa persistance dans les populations de *Cx. pipiens* et de sa faible pathogénicité. Pour mieux comprendre la biologie du CpDV, il serait utile d'étudier sa spécificité en termes d'hôtes. Comme ils sont génétiquement plus proches des ambidensovirus lépidoptères (Baquerizo-Audiot et al., 2009 ; Cotmore et al., 2014), il est possible qu'ils soient infectant pour ce groupe d'insecte. Très récemment, un densovirus génétiquement similaire au CpDV a été découvert dans des populations naturelles de Drosophiles proches de Paris montrant que ce virus n'est peut-être pas spécifique des *Culex* au sein des Diptères (Kapun et al., 2018). Les transfections de cultures cellulaires par le CpDV avaient par ailleurs montré que ce virus étaient capables de s'amplifier dans les cellules d'Anophèles suggérant également que leur spécificité n'était pas stricte (Jousset et al., 2000). Nos résultats préliminaires d'infection expérimentale ont également montré que les CpDV (clade 1 ou 2) n'entrainent pas de mortalité chez les larves d'*Ae. albopictus*. Des études futures sur Anophèles, certains lépidoptères ravageurs et la drosophile pourraient permettre de préciser le spectre d'hôtes de CpDV.

Les récent progrès de la technologie de séquençage et des techniques de biologie moléculaire ont permis aux chercheurs de révéler l'ubiquité et la diversité des virus. Malgré ces progrès, la découverte de virus s'est surtout concentrée sur les virus de vertébrés. Les arthropodes avec leur histoire évolutive ancienne représentent 80% de la diversité animale sur notre planète. En supposant une corrélation entre la diversité des hôtes et la diversité des virus, cela pourrait signifier que nous sommes encore inconscients de la plupart de la diversité des virus existants. De plus, même les virus connus ne sont que peu étudiés en termes d'écologie virale. L'omniprésence de l'endosymbiose bactérienne chez

les arthropodes et la capacité des arthropodes à soutenir des symbiotes (parfois multiples) et de leur « déléguer des fonctions » suggèrent que leurs interactions avec leurs virus pourraient également être de nature symbiotique, allant des interactions durables de type mutualiste à celles de type antagoniste. Cette nouvelle approche conceptuelle des virus, tout en présentant de nombreux défis, pourrait répondre à un large éventail de questions sur l'écologie et l'évolution des interactions hôte-microbiote. Les densovirus, aussi anciens et divers que leurs hôtes arthropodes, sont omniprésents dans la nature et ont des petits génomes faciles à manipuler. Ils représentent donc de bons modèles pour étudier les interactions hôte-virus-bactéries. À l'intersection de nombreux domaines, nos résultats, bien qu'ils aient pu soulever plus de questions qu'ils n'en aient répondu, ont suggéré que le système CpDV-*Cx. pipiens*-*Wolbachia* pourrait être un système modèle pertinent pour étudier les interactions écologiques entre ces partenaires et leur évolution conjointe tant dans la nature qu'au laboratoire.

Appendix A:

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Appendix B:

Jury Reports

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Referee report on the PhD thesis of Mine ALTINLI «Ecology and evolution of *Cupex pipiens*-*Wolbachia*-Densovirus tripartite interactions»

Pr. Yannick Outreman
Agrocampus Ouest – Rennes, France

Mine ALTINLI submitted the doctoral thesis dealing with the evolution and ecology of a tripartite interaction including an insect, a bacterial symbiont and ssDNA viruses. Given that bacterial symbioses are ubiquitous in insects and that those arthropods are often infected with viruses, Mine ALTINLI asked for a very interesting, innovative and original research questions: (1) are there some interactions between viruses and bacterial symbionts within insect individuals/populations and (2) how may evolve an ecological trio composed of an arthropod, a bacterial symbiont and viruses. Viruses have long been considered as microbes causing mortality in infected insect populations. However, interactions between insect and viruses might have be of symbiotic nature, intimidate long-term associations with effects on host individuals ranging from mutualistic to antagonistic. In this context, the PhD work focused on prevalence and diversity of densoviruses in the *Culex pipiens* mosquito species (CpDV) and their interactions with the symbiotic bacteria *Wolbachia* harbored by mosquitos. The topic of the thesis is highly relevant for all studies on evolutionary ecology of interspecific interactions, a very important field of biological research.

Quality of the manuscript

The PhD manuscript is mainly written in English, and the results are presented in the form of chapters written as papers that have been or will be submitted/accepted. The report, of high quality, consists of seven chapters, covers 158 pages, including the introduction, four papers, the discussion and a French summary of the thesis. The overall style is clear and the extent of the thesis corresponds to its significance and amount of new knowledge it details, especially considering the fact that results of the thesis are published or submitted in renowned journals.

Introduction section

The first part, the Introduction, gives a good literature review covering the essential background of the thesis and details biological models studied during three years. The main goals of the PhD work are also described but too succinctly. Mine ALTINLI should have detailed the research questions included in her thesis work and the link between those issues.

Results section

The result section contains four subsequent parts, each presenting an article written by the candidate as first author. The first manuscript, not submitted, presents investigation of the CpDV prevalence in natural populations using specific PCR-based diagnostic test and considering both past and contemporary samples. Also, by sequencing amplicons from virus genome, Mine ALTINLI analysed dispersal of CpDVs and their evolution within mosquito's populations. A high prevalence

and high diversity of CpDVs were found in studied populations. Highly significant migration paths and lack of geographical structure in CpDVs diversity suggest efficient dispersal worldwide.

Given high prevalence and high dispersal of CpDV viruses in natural populations, second part asked for two main factors influencing infection dynamics of densoviruses within *C. pipiens* populations: (1) the CpDVs transmission routes and (2) nature of their interactions with mosquito hosts. If the effect of densoviruses on host fitness is shortly presented in an Appendix (Appendix A, pp 75-77), the vertical transmission of CpDV is detailed in a paper submitted to *Environmental Microbiology*. Results show that CpDV are vertically transmitted via transovarian path along with the endosymbiont *Wolbachia*. Rate of transmission is highly variable at the individual level but not at the mosquito line one and interestingly, is marginally lowest when hosts are uninfected with *Wolbachia*. Also, the density of CpDV in host's ovaries would be controlled by maternally transmitted cytoplasmic factors: *Wolbachia* strains would affect density of CpDV.

The third Result's part present studies focused on *Wolbachia* and CpDV prevalence and diversity in Turkey. Firstly, a published paper details the study focusing on the *Wolbachia* diversity and cytoplasmic incompatibility in *C. pipiens* populations in Turkey. Within this country, all individuals were infected with *Wolbachia* from three different groups with varying frequencies (I, II and IV). The most widespread groups, I and II, induce reciprocal computability between hosts and this would explain their coexistence. In addition, contrary to expectations, coexistence of bidirectional incompatability inducing strains (II and IV) has been observed in one site. Interestingly, mosquito males infected with *Wolbachia* of group IV may be used to sterilise mosquito females infected with *Wolbachia* from the two other groups. Secondly, Mine ALTINLI details a study that focused on the prevalence of CpDV and diversity in natural populations of *C. pipiens* in Turkey. About 60% of individuals were infected with the CpDV, an infection rate that varies between mosquito life stages and host population locations. The *Wolbachia* group harboured by the mosquitoes has not effect on densoviruses prevalence. CpDV diversity has been found in Western-North Turkey only and there is no clear link between CpDV and *Wolbachia* diversity.

Finally, to test whether *Wolbachia* could be a major determinant of CpDV dynamics in mosquito populations, the fourth Result's part present a very interesting study focused on the influence of the bacterial endosymbiont *Wolbachia* on prevalence, viral load and diversity of CpDV. Reciprocal interactions between those endosymbionts are suggested from results: (1) *Wolbachia* groups influence prevalence of CpDV in Tunisian mosquito populations but not their load; (2) there is a positive correlation between CpDV and *Wolbachia* loads; (3) group variation in *Wolbachia* would influence distribution of CpDV variants in *C. pipiens* populations, a distribution covariation explained by co-vertical transmission of these microbial endosymbionts.

Discussion section

In the final chapter, a general conclusion and perspectives are offered. In the discussion section of each manuscript, plus this general discussion, Mine ALTINLI clearly demonstrates by her writing that she has thought deeply about the results and attempts to interpret them in regards to the work done by others. This section is very much appreciated and summarises with clarity results obtained. Besides, areas of further research suggested by the findings are also clearly proposed and there clearly remains much to be done in this very interesting field of research. Without any doubt, this thesis will incite further studies on other tripartite interaction between arthropods, bacterial symbionts and viruses.

Recommendation

After carefully reading and studying Mine ALTINLI's thesis, I am fully convinced it represents a significant and original contribution to the scientific field it deals with. The thesis contains original results and ideas, and therefore is a quite important publication. Mine ALTINLI does highlight the novel findings of her studies, and at times indicates their limitations, providing new relevant perspectives. Overall, Mine ALTINLI has demonstrated excellent scientific rigour in her writing and the report shows that Mine ALTINLI masters a number of field, experimental and analytical techniques and academic fields for answering addressed issues, proving to be skilled scientist.

En conclusion, ma recommandation pour l'Université de Montpellier est d'accepter sans aucune réserve que la thèse de Mine ALTINLI soit soutenue.

Yannick OUTREMAN

Rennes, 28th October 2018

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Objet : Rapport d'évaluation du manuscrit de thèse de doctorat de Mine Altinli

Le Service des Études Doctorales,

I read with pleasure the thesis of Mine Altinli, on the evolutionary and symbiotic relationships between Culex mosquitoes, Wolbachia and densoviruses. The Introduction of the thesis presents and interesting and insightful overview of the relevant topics: the concept of symbiosis, the interactions between symbionts, a summary of different phylogenetic groups of DNA viruses including CpDV, a review of the biology of Wolbachia as a symbiont including the effects on vertebrate pathogen transmission, and finally framing the subject of the thesis, which is the tripartite interaction between Cx. pipiens, CpDV, and Wolbachia.

The second chapter, which will be submitted before the end of 2018, describes the natural distribution of CpDV in Cx pipiens populations on all main continents. A diagnostic PCR test was designed, and about half of all samples tested, in most global populations, were positive for CpDV carriage. Two amplicons were amplified and sequenced for a subset of samples, which

was used to infer evolutionary histories of 3 CpDV lineages with distinct inferred recombinational origins. This part provides a valuable descriptive substrate for the following sections;

Chapter 3 describes the modes of transmission and persistence of CdDV in laboratory lines of Cx pipiens. Levels of both microbes displayed variation between lines, and crosses were established to test interaction. Only Wolbachia strain but not Wolbachia abundance or host genotype influenced CpDV abundance. Large variation was observed in CpDV vertical transmission rates by individual mosquitoes regardless of Wolbachia strain or host genetic line. After curing of Wolbachia, CpDV abundance levels per individual decreased transiently but then returned to previous levels, but CpDV vertical transmission rate per female was reduced. It is interesting how low the vertical transmission rate is, which is likely balanced by horizontal transmission from infected individuals to maintain persistent infection of the line. It is also interesting that the levels of the two microbes seem largely independent, and the transient decrease in CpDV may be due to perturbed microbiome and/or physiology from antibiotic or loss of Wolbachia contribution.

Chapter 4 is a study of CpDV and Wolbachia in the population in Turkey, of which the Wolbachia part was published in 2018. All of the samples carried Wolbachia wPip strains, and three strains were represented. There was only one strain per individual. Sympatric strain distribution was determined, and cytoplasmic incompatibility (CI) was tested between strains. CpDV levels were also tested in samples from Turkey, and most populations were positive. An interesting observation was a potential correlation between wPip strain and CpDV clade. In addition, results on adult carriage and possible mortality or other explanations merit further examination.

Chapter 5 synthesizes some of the previous studies in a zone in Tunisia with Cx pipiens populations carrying CpDV as well as incompatible wPip strains. The results indicate dependence of wPip and CpDV on several levels, both wPip strain and CpDV prevalence, and

also positive correlation of loads of both microbes per individual mosquito. For the latter observation, I wonder if dual-microbe load is a surrogate measure of individual overall fitness, or maybe size. Finally, two clades of CpDV were identified by sequencing, which the distribution correlated with wPip strain. This section is very interesting and when prepared for publication will make an important contribution to the understanding of the ecology of the multiple microbial "-omes", bacterial and viral, carried by animals. It is not yet part of the general paradigm that the virome as well as bacterial microbiome could include interactions with the host that range from commensal to pathogenic. The outcome will probably be context-dependent, according to microbial interactions with host immunity and physiology, and also ecological interactions of the microbes with each other within the host.

In short, the results of this thesis work seemed to me extremely interesting, of a high scientific and technical level, and I can express an opinion entirely favorable to the defense of this thesis by Mine Altinli on November 28. It will be a great pleasure for me to participate.

Kenneth Vernick

Ecology and Evolution of *Culex pipiens* - *Wolbachia* - Densovirus Tripartite Interactions

Virus discovery has long depended on the observation of outbreaks, and the research to understand their pathogenicity depended on their culture. The advances of sequencing technology and molecular biology techniques allowed researchers to discover the ubiquity and the diversity of viruses. Despite these advances, virus discovery remains mainly focused on vertebrate viruses. Furthermore, even the known diversity of these viruses is poorly studied in term of virus ecology. Arthropods with their ancient evolutionary history represent 80% of animal diversity on our planet. The ubiquity of endosymbiosis in arthropods and their ability to support (sometimes multiple) symbionts suggest that their interactions with their viruses might be of symbiotic nature, intimate long-term associations with outcomes ranging from mutualistic to antagonistic. In this context, this PhD dissertation focuses on a mosquito densovirus, *Culex pipiens* densovirus (CpDV), to investigate their prevalence and diversity in natural populations of *Culex pipiens* mosquitoes and their interactions with endosymbiotic bacteria *Wolbachia* in a natural host-bacteria-virus system (*Cx. pipiens* - *Wolbachia* - CpDV) both in natural populations and laboratory colonies. We reveal a high prevalence and diversity of CpDV in worldwide *Cx. pipiens* populations. Moreover, CpDV persistently infects laboratory lines and are vertically transmitted along with their hosts' endosymbiont *Wolbachia*. We reveal high CpDV prevalence and diversity in Turkey and Tunisia and further focus on these two countries to investigate *Wolbachia*-CpDV interactions in natural populations. In Tunisia, in a narrow contact zone where two genetically distinct *Wolbachia* groups coexist, different *Wolbachia* groups influences CpDV prevalence and diversity. Moreover, we show a positive correlation between *Wolbachia* density and CpDV density. Overall, our results suggests that CpDV-*Cx.pipiens* - *Wolbachia* system is a good model system to study the ecological interactions between these partners and their evolution together both in the nature and in laboratory conditions. Densoviruses with their diversity and long-term evolution with their hosts, and their easily manipulated small genomes, are good models to study complex host-virus-bacteria interactions as well as virus ecology and evolution in a symbiotic context.

Écologie et évolution des interactions tripartites entre *Culex pipiens*, *Wolbachia* et les Densovirus

Les récents progrès des technologies de séquençage et des techniques de biologie moléculaire ont permis aux chercheurs de révéler l'ubiquité et la diversité des virus. Malgré ces progrès, la découverte des virus s'est surtout concentrée sur les virus de vertébrés. De plus, les virus connus sont très peu étudiés en terme d'écologie virale. Les arthropodes de par leur histoire évolutive ancienne représentent 80% de la diversité animale sur notre planète. L'omniprésence de l'endosymbiose chez les arthropodes et leur capacité d'héberger des symbiotes (parfois multiples) suggèrent que leurs interactions avec leurs virus pourraient également être de nature symbiotique, allant des interactions durables de type mutualiste à celles de type antagoniste. Dans ce contexte, mon travail de thèse s'est concentré sur un densovirus de moustiques, le *Culex pipiens* densovirus (CpDV), en étudiant leur prévalence et leur diversité dans les populations naturelles de moustiques *Culex pipiens* et leurs interactions avec les bactéries endosymbiotiques *Wolbachia* dans un système naturel hôte-bactérie-virus (*Cx. pipiens* - *Wolbachia* - CpDV), tant dans des populations naturelles que des colonies de laboratoire. Nous avons révélé une forte prévalence et une grande diversité de CpDV dans les populations mondiales de *Cx. pipiens*. De plus, le CpDV infecte de façon persistante les lignées de laboratoire et est transmis verticalement avec l'endosymbiote *Wolbachia*. Nous montrons une prévalence et une diversité élevées du CpDV en Turquie et en Tunisie, et nous nous sommes concentrés davantage sur ces deux pays pour étudier les interactions *Wolbachia*-CpDV dans les populations naturelles. En Tunisie, dans une zone de contact étroite où deux groupes de *Wolbachia* génétiquement distincts coexistent, ces différents groupes de *Wolbachia* influencent la prévalence et la diversité du CpDV. Nous montrons également une corrélation positive entre la densité de *Wolbachia* et la densité de CpDV. D'une manière globale, nos résultats suggèrent que le système CpDV-*Cx.pipiens* - *Wolbachia* et plus généralement les densovirus sont de bons modèles pour étudier les interactions écologiques entre ces partenaires et leur évolution dans la nature et au laboratoire.