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Interaction of novel natural RNA viruses with Anopheles malaria vectors

Ferdinand Nanfack Minkeu

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Sorbonne Université

Ecole doctorale Complexité du Vivant

Unité de Génétique et Génomique des Insectes Vecteurs

Interaction of novel natural RNA viruses with Anopheles malaria vectors

Interaction entre les virus naturels à ARN et les anophèles,
vecteurs du paludisme

Par Ferdinand NANFACK MINKEU

Thèse de doctorat de Science du Vivant

Dirigée par Dr Kenneth Vernick

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For Maxime Ndong Nanfack, my dear son, you gave me energy to finish this thesis. I hope that it will be an example for you, never stop reaching for your objectives.

‘Nothing is more dangerous than an idea, when you have only one idea’
“Rien n'est plus dangereux qu'une idée, quand on n'a qu'une idée”
Alain (*Émile-Auguste Chartier*, 1868-1951)

SUMMARY

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ABBREVIATIONS

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A

aa – amino acids

Ae – *Aedes*

AGO2 – Argonaute 2

AMP – Antimicrobial peptide

An – *Anopheles*

AnCV – *Anopheles C* virus

AnCPV – *Anopheles cypovirus*

AgDNV – *Anopheles gambiae* densovirus

APL1- *Anopheles Plasmodium*-responsive *leucine*-rich repeat 1

B

BHK – Baby hamster kidney cells

BSA – Bovine Serum Albumin

C

cDNA – complementary DNA

CHIKV – Chikungunya Virus

CLIP – Clip domain serine protease

CPE – Cytopathic effect

Cx– *Culex*

D

DAPI - 4',6-Diamidino-2-Phenylindole

DCV – *Drosophila C* Virus

DENV – Dengue Virus

DMEM – Dulbecco's minimum essential media

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

Dpi – Days post infection

ds – double stranded

E

EDTA – Ethylenediaminetetraacetic acid

EEEV – Eastern Equine Encephalitis Virus

EVE – endogenous viral element

F

FCS – Fetal Calf Serum

FHV – Flock House Virus

G

GFP – Green fluorescent protein

GO – gene ontology

H

HOP – Hopscotch

I

IFA – immunofluorescence assay

IMD – Immune Deficiency

ABBREVIATIONS

IM – intramuscular

IP - intraperitoneal

ITN – Insecticide treated bed net

IRS – Indoor residual spraying

ISV– Insect specific virus

J

JAK/STAT – Janus Kinase/Signal Transducer and Activator of Transcription

JEV – Japanese Encephalitis Virus

K

KD – knock down

KO – knock out

L

LRIM – Leucine rich immune gene

LRR – leucine rich repeat

M

miRNA – microRNA

miRNP – microRNA ribonucleoprotein complex

mRNA – messenger RNA

N

nt – nucleotide

nsP – non structural protein

O

ONNV – O'nyong-nyong Virus

ORF – Open reading frame

P

PBS – phosphate buffered saline

PCI – phenol/chloroform/iso-amyl alcohol

PCR – polymerase chain reaction

PGRP – Peptidoglycan recognition protein

PI – Post infection

PIWI – P element induced wimpy testis

PO – Phenoloxidase

PRR – Pathogen recognition receptor

Q

Qrt-PCR – Quantitative real time PCR

R

raSiRNA – repeat associated short interfering RNA

RDRP – RNA dependent RNA polymerase

RISC – RNA induced silencing complex

RNA – ribonucleic acid

RNase - ribonuclease

RNAi – RNA interference

Rpm – revolutions per minute

RT – room temperature

S

ss –single stranded

ABBREVIATIONS

SFV – Semliki Forest Virus

SINV – Sindbis Virus

siRNA – short interfering RNA

sp – species

T

TEP – Thioester containing protein

TLR – Toll-like receptor

T_m – primer melting temperature

U

UPD – Unpaired

V

VERO – African Green monkey kidney cells

viRNA – virus specific short interfering RNA

VEEV – Venezuelan Equine Encephalitis Virus

W

WEEV – Western Equine Encephalitis Virus

WNV – West Nile Virus

WHO – World Health Organization

Y

YFV – Yellow Fever Virus

Z

ZIKV – Zika virus

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Les anophèles sont les principaux vecteurs du parasite *Plasmodium* responsable du paludisme humain dans le monde, où l'Afrique sub-saharienne paye le plus lourd tribut. Le paludisme est une parasitose due au *Plasmodium* qui a touché plus de 200 millions de personnes et en a tué plus de 400 000 en 2016. Les moustiques du genre *Anopheles* transmettent aussi le virus O'nyong nyong (ONNV, *Togaviridae*, *Alphavirus*) qui cause une arbovirose ayant touché plus de 2 millions de personnes en Afrique entre 1959 et 1960 (Williams *et al.*, 1965). La plupart des études relatives aux interactions anophèles-pathogènes se sont focalisées sur les interactions entre *Plasmodium* et *Anopheles*. Cependant une faible caractérisation des virus d'anophèle peut être notée dans la littérature. De plus, le virome d'anophèle s'avère diversifié et mal caractérisé (Colmant *et al.*, 2017a; Colmant *et al.*, 2017b; Nanfack Minkeu and Vernick, 2018). Les virus d'anophèles identifiés ces dernières années par des analyses de métagénomique sont classifiés comme des virus spécifiques d'insectes (VSIs) (Carissimo *et al.*, 2016; Fauver *et al.*, 2016; Nanfack Minkeu and Vernick, 2018). Ces derniers se répliquant uniquement chez les insectes n'ont pas fait l'objet d'études plus poussées concernant leurs impacts sur la biologie et l'immunité de l'anophèle ou d'une possible transmission aux hôtes vertébrés. Du fait de l'émergence et/ou la ré-émergence récentes de viroses telles que ZIKA et Chikungunya, aussi transmises par des moustiques, nous avons entrepris de rechercher et de caractériser de nouveaux virus pouvant être transmis par l'anophèle, et ce afin de prévenir l'émergence de potentielles nouvelles arboviroses transmises par cette espèce de moustique (Carissimo *et al.*, 2016). Ce travail est d'autant plus important du fait que les symptômes cliniques entre paludisme et arboviroses sont assez proches, masquant ainsi l'identification de nouvelles arboviroses. De plus, du fait que la plupart des arboviroses sont transmises par les genres *Aedes* et *Culex*, les praticiens et chercheurs présentent un faible intérêt pour les arbovirus et VSIs potentiellement transmis par l'anophèle. Ce faible intérêt a conduit à une faible connaissance des virus d'anophèles et de leurs réponses antivirales. Les études de réponses antivirales menées chez un autre modèle, la mouche *Drosophila melanogaster* utilisant le virus C de la drosophile ou « *Drosophila C virus* » (DCV) ont permis des avancées majeures en médecine. De ce fait un modèle d'étude similaire chez l'anophèle serait d'un apport significatif au regard, par exemple, de la haute pathogénicité d'ONNV. Par ailleurs, face aux dégâts environnementaux et sur la santé humaine causés par l'utilisation des insecticides contre les vecteurs responsables du paludisme et des arboviroses (ONN, Zika, dengue, chikungunya,

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Encéphalite japonaise, etc...) ; la para-transgénèse ciblant les virus identifiés chez l'anophèle pourrait constituer un outil supplémentaire dans l'arsenal de lutte anti-vectorielle (Iturbe-Ormaetxe et al., 2011; Ren and Rasgon, 2010).

Le but de ce travail de thèse était d'explorer la flore virale des anophèles et de caractériser deux de ses nouveaux virus, *Anopheles C virus* (AnCV) et *Anopheles Cypovirus* (AnCPV), récemment identifiés chez *Anopheles coluzzii*, membre du complexe *An. gambiae* et l'un des vecteurs majeurs du paludisme en Afrique subsaharienne (Carissimo et al., 2016). Le premier est un dicistrovirus proche du DCV, et le second, AnCPV, est un virus à ARN double brin de la famille des Reoviridés incluant également l'arbovirus responsable de la fièvre catarrhale ovine (bluetongue virus). Plus précisément, le projet visait : à explorer le virome des anophèles, de faire une description biologique de ces virus, d'étudier les réponses antivirales chez l'anophèle, de comprendre comment ces différents virus interagissent au sein de l'anophèle, de générer un modèle pour faciliter leurs études, d'étudier les variations génomiques chez les espèces anophéliennes et enfin de comprendre les facteurs influençant la co-évolution virus/anophèle.

Au cours de ce travail, nous avons entrepris d'étudier le comportement de ces virus chez *An. coluzzii* par transcription inverse suivie d'une réaction de polymérisation en chaîne (RT-PCR). Cette première approche nous a permis de détecter une variation de la prévalence d'*Anopheles C virus* et d'*Anopheles Cypovirus* dans différentes lignées anophéliennes et à différents stades biologiques. Presque toutes les lignées d'*An. gambiae* sont co-infectées, et ce statut entraîne une difficulté supplémentaire à la caractérisation d'un seul virus. Nous avons identifié un mécanisme intra-embryonnaire dans la transmission verticale de ces virus chez *An. coluzzii*, Ngousso. Pour affiner la détection de ces virus, des PCR quantitatives en Taqman duplex ont été développées et ont permis de mettre en évidence une corrélation négative entre AnCPV et AnCV chez *An. coluzzii*. En effet des anophèles hautement infectés à l'un des virus sont faiblement ou non-infectés à l'autre. Ce type d'interaction entre deux virus de famille différente s'appelle interférence hétérologue par opposition à l'interférence homologue entre virus de la même famille. Notre étude est l'une des rares études *in-vivo* à montrer une interférence virale chez des moustiques, la plupart étant des études *in-vitro*. Dans ces derniers cas, le virus sindbis (*Togaviridae*, *Alphavirus*) réduit la répllication du virus de l'encéphalite équine vénézuélienne dans la cellule C7/10 d'*Ae. albopictus*

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(Nasar et al., 2015). Le virus spécifique d'insecte, Nhumirim virus empêche la réplication des arbovirus dengue et du virus du Nil Occidental (West Nile virus) dans les cellules des culicidés (Goenaga et al., 2015; Zhang et al., 2017).

Les interactions entre virus ou micro-organismes sont au cœur des nombreuses études et permettent d'envisager l'utilisation des symbiotes pour combattre des micro-organismes pathogènes ou les hôtes. Cette approche nommée paratransgénèse peut être utilisée de différentes manières en fonction du but visé. Par exemple, la para-transgénèse est envisagée pour combattre les parasites du paludisme. Ainsi, chez *An. stephensi*, des bactéries du genre *Asaia* génétiquement modifiées pourraient diminuer l'infection à *P. berghei* (Capone et al., 2013). Un autre but recherché en paratransgénèse peut être l'élimination du moustique vecteur par l'un de ses symbiotes. Or dans ce travail, nous démontrons une diminution de la durée de vie des populations d'*An. stephensi* infectées par AnCPV. De même, *Anopheles* densovirus (AgDENV, *Densovirus*, *Parvoviridae*) diminue la durée de vie des populations d'*An. gambiae* (Ren et al., 2008; Ren and Rasgon, 2010). Ces résultats montrent que les virus peuvent être utilisés pour combattre leurs hôtes. De plus, les virus non pathogènes à l'homme comme les VSIs pourraient également être utilisés pour combattre des arbovirus. Cette hypothèse pourrait être testée pour la première fois chez les anophèles en étudiant les interactions entre AnCV/AnCPV et ONNV chez des lignées anophéliennes.

Au cours de ce travail nous avons également mené des études fonctionnelles par des approches d'interférence à l'ARN pour caractériser les réponses antivirales chez *An. coluzzii* Ngousso. Ces études ont montré un contrôle d'AnCPV par la voie immunitaire de signalisation Toll, et ce, en absence ou faible niveau d'infection d'AnCV chez *An. coluzzii*. En plus de Toll, ces études ont également montré l'implication de la voie JAK/STAT sur le contrôle de la prévalence d'AnCPV chez les anophèles co-infectés. Nos résultats obtenus avec Cactus corroborent ceux obtenus par Xi et collaborateurs qui ont montré que l'extinction de cactus a entraîné une réduction de la charge virale de la dengue chez *Ae. aegypti* (Xi et al., 2008). Nous confirmons ici le rôle de la voie JAK/STAT dans l'infection virale chez les anophèles précédemment mis en évidence par Carissimo et collaborateurs sur le contrôle de la charge virale d'ONN (Carissimo et al., 2015b). Cependant, nos résultats montrent un rôle pro-viral de JAK/STAT et alors que ceux de Carissimo montrent plutôt

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un rôle antiviral de cette voie ; cette différence peut être due à la famille virale (*Reoviridae* contre *Togaviridae*) et le type d'infection (disséminée contre primaire).

Par contre, l'extinction d'AGO2 associé à la voie de l'ARN interférence (RNAi) n'a pas eu d'influence sur les prévalences et les charges virales de deux virus, suggérant l'existence potentielle des surpresseurs de cette voie par ces virus. En effet, le DCV et le virus de la paralysie du criquet (CrPV) présentent des inhibiteurs ou surpresseurs du RNAi nommés respectivement DCV-1A et CrPV-1A (Nayak et al., 2010; van Rij et al., 2006).

Au cours de cette thèse, nous avons mis en place un modèle d'étude utilisant du sang infecté par ces virus et la lignée *An. stephensi* qui en est dépourvue pour mieux caractériser les infections de ces virus chez l'anophèle. Grâce à ce modèle, et du fait que seul AnCPV infectait *An. stephensi*, nous avons entrepris un séquençage d'ARN (RNAseq) afin de caractériser le transcriptome d'*An. stephensi* pendant son infection primaire et disséminée à AnCPV. Ce dernier régule l'expression de plusieurs gènes chez ce vecteur dont plusieurs ne sont pas connus/caractérisés. Parmi ceux qui sont connus, certains sont impliqués dans des voies métaboliques des macromolécules et d'autres dans la réponse immunitaire de l'anophèle. A notre connaissance, c'est la première fois que le transcriptome d'un moustique est généré lors de son infection à un VSI ouvrant ainsi des perspectives dans la compréhension des interactions entre virus et moustique. La suite de ce travail consistera à confirmer nos résultats de RNAseq par des qPCR quantitatives, puis de procéder à des études de génomiques fonctionnelles afin de mieux comprendre la réponse d'anophèle lors de son infection à AnCPV.

En outre, nous avons pu montrer qu'AnCPV peut être transmis horizontalement, potentiellement comme un arbovirus, de l'anophèle au lapin et réciproquement, amplifiant la nécessité de caractériser plus de virus d'anophèles, considérés comme des VSIs, afin de vérifier leurs transmissions aux vertébrés. Ce résultat est le premier du type chez l'anophèle et montre bien que l'anophèle, en plus de transmettre des parasites du genre *Plasmodium*, peut également transmettre des virus autres qu'ONNV. De plus, notre étude rétrospective révèle l'implication d'anophèle dans des épidémies d'arboviroses et montre que les virus d'anophèle peuvent être regroupés en arbovirus, probables arbovirus et virus spécifiques d'insectes. Des résultats similaires ont été obtenus chez des moustiques du genre *Culex* et *Aedes* par d'autres auteurs (Atoni et al., 2018; Zakrzewski et al., 2018). Néanmoins, il reste difficile d'avoir une idée du nombre de VSI et

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d'arbovirus chez les *Culicinae*; notre étude rétrospective donne un aperçu quantitatif chez les *Anophelinae*. Le défi actuel est de mieux comprendre pourquoi les arboviroses les plus mortelles qui sont la dengue, la fièvre jaune, le zika, la fièvre de la vallée du Rift, l'encéphalite japonaise et d'autres sont majoritairement transmises par *Culex* et *Aedes* (Cunha et al., 2017; Dwibedi et al., 2015; Patterson, 1992; Tambo et al., 2016). Des études de compétence vectorielle et génomique fonctionnelle apporteront quelques réponses sur ces différences de transmission d'arbovirus entre les différents *Culicidae*.

Par ailleurs, nous avons entrepris au cours de ce travail des études de métagénomique et d'assemblage *de novo* qui ont démontré qu'AnCV avait des mutations mineures alors qu'AnCPV présentait une diversité génétique intra et inter hôte, avec des polymorphismes fixés d'un hôte à un autre. Nos résultats sont en accord avec des études faites sur plusieurs virus à ARN dont le virus du Chikungunya (CHIKV) et se justifieraient par l'incapacité des ARN polymérases à corriger leurs erreurs de réplication entraînant des taux élevés de mutation et recombinaison chez ces virus. En l'occurrence dans le cas du CHIKV, sa variabilité génétique lui a permis de s'adapter à *Ae. albopictus* afin de provoquer des épidémies dans plusieurs pays de l'Océan Indien (Kek et al., 2014; Schuffenecker et al., 2006). La variabilité génétique permet également aux arbovirus d'échapper aux systèmes immunitaires des hôtes. La variabilité génétique devrait être caractérisée chez tous les virus car elle joue un rôle majeur dans l'émergence de nouvelles arboviroses. Les autres facteurs impliqués dans l'émergence des arboviroses sont les changements climatiques et les comportements anthropologiques tels que les voyages, la construction des barrages, les déforestations, les irrigations et bien d'autres. De plus, les variations génétiques, les recombinaisons et les réassortiments (virus segmentés) sont complètement ou partiellement nécessaires à l'évolution des virus. A titre d'exemple, l'encéphalite équine de l'Ouest des états unis (WEE) a résulté d'une recombinaison génétique entre l'encéphalite équine de l'Est (EEE) et Sindbis virus (Hahn et al., 1988).

L'exploration du virome d'*An. stephensi* a aussi été menée au cours de ce travail par des approches de métagénomique et deux virus à ARN jamais décrits chez cette espèce anophélienne ont été découverts, il s'agit d'un « chaq-like virus » et d'un « partiti-like virus ». Si le premier est très peu documenté, le second est très présent chez les plantes, les champignons, les protozoaires et ses formes d'ADN ont été retrouvées chez *An. stephensi* dans le cadre de ce travail. Les formes d'ADN

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des non-rétrovirus contribuent à leurs persistances dans leurs hôtes (Goic et al., 2013). Ces formes d'ADN produites par des reverse transcriptases peuvent être intégrées dans les génomes (endogénéisation) de leurs hôtes entraînant une coévolution (hôte/virus). Les séquences virales intégrées aux génomes de leurs hôtes sont rares et sont nommées des éléments viraux endogènes (EVEs) ou des séquences intégrées des virus à ARN non-rétroviraux pour les non-rétrovirus (Lequime and Lambrechts, 2017; Palatini et al., 2017; Suzuki et al., 2017). Chez les anophèles, les intégrations virales des non-rétrovirus ont été décrites chez les familles de *Rhabdoviridae*, *Flaviviridae*, *Reoviridae*, *Bunyaviridae* et aucune intégration ou forme d'ADN n'était encore décrite chez les *Partitiviridae* (Lequime and Lambrechts, 2017; Palatini et al., 2017; Suzuki et al., 2017).

Nos analyses *in silico* et la technique d'hybridation moléculaire *in situ* (FISH) ont montré une non-intégration du partiti-like virus (PV) dans le génome d'anophèle. Cette non-intégration du PV pourrait s'expliquer par un processus d'endogénéisation inachevée ou un déficit d'intégrase. Les mécanismes d'endogénéisation sont mal connus et la plupart des études sont faites chez les rétrovirus. Nos résultats sur ces formes d'ADN d'un partiti-like virus, en plus d'autres études sur les intégrations virales des non-rétrovirus, renforcent l'intérêt d'étudier les intégrations virales et de l'élargir aux autres familles de virus, ouvrant ainsi de nouvelles perspectives pour la paleovirologie. Cette dernière étudie l'évolution et l'influence des anciens virus sur la biologie de leurs hôtes (Patel et al., 2011).

Nos études nous ont permis de mettre en évidence : la variation en prévalence d'AnCV et AnCPV, une corrélation négative chez *An. coluzzii* Ngousso entre les deux virus, la pathogénicité d'AnCPV chez *An. stephensi* après un repas de sang infectieux. L'implication des voies de Toll et JAK/STAT dans le contrôle d'AnCPV qui présente un polymorphisme génétique et est transmissible comme un arbovirus. Nous avons découvert des virus chez *An. stephensi* et l'un d'eux présente des formes d'ADN. Dans l'ensemble, ce travail de thèse met à la disposition de la communauté scientifique des outils complémentaires pour étudier les interactions entre virus et vecteurs du paludisme. Il contribue à la compréhension des réponses virales chez les anophèles. Nous soulignons le fait que certains virus décrits comme VSIs pourraient être potentiellement des arbovirus. Il est donc nécessaire de continuer à rechercher des nouveaux virus par des approches méta-génomiques ou autres méthodes, mais il est surtout nécessaire de les caractériser afin de comprendre leurs

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évolutions, leurs interactions avec d'autres virus pathogéniques existants et leurs impacts sur la biologie des hôtes.

L'une des difficultés majeures de ce projet a résidé dans l'échec de l'isolement/purification de ces virus malgré plusieurs tentatives sur différentes lignées cellulaires. L'autre difficulté a été l'incapacité à maintenir/trouver des lignées anophéliennes non-infectées à nos virus.

Nous envisageons de caractériser la variabilité génétique d'AnCPV afin d'explorer l'évolution de ce virus lors de son adaptation à une autre espèce d'anophèle. Nous essaierons de cultiver les virus identifiés chez *An. stephensi* afin de les isoler et d'obtenir le génome complet pour des études ultérieures de variabilité génétique entre les différents hôtes. Nous tenterons de localiser les potentielles formes d'ADN de ces virus à ARN (non-rétrovirus) et d'identifier les interactions qu'ils entretiennent avec les « petits » ARN de l'anophèle impliqués dans le contrôle des EVEs et des formes d'ADN.

Mots clés : anophèle, virus, interactions, métagénomique, mutation, voies immunitaires

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1.1 MALARIA VECTORS

Malaria is a disease mainly found in tropical countries and is caused by protozoan parasites belonging to the genus *Plasmodium* that infect humans through the bite of infected female mosquito of the genus *Anopheles*. In 2016, malaria touched more than 200 million people and killed more than 400 000 persons globally (WHO, 2017). Sub-Saharan Africa still remains the most affected region with 90% of total morbidity and mortality recorded in 2016 (WHO, 2017). The South-East Asia region is the second most affected region of the world with 7% of malaria cases and 6% of malaria deaths in 2016. *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi* are the five species responsible for human malaria (WHO, 2017). *Anopheles* malaria vectors belong to the sub-family *Anophelinae* in the family *Culicidae*. The latter also includes the sub-family *Culicinae* which includes the *Culex* and *Aedes* genera. *Anopheles*, *Culex* and *Aedes* all belong to the *Diptera* order, class *Insecta*, sub-phylum *Mandibulata* and phylum *Arthropoda*. Based on phylogenetic studies, the divergence between *Anophelinae* and *Culicinae* occurred around 226 million years ago (Reidenbach et al., 2009).

Of the 465 *Anopheles* reported in the world, only 70 *Anopheles* species are involved in the transmission of human *Plasmodium* and 41 are considered to be dominant vectors (Sinka et al., 2012). The main *Anopheles* species complexes (Fig. 1) in Africa are *Anopheles nili*, *An. moucheti*, *An. funestus* and *An. gambiae* (Antonio-Nkondjio et al., 2006; Sinka et al., 2012). In Southeast Asia, the major malaria vectors are *Anopheles dirus sensu lato*, *An. minimus s.l.*, *An. epiroticus*, *An. stephensi* (Obsomer et al., 2013; Sinka et al., 2012; Trung et al., 2004). Most *Anopheles* species are not human malaria vectors, because their biting behavioral preference for animals means they are not exposed to human *Plasmodium* infection (Vizioli et al., 2001). In addition to physiological susceptibility or permissiveness for malaria parasites (vector competence), malaria vectors must be able to transmit the parasite (vector capacity). Knowledge of the life cycle of *Anopheles* is crucial to better understand both competence and vectorial capacity to pathogens.

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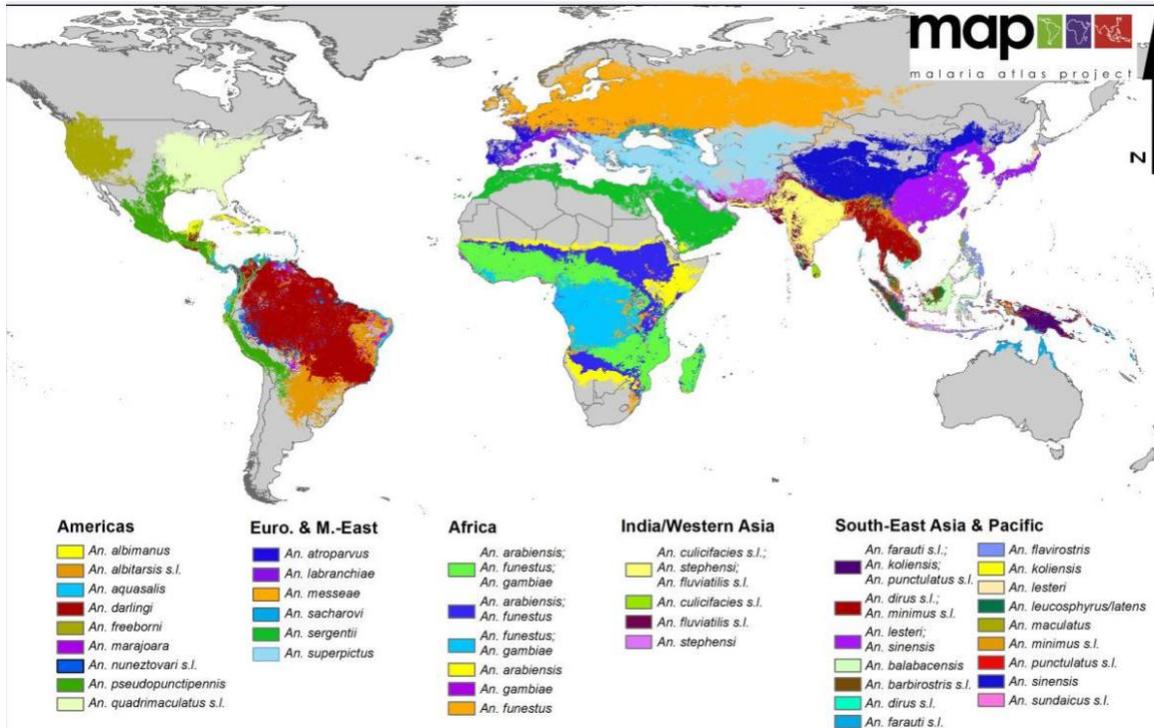


Figure 1 : The global distribution map of the main malaria vector species (Sinka et al., 2012).

1.1.1 Life cycle of malaria vectors and biting/resting behaviors

The *Anopheles* life cycle is characterized by aquatic and terrestrial phases and its duration depends mainly on temperature, microorganisms, water salinity and turbidity, light exposure time, mineral salts (Beck-Johnson et al., 2013; White et al., 2013). Like all mosquito species, *Anopheles* mosquitoes start their life in an aquatic environment. Two days after a blood meal, *Anopheles* females lay their eggs on the surface of water, which may be permanent water bodies as in the case of *An. coluzzii* or temporary such as rain puddles, as is often observed for *An. gambiae*. The eggs hatch within 2-3 days to produce larvae. There are 4 larval stages (L1 to L4) where the first instar undergoes three molts between 7 and 10 days (d) to become the fourth instar. The larvae develop into pupae, from which an adult mosquito (male or female) called imago emerges and starts its aerial life. Males and females copulate and only females bite and take a blood meal, which is required for egg maturation. The trophic behavior of mosquito females and their attraction to humans (anthropophily) or animals (zoophily) varies amongst *Anopheles* species. All malaria

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vectors must be at least partially anthropophilic, or else they cannot maintain transmission of a human pathogen, although most are opportunistic and also feed on animals, depending on host availability (Bashar et al., 2012; Waite et al., 2017). Species that feed preferentially on animals (zoophilic) are not malaria vectors. Some species such as *An. albimanus* Wiedemann, a major malaria vector in Central and South America, bites mainly outside (exophagous) (Molez et al., 1998). Others such as *Anopheles darlingi*, an important vector of human malaria in South America tends to bite indoors (endophagous) (Gonzalez et al., 2007). After a blood meal, mosquitoes rest outdoors during blood digestion and egg production. Resting may be indoors (endophilic) or outdoors (exophilic). *Anopheles* species can modify their biting and resting behaviors in evolutionary response to the presence of insecticide impregnated bed nets, indoor residual spraying of insecticides, or the absence of preferential host in one location (Paupy et al., 2013; Sougoufara et al., 2014). The life cycle of *Anopheles* is illustrated below.

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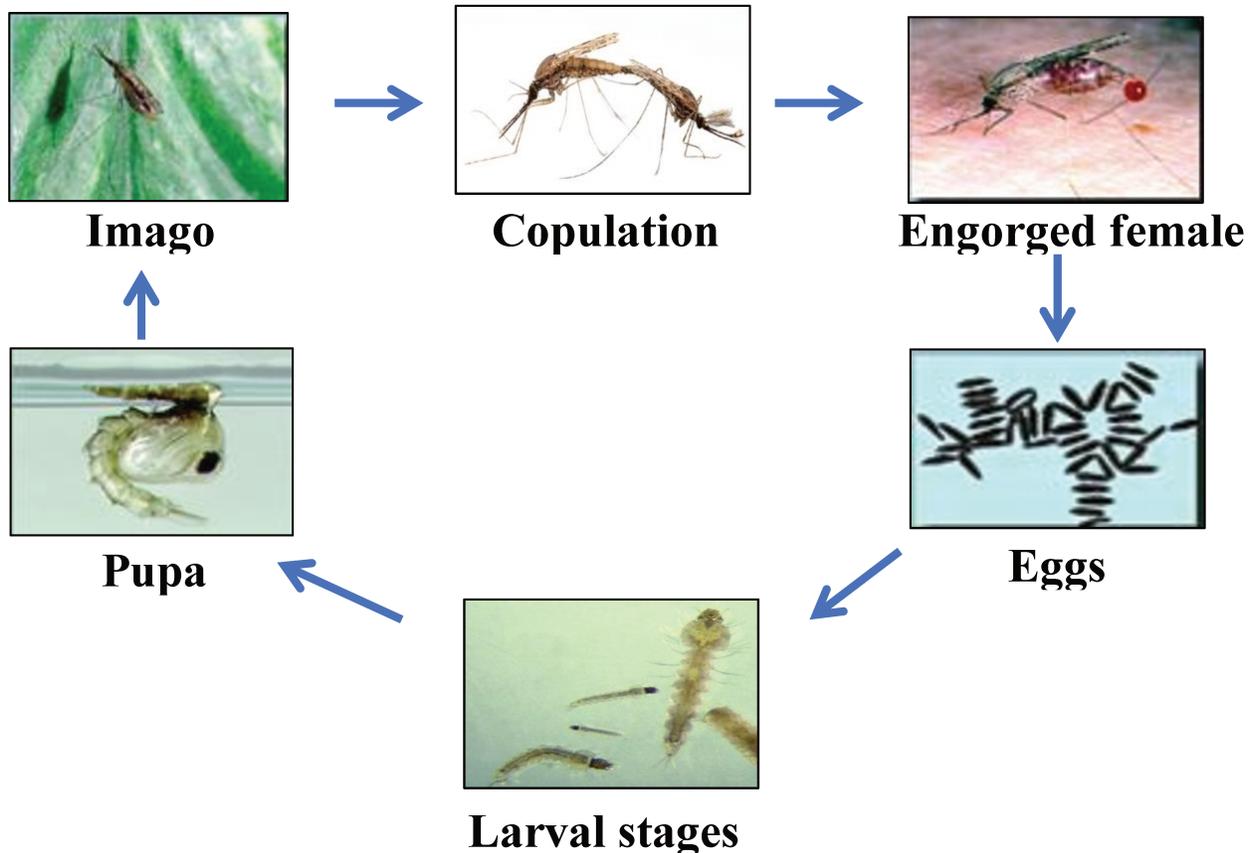


Figure 2: The *Anopheles* life cycle. Following copulation, females ingest a blood meal, required for egg development. Eggs are laid in water where they hatch and where larval and pupal development is completed. Adults emerge from pupae to complete the cycle.

1.1.2 Competence of malaria vectors

Competence of *Anopheles* for *Plasmodium* can be defined as the physiological ability of *Anopheles* species to acquire, replicate and transmit malaria parasites. Competence, susceptibility or permissiveness for malaria infection means that *Anopheles* is able to support *Plasmodium* development from gametocytes to sporozoites. To initiate the sporogonic cycle, *Plasmodium* gametocytes are ingested from an infected person during the blood meal (Fig. 3). Within the

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Anopheles midgut, gametogenesis and fertilization produce zygotes and then ookinetes. This latter motile stage crosses the midgut epithelium and reaches the basal side of the midgut, where it forms a protected capsule called the oocyst. The oocyst stage undergoes numerous cell divisions that result in the formation of thousands of sporozoites. The oocyst ruptures when it becomes mature, the sporozoites are released into the mosquito hemocoel, and subsequently some of them invade the salivary glands. Once in this organ, the sporozoites can be transmitted to a human host during a subsequent blood meal. The mosquito host is the definitive host of *Plasmodium sp.*, because the sexual stage (mating) occurs in the mosquito. This probably means that, evolutionarily, the insect is the more ancient host of a *Plasmodium* ancestor, and the vertebrate host is more recent.

Vector competence is influenced by many parameters such as environmental factors (temperature, mosquito midgut microbiome), genetic factors (parasites and hosts), and physiological factors (hosts and parasites) (Cohuet et al., 2010). In terms of host-parasite interactions, some parasite species are unable to infect the midgut or the salivary glands of some *Anopheles* and are therefore not transmitted. The salivary glands of *An. stephensi* are resistant to *P. vivax* infection when they are treated with mannose, N-acetyl-galactosamine and lactose, indicating that carbohydrate moieties are involved in *An. stephensi* competence to *P. vivax* (Basseri et al., 2008). In *An. gambiae* the ookinetes of *P. cynomolgi* are encapsulated between the midgut epithelial and basal lamina, killing the ookinetes and blocking transmission of this simian parasite (Collins et al., 1986).

An immune protein called thioester containing protein-1 (TEP1) is a glycoprotein secreted by *Anopheles* hemocytes into the hemolymph. TEP1 mediates killing of *P. berghei* ookinetes by binding to their surface (Blandin et al., 2004). RNAi mediated silencing of TEP1 increases the number of oocysts in a susceptible strain of *An. gambiae* and abolishes *Plasmodium* ookinete melanization in a refractory strain, which becomes susceptible in the TEP1-silenced background (Blandin et al., 2004). *Anopheles Plasmodium*-responsive leucine-rich repeat 1 (APL1) is a family of 3 paralogs called APL1A, APL1B and APL1C in *An. gambiae*. These 3 paralogs are located on the chromosome arm 2L of *An. gambiae* in a genomic region named *Plasmodium*-resistance island (PRI) that carries a major locus for resistance to natural *P. falciparum* infection, as detected by genetic linkage mapping (Riehle et al., 2006). Functional analysis by RNAi-mediated gene silencing showed that only one paralog, APL1A protects against infection with *P. falciparum*,

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while only paralog APL1C confers protection against the rodent malaria parasites, *P. berghei* and *P. yoelii* (Mitri et al., 2009; Riehle et al., 2008).

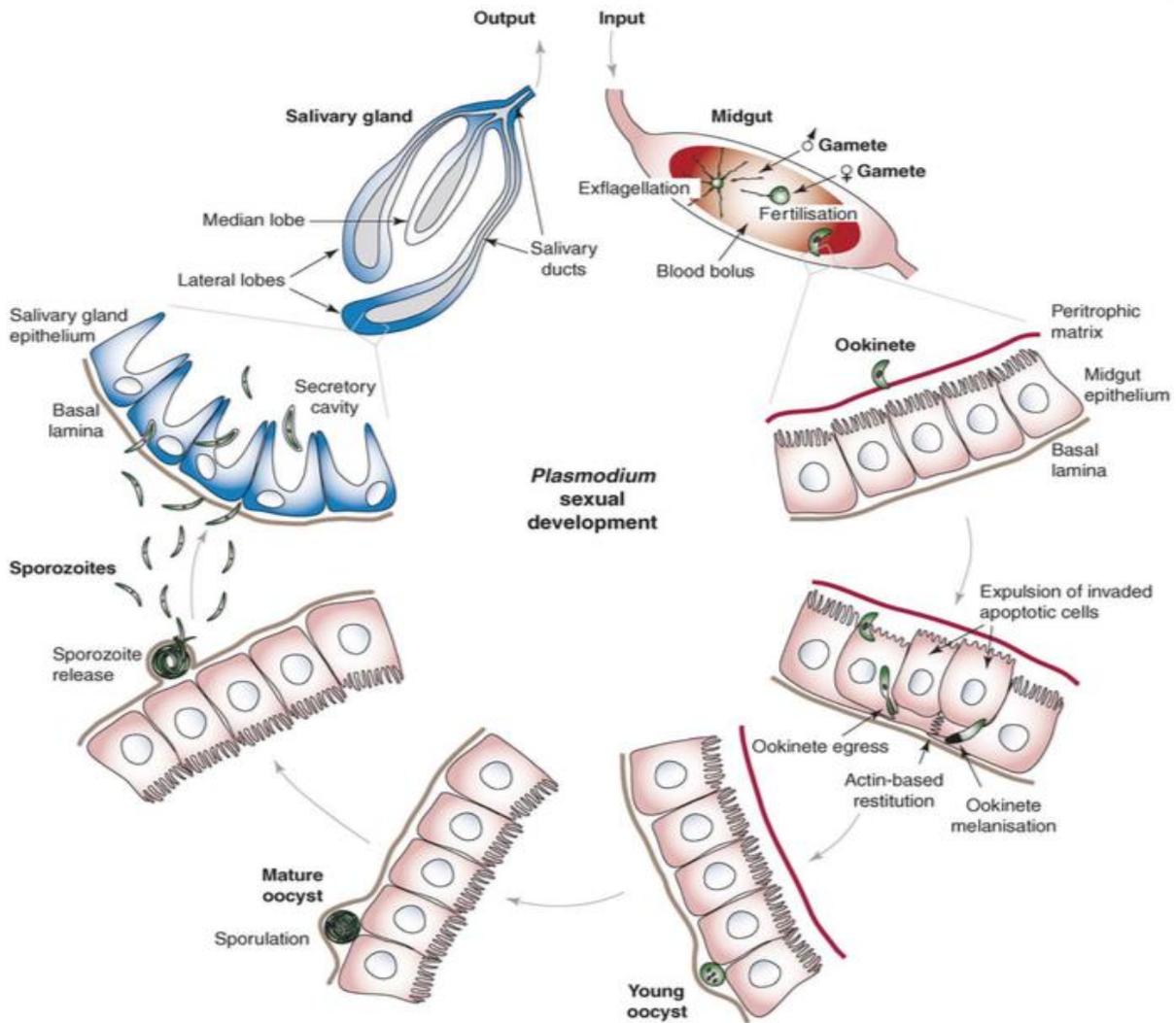


Figure 3 : Sporogonic cycle of *Plasmodium* in the mosquito host. *Plasmodium* undergoes sexual development in the mosquito host, called the sporogonic cycle, or sporogony. Gametocytes are ingested in infected blood. A zygote is formed after fertilization and develops into an ookinete, which crosses the midgut epithelium and continues to develop as an oocyst. Sporozoites are released from the oocysts, circulate in the hemolymph and reach the salivary glands to be transmitted when a vertebrate host is next bitten. Input refers to infected vertebrates from which the blood meal is ingested; while output refers to uninfected vertebrates which will be bitten by an infected mosquito (Vlachou et al., 2006).

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1.1.3 Vectorial capacity

By definition, vectorial capacity is the mean of future inoculations that arise from a currently infective case, provided that all vectors biting that case become infected. This notion highlights the fact that a vector may be competent to *Plasmodium* species but it could be a poor vector when taking into account real-world parameters such as environment and behavior. Vectorial Capacity (C) can be estimated by the MacDonal formula below (Cohuet et al., 2010).

$$C = ma^2 p^n / -lnp$$

Where m is vector density, a is the human biting rate per day or hour, p is vector longevity (the survival probability), n is the duration (days) of infection or the duration of sporogonic cycle.

By this formula, an effective vector must live long enough to complete at least one sporogonic cycle. High vector population density and high biting rate are other qualities of an effective vector. For instance, the *An. gambiae* complex is the most abundant anopheline species in many African villages and it has a parity rate higher than 70%. This vector is involved in the transmission of at least *P. falciparum*, *P. malariae* and *P. ovale* (Bigoga et al., 2012). Besides this important vector in Africa, there are secondary or local vectors such as *An. paludis*, *An. mascarensis*, *An. pharoensis*, *An. wellcomei*, *An. coustani*, *An. ziemanni*, *An. ovengensis*, *An. hancocki*, *An. marshallii*, *An. carnevalei*, *An. flavicosta* and *An. melas* (Antonio-Nkondjio et al., 2006; Bigoga et al., 2012). They are secondary vectors because of their low density, their seasonal presence and their weak preference for human biting: however, in some areas, they could be locally important vectors. For this reason, vector control must target all malaria vectors.

1.1.4 *Anopheles coluzzii* and *Anopheles stephensi*

Anopheles coluzzii is the former M molecular form of *An. gambiae sensu stricto* in the *An. gambiae* complex or *An. gambiae sensu lato*. This complex is one of the most important African malaria vectors, because of its density, its competence and vectorial capacity and its anthropophily. It is responsible for at least 80% of *P. falciparum* transmission in much of Sub-Saharan Africa (Abraham et al., 2017; Bigoga et al., 2012). *P. falciparum* is the most virulent parasite of human malaria and is responsible of the most morbidity and mortality rates reported in Africa. The *An. gambiae* complex has 8 members: *An. melas* Theobald, *An. merus* Dönitz, *An. bwambae* White,

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An. quadriannulatus Theobald, *An. amharicus* Hunt, Wilkerson & Coetzee sp.n., *An. coluzzii* Coetzee & Wilkerson sp.n, *An. arabiensis* Patton, *An. gambiae* Giles (Coetzee et al., 2013). The complex members are not morphologically distinguishable; they are distributed in different ecological zones but some are sympatric in many areas. A diagnostic PCR assay based on a short interspersed element, SINE200, that is repetitive and widespread in the *An. gambiae* genome permits the molecular identification of *An. quadriannulatus*, *An. melas*, *An. arabiensis*, *An. gambiae* and *An. coluzzii* (Santolamazza et al., 2008). *An. coluzzii* is both an endo and exophagic vector and is involved in 90% of malaria transmission in the Southern region of Cameroon (Bigoga et al., 2012).

Anopheles stephensi is a major human malaria vector in India and it is present in many other Asian and middle-Eastern countries, such as Pakistan, Afghanistan, Iran, Iraq, Bahrain, Oman and Saudi Arabia, Bangladesh, China and Myanmar and Sri Lanka, thus widely distributed in Asia (Surendran et al., 2018; Thomas et al., 2017). In addition to molecular tools based on cytochrome oxidase and the nuclear internally transcribed spacer 2 of the ribosomal RNA gene (ITS2), larval and adult morphologies are also used to distinguish *An. stephensi* from other *Anopheles species* (Surendran et al., 2018). Morphological traits used for characterization include egg ridges, spiracular index, thoracic length and cuticular hydrocarbon profiles. *An. stephensi* exists as three biotypes: *An. stephensi stephensi* (type form), variety *mysorensis* and ‘intermediate’ form (Surendran et al., 2018). These three biotypes have different ecological, behavioral and mating characteristics. The type-form displays high vectorial capacity for malaria parasites, while the *mysorensis*-form is a poor or non-vector. The vectorial capacity of the intermediate form is at present unknown (Surendran et al., 2018). *An. stephensi* is an anthropo-zoophagic vector that can bite cattle and humans depending on availability of hosts and it is involved in the transmission of *Plasmodium vivax* and *P. falciparum* (Thomas et al., 2017).

1.1.5 Malaria vector control

Control of malaria vectors can be done at both larval and adult stages. Larval stage management is complicated because some *Anopheles* complexes have specific breeding habitats. For instance, *An. gambiae s.l.*, larvae breed in temporary water, such as rain puddles or those that form in animal foot prints or tire tracks, whereas *An. funestus s.l.*, prefer permanent breeding sites with vegetation

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(Mattah et al., 2017). Larval control could be achieved by eliminating breeding sites or vessels in which breeding sites could form and by using petroleum oil, larvicides, larval predators, or the introduction of fungal pathogens that kill larvae (Walker and Lynch, 2007). In current practice, most vector control is based on the control of adults by insecticides use through indoor residual spraying or bednets. The main classes of insecticide involved in vector control are pyrethroids, carbamates, organophosphates, and organochlorides.

1.1.5.1 Insecticides for malaria vector control

Insecticides used for wall spraying are delivered by vaporizing water dispersible powders of the residual insecticide to the interior surfaces of walls, ceilings and roofs of human dwelling. Pyrethroids are largely used for indoor residual spraying (IRS) because of low human toxicity, while carbamates and organophosphates are being used more frequently in areas where pyrethroid resistance has developed (Tangena et al., 2013). However, the effectiveness of spraying houses in vector control campaigns depends on the vector resting behaviors. For example, *An. arabiensis* feeds and rests both indoors and outdoors which means that IRS will not be suitable for control of the outdoor feeding/resting portion of the populations (Gordicho et al., 2014). In addition, house spraying may promote the selection of exophilic populations.

Insecticide treated nets (ITNs) provide both a physical barrier from biting mosquitoes and also kill or repel endophagous mosquitoes. ITNs are prepared by dipping nets in pyrethroids contained in a plastic bowl or dustbin. Pyrethroids are the only insecticide approved for ITNs because of their low toxicity to humans. Washing ITNs decreases the insecticide efficiency, although nets treated with some pyrethroids such as alpha-cypermethrin can remain effective after five washes. The use of long-lasting insecticidal nets (LLINs) reduces the need to renew the impregnation (Kleinschmidt et al., 2018).

The efficiency of vector control by insecticides is hampered by widespread insecticide resistance of malaria vectors to the main insecticide classes (Gnanguenon et al., 2015; Ibrahim et al., 2018; Mitchell et al., 2012). Therefore, the discovery of new insecticides is needed to improve vector control with insecticides (Hemingway et al., 2016). In addition, alternative tools are needed to support insecticides. Transgenesis and paratransgenesis are potential tools against malaria vectors.

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1.1.5.2 Transgenesis and paratransgenesis

Transgenesis in malaria control would involve introducing an exogenous gene into *Anopheles* in order to prevent or reduce the sporogonic development of *Plasmodium* (Coutinho-Abreu et al., 2010). The donor plasmid that carries the exogenous gene is generally composed of a transposable element (such as piggy Bac, Hermes, Mariner), a reporter gene such as green fluorescent protein (GFP) or red fluorescent protein (RFP), in order to identify successful transgenesis and a promoter that can be tissue specific such as carboxypeptidase (midgut specific) apyrase (salivary gland specific), vitellogenin (fat body specific), 3xP3 (eye and nerve cell specific) (Chen et al., 2007; Volohonsky et al., 2017). For instance, a *piggyBac* vector with GFP and the synthetic salivary gland and midgut peptide, SM1, under control of carboxypeptidase promoter (to drive midgut expression) injected in embryos of *An. stephensi* produced a transgenic line with green eyes that displayed a reduction of 81.6% of *P. berghei* oocysts as compared to the wild type line (Ito et al., 2002). High mortality is common during embryo microinjection. Transgenic larvae can be selected by using the complex object parametric analyzer and sorter (COPAS) system (Volohonsky et al., 2015). The antibiotic puromycin can also be used to select transgenic larvae, killing individuals lacking the introduced resistance gene. However, the selection fails to distinguish heterozygous from homozygous larvae (Volohonsky et al., 2015). For the transgenic strategy to succeed, mosquitoes must have the same fitness as wild type mosquitoes.

Paratransgenesis in mosquitoes is developed with the aim to reduce vector competence by using engineered symbiotic microorganisms. Paratransgenic mosquitoes should have almost no fitness cost, and symbionts should be specific to the mosquito species or strain to avoid off-target effects on other organisms mosquitoes. Bacteria, fungi and viruses are possible symbiotic microorganism candidates to develop paratransgenesis in *Anopheles* mosquitoes against malaria (Fang et al., 2011; Favia et al., 2007). *Anopheles gambiae* densovirus (AgDNV) is a virus that infects *An. gambiae* larvae and adults with no detectable effect on their life span (Ren et al., 2008). In one study, AgDNV prevalence in laboratory mosquitoes was 62%, maintained by vertical transmission. Recombinant AgDNV is efficiently transmitted and persistent at least to three generations, suggesting that it could drive expression of exogenous genes. Therefore, AgDNV could be a candidate for viral paratransgenesis in *Anopheles* control. In addition to this virus, a gram-negative, aerobic and rod-shaped bacteria named *Asaia* belonging to the family *Acetobacteraceae*, has

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characteristics suitable for paratransgenesis in malaria control. It is cultured at low pH (~3) and 30°C, which varies according to species. *Asaia* is present in plants and *Anopheles* species such as *An. maculipennis*, *An. gambiae*, *An. arabiensis*, *An. funestus*, and *An. stephensi* (Favia et al., 2007; Yamada et al., 2000). In *An. stephensi*, *Asaia bogorensis* and *Asaia siamensis* are the most abundant bacteria where they infect males and females and they are present in pre-adult and adult stages (Favia et al., 2007). *Asaia* sp. is well disseminated in *An. stephensi* three weeks after infection with high density in the salivary glands and the midgut. It is maintained in mosquitoes by vertical transmission and venereal transmission (Favia et al., 2007). It is possible to transfer *Asaia* from infected to non-infected colonies. *Asaia* sp. load increases after a blood meal in *An. stephensi*, and *P. berghei* infection does not affect its motility or reproduction (Capone et al., 2013). In addition to *Asaia* spp, the genetic modification of another bacteria, *Pantoea agglomerans* to carry *E. coli* hemolysin has been shown to reduce the development of *P. falciparum* and *P. berghei* by up to 98% in *An. gambiae* (Wang et al., 2012). The recombinant *P. agglomerans* expresses anti-*Plasmodium* molecules, such as SM1 peptide, scorpine, *Plasmodium* enolase-plasminogen interaction peptide, Shiva1 and others (Wang et al., 2012).

1.2 Virus infection and evolution

Viral infections partially depend on the structure of viruses and hosts. This section will focus on virus discovery in mosquitoes and the quantification, structure, replication and genomic variation of mosquito viruses.

1.2.1 Mosquito virus discovery

Many mosquito viruses are probably insect-specific viruses (ISVs), and not pathogenic for humans, while arboviruses infect both arthropod vector and vertebrate hosts. People infected with arboviruses can often be asymptomatic. Symptomatic cases share the same symptoms with diseases such as malaria, flu, and others, leading to misdiagnosis. Yellow Fever virus (YFV) was one of the first viruses to be classified as an arbovirus. YFV is a *Flavivirus* that causes a haemorrhagic disease and is transmitted by infected *Aedes* mosquitoes. Yellow Fever killed 435 people in Brazil within 6 months and 3,240 suspected cases were reported between 2016 and 2017 (Fischer et al., 2017). Historically, YFV was thought to be microbial in origin when, after filtration to remove bacteria, infected blood with YFV was still infective to monkeys. Adrian Stokes who

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worked on YFV died in 1927 after being bitten by an infected monkey. The YFV Asibi strain was isolated in 1927 by Rockefeller Foundation in Lagos, Nigeria after passage into monkeys. The Asibi strain was used to make attenuated 17D vaccine against YFV (Galler et al., 1997). Today, arboviruses or arthropod-borne viruses are typically isolated by passage into vertebrate cell lines. For ISVs that replicate only in insects, insect cells are used for culturing. Molecular and bioinformatic tools such as PCR and sequencing are used to characterize their genomic organization. Ultracentrifugation on sucrose gradients is also very useful in virus purification (Susevich et al., 2017).

Outbreaks cause the discovery of arboviruses, and new or unknown viruses are discovered by metagenomic approaches (Varghese and van Rij, 2018). The discovery of viruses may be followed by their isolation, structural characterization by microscopy and production of antibodies as a tool for a deeper study. Infectious clones are good alternatives in absence of virus isolation and they are generated with reverse genetic tools. For instance, the infectious clone of YFV Asibi strain exists permitting a great number of studies on this virus (McElroy et al., 2005).

1.2.2 Mosquito virus quantification and quantitative real time PCR (Qrt-PCR)

Many tools are available to quantify mosquito viruses and the choice depends on the study goals. To quantify the infectious virus particles, cell cultures followed by serological assays such as Fluorescent Focus Assay (FFA) and Enzyme-Linked Immunosorbent Assay (ELISA) are common but these techniques are limited by plaque forming ability and availability of antibodies respectively (Martinez-de la Puente et al., 2018; Payne et al., 2006). Most ISVs do not form plaques during their culture and specific antibodies are lacking. Therefore another molecular tool, such as quantitative real time PCR (Qrt-PCR) or qPCR is useful as an alternative for quantifying these ISVs (Mackay et al., 2002). The technique permits to follow the amplification of nucleic acid in real-time by using specific primers, as in PCR, but with the read-out obtained by integration of a fluorescent dye like SYBR Green into the amplified double stranded DNA. It is used to quickly detect and measure viruses with a good sensitivity and specificity (Bustin et al., 2009; Mackay et al., 2002). A more specific approach to qPCR is Taqman whereby probes are attached to primers and can be multiplexed (Tajadini et al., 2014). A qPCR assay can be absolute with a standard curve or comparative with a reference gene or housekeeping gene as ribosomal protein S7 (*rpS7*) in *Anopheles* species (Lee et al., 2012). During qPCR amplification, the amount of fluorescence

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emitted (SYBR Green or Taqman) is proportional to the amount of amplified nucleic acid. This latter increases during each qPCR cycle and the cycle in which fluorescence can first be detected above a minimum threshold is called quantitation cycle (C_q) or cycle threshold (C_t). This latter is the number of cycles required for the fluorescent signal to cross the threshold. Samples with lower C_q or C_t have more targets present in a sample and their fluorescence are detected earlier. The main phases of qPCR amplification curve are (Fig.4): the initial phase, exponential phase and plateau where there is almost no increase in product yield or amplicon. During the initiation phase, fluorescence emission is not yet detected whereas in the exponential phase, the fluorescence of amplicons is detected (Mackay et al., 2002).

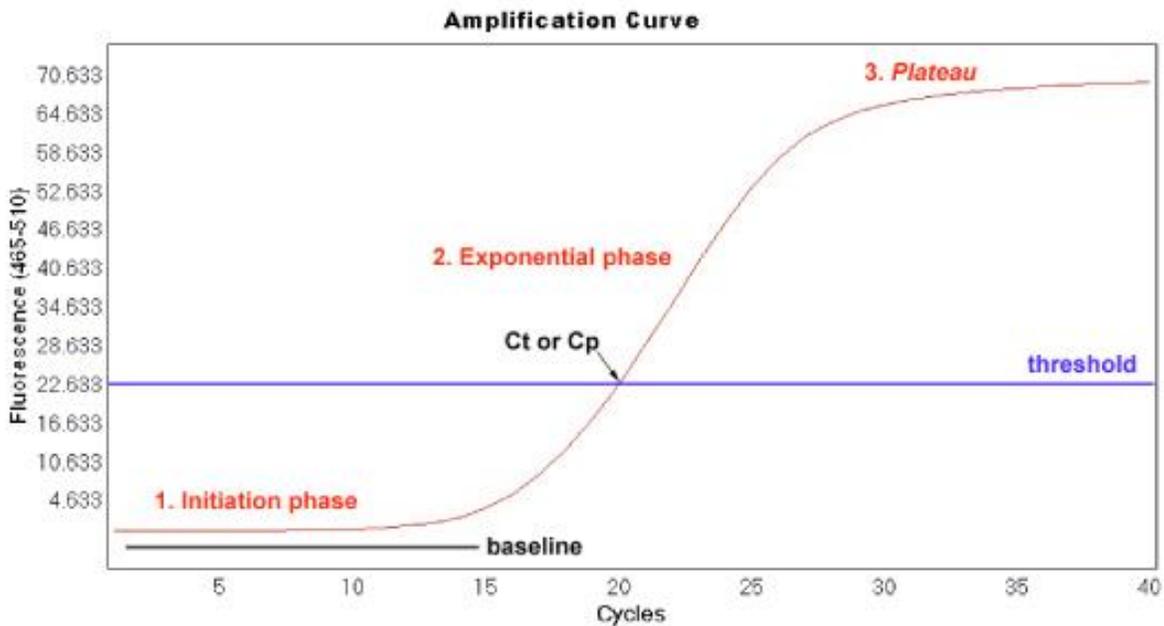


Figure 4: The main phases of qPCR amplification curve. The Y-axis represents fluorescence and the X-axis represents the number of cycles. The baseline represents the PCR cycles in which a reporter fluorescent signal is accumulating but is below the limits of detection. The C_t represents the point where the increase in fluorescence becomes higher than the background, and the threshold is set by the software which is using for analyses (Mackay et al., 2002) <https://www.highveld.com/pcr/real-time-pcr-quantification-analysis.html>.

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1.2.3 Structure and replication of viruses

Viruses are small obligate intracellular microbes. Their specific structure and mode of replication depend on the virus family to which they belong. In general, viruses comprise a nucleic acid (genome), a capsid and in some an envelope. The genome can be DNA or RNA, which may be single stranded (ss) or double stranded (ds), linear or circular and one segment or more. The capsid protects the viral genome from the external environment and plays a role in non-enveloped virus attachment to specific receptors exposed on the host cell. A virion is an infectious virus particle that contains the genome and an outer shell of protein. Capsids are composed of subunits made of structural proteins. Spherical capsids have an icosahedral structure and the helical capsids have an elongated capsid structure (Prasad and Schmid, 2012). The envelope is derived from modified host cell membranes as virus buds from the cell. Viral envelopes consist of a lipid bilayer and membrane-associated proteins.

Bluetongue virus (BTV) belonging to the genus *Orbivirus* within the family *Reoviridae* is used here as an example. Bluetongue virus is a non-enveloped virus and its genome is double stranded RNA comprising 10 linear segments (Fig. 5) which encode viral proteins (VP) and non-structural proteins (NS) (Auguste et al., 2015; Van Dijk and Huisman, 1988). Bluetongue virions have icosahedral capsids which attach to sialic acid of clathrin to enter into vertebrate cells by endocytosis (Fig. 6). Once inside cells, the endosome liberates uncoated virion into the cytoplasm. The liberated core is comprised of VP1, VP4 and VP6 and induces transcription and translation to produce non-structural proteins and to egress via NS3 from viral inclusion bodies (Albina, 2007; Mohl and Roy, 2014). Viral inclusion bodies are considered to be sites of viral assembly and they are punctuated perinuclear globules mainly composed of NS2. Virion maturation is completed by the association between the core and the viral proteins VP2 and VP5, and virions are released by cell lysis (Mohl and Roy, 2014).

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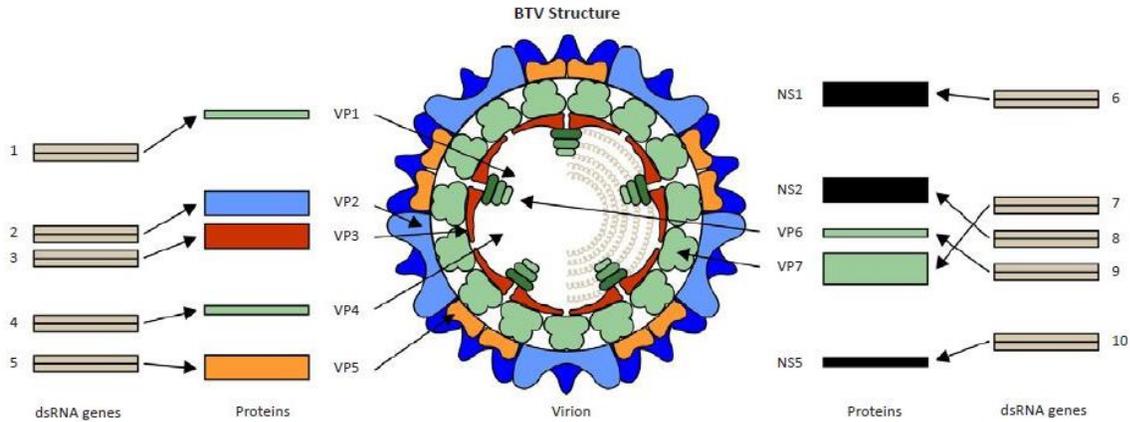


Figure 5: Structure of the bluetongue virus genome. It composed of 10 segments encoding viral and structural proteins. VP1 to VP7 are viral proteins and NS1 to NS5 are non-structural proteins. VP2 and VP5 form the outer capsid, while VP1, VP3, VP4, VP6 and VP7 the core (Verwoerd, 2012).

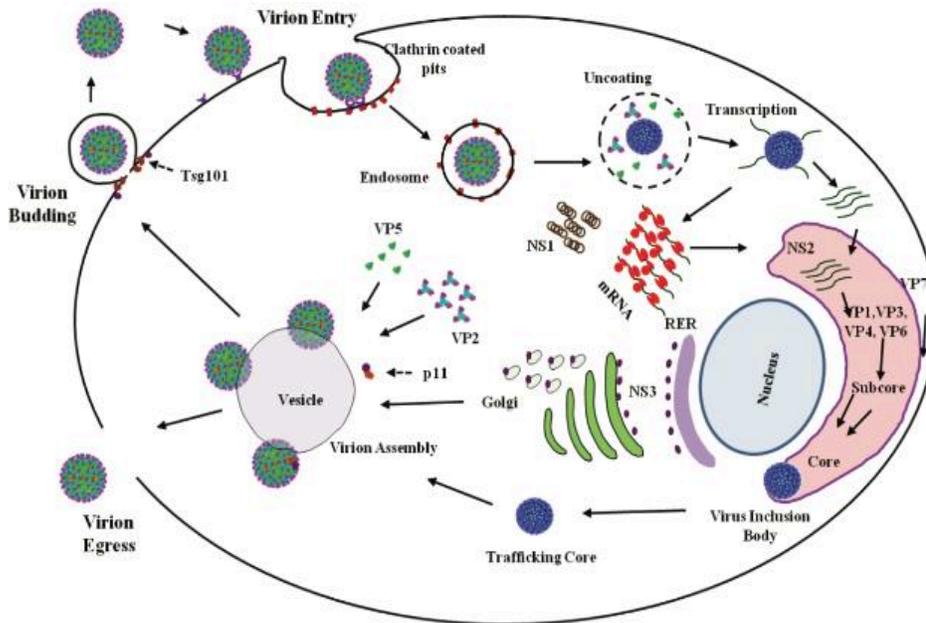


Figure 6: Replication of bluetongue virus genome. VP2 and VP5 play a role in virus trafficking and they are main constituents of outer capsid whereas inner capsid or core is mainly made of VP7. Bluetongue enters into cells by endocytosis, and its uncoating is necessary for replication before virus is released by cell lysis for further dissemination (Mohl and Roy, 2014).

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1.2.4 Genomic variation and evolution

Genomic variation, that is, changes in the genome sequence, is influenced by genetic and environmental factors. Variation is high in RNA viruses. Indeed, viral RNA polymerases are error-prone during replication, which lead to a mutation rate that is higher than the mutation rate in DNA viruses. Unlike DNA polymerases that can correct errors made during replication, RNA polymerases have no proofreading ability. Based on RNA-dependent RNA polymerase (RdRp), the estimated mutation rate for RNA viruses is around one mutation per genome, per replication (Holmes, 2009). The mutational distribution in RNA viruses is not very clear for many viral populations but there is an accumulation of some mutations from the parental genome (which acts as template) to their offspring genomes. The different types of mutation are substitution (exchange between two bases), insertion (extra sequence) and deletion (loss of sequence). Synonymous mutations have no impact on the protein sequence, while non-synonymous mutations result in changes in the protein sequence. For instance, the Zika virus strain responsible for outbreak in America in 2015 has an alanine-to-valine amino acid substitution (non-synonymous) in the nsP1 protein as compared to the FSS13025 strain purified in Cambodia in 2010, leading to high infectivity in *Ae. aegypti* (Liu et al., 2017). In arboviruses, non-synonymous mutations in genes encoding viral proteins can cause antigenic variation, allowing viruses to escape vertebrate immune responses (Palmer et al., 2018). In Madagascar, the flavivirus West Nile virus (WNV) displayed several antigenic variants in birds and in humans, and high heterogeneity between *Culex*, *Aedes* and *Anopheles* mosquitoes (Morvan et al., 1990).

Mutations produce many variants and the most abundant has the highest ability to survive and replicate. Some variants become dominant with time while others disappear leading to a 'new' population showing the important roles of selection and mutation in virus evolution and adaptation. The mutated forms can be more resistant against current or changing environmental conditions, explaining why some virus genotypes are present in specific area (Lauring and Andino, 2010; Tabachnick, 2016). For instance, there are five genotypes of YFV that are present in Africa and two in South America. The nucleotide difference between the African and American genotypes is around 15% and African YFV is believed to be the ancestor of American strains. Yellow fever virus is proposed to have originated from Africa and spread to the Americas during slavery 400 to 500 years ago (Tabachnick, 2016).

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Genomic variation can modulate the fitness of a virus in a vector, affecting the vector competence. For example, the substitution of alanine by valine in the glycoprotein E1 (E1-A226V) of Chikungunya virus increased its infectivity to *Ae. albopictus* during 2005-2006 outbreak in Mayotte, Seychelles, Comoros Island, Reunion (Schuffenecker et al., 2006). In the beginning (March-May 2005) of the outbreak the glycoprotein E1 had alanine at position 226, while by the end of the outbreak (December 2005), valine had replaced alanine (E1-A226V) (Schuffenecker et al., 2006). In the absence of *Ae. aegypti*, this mutation allows *Ae. albopictus* to serve as the main vector of CHIKV, highlighting the adaptability of RNA viruses to different hosts. The mutation E1-A226V affects dissemination to the salivary glands and has no effect on viral load (Tsetsarkin et al., 2007). This last example shows that selection, evolution and adaptation of arboviruses play a major role in outbreaks and virus evolution depends on mosquito species. There is more diversification of virus in mosquitoes with variation according to tissues because purifying selection is weaker than in vertebrates (Grubaugh et al., 2016). Further studies will be needed to assess the impact of genomic variation of ISVs into different hosts and verify the assumption that “ISVs are evolutionary precursors to arboviruses” (Junglen et al., 2017).

1.2.5 Maintenance of viruses in mosquitoes

Vertical or hereditary transmission, that is, the passage of virus from parents to progeny, plays a crucial role in the maintenance of virus in mosquitoes (Lequime et al., 2016). The presence of viruses in the ovaries and larval stages of mosquitoes are indicators of their vertical transmission. Rift valley fever virus (RVFV) is present in the pupae of *Ae. lineatopennis* suggesting that vertical transmission is involved in its maintenance but the transmission route is unknown (Linthicum et al., 1985). Virus routes in mosquitoes can be inside eggs (trans-ovarial) or in seminal fluids or on the egg surface (trans-ovum) (Linthicum et al., 1985; Peter 1973 ; Sikorowski et al., 1973).

Insect specific viruses are maintained in *Anopheles* species by vertical transmission (Carissimo et al., 2016) and some arboviruses such as dengue virus (*Aedes aegypti*), Zika virus (*Ae. aegypti* and *Ae. albopictus*), and West Nile virus (*Culex tritaeniorhynchus*, *Ae. aegypti* and *Ae. albopictus*) are also vertically transmitted (Baqar et al., 1993; Ciota et al., 2017; Gutierrez-Bugallo et al., 2017). Moreover, DNA forms of CHIKV arbovirus are involved in its tolerance and its maintenance in

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Aedes mosquitoes (Goic et al., 2016). Integration of DNA forms into the mosquito genome can also be a mechanism for vertical transmission to offspring.

Horizontal transmission (HT) refers to all other modes of non-parental transmission, comprising venereal and transmission to and from vertebrates (Lequime et al., 2016). Venereal transmission of viruses between *Anopheles* mosquitoes means that viruses are transmitted by infected mosquitoes to uninfected ones during mating. This is the case for *An. gambiae* densovirus, which is transmitted from infected males to non-infected females during mating (Barik et al., 2016). The acquisition of viruses by *Anopheles* from infected people and their transmission to non-infected *Anopheles* is another type of HT. Cannibalism is a type of HT observed among *Anopheles* larvae where non-infected larvae eat the infected ones and become infected (Carissimo et al., 2016).

1.3 Antiviral responses in mosquitoes

Antiviral responses are poorly studied in *Anopheles* species while they are quite well characterized in *Drosophila*, because of its robustness as a laboratory model system. *Drosophila* viruses such as *Drosophila C* virus and Cricket paralysis virus have been used for studies in this system. A few studies have been carried out in *Aedes* and *Culex* mosquitoes. In insects, there are no specific antibodies for antigens as in vertebrates. Therefore, the immune response in insects is based on the cellular and humoral responses of innate immunity. Nevertheless, prior to encountering these responses, viruses must cope with the tissue barriers of mosquitoes, which represent the first line of defense.

1.3.1 Mosquito barriers to viral infections

To be transmitted to vertebrates by a given mosquito, a virus must overcome tissue barriers. Tissue barriers are also involved in the vertical transmission of viruses in mosquitoes and play essential role in mosquito competence for a virus. The extrinsic incubation period (EIP) defines the time between ingestion of virus, replication in mosquitoes and transmission of the virus to vertebrates (Asgari, 2014). Virus infection starts in the mosquito midgut and spreads within the midgut epithelium. The mosquito midgut is composed of epithelial cells surrounded by the basal lamina matrix and it can be divided into foregut, anterior midgut, posterior midgut and hindgut (Fig. 7) (Franz et al., 2015). A susceptible strain of *Culex tarsalis* has more specific receptors in the midgut

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brush border membrane for binding western equine encephalitis virus (WEEV) as compared to a resistant strain (Houk et al., 1990).

In *Aedes taeniorhynchus*, endemic and enzootic strains of Venezuelan equine encephalitis virus (VEEV) infect the anterior midgut but only the posterior midgut is infected by the endemic strain, leading to a better infection (Smith et al., 2008). The midgut infection is therefore the site of primary infection, followed by dissemination to and replication in secondary tissues such as nerve tissue, hemocytes, ovaries, fat body, legs and salivary glands. Dissemination requires that viruses escape the midgut infection barrier. From the salivary glands, viruses can then be transmitted by salivation during to bite to non-infected vertebrates. Salivary gland lobes are surrounded by epithelial cells bounded by a basal lamina, and the lateral lobes can be separated into proximal and distal regions (Franz et al., 2015; Vega-Rua et al., 2015). These latter regions are the main targets for virus invasion into the salivary glands (Franz et al., 2015). The salivary glands of *An. stephensi* adults are refractory to Rift Valley fever virus infection. It was proposed that anopheline mosquitoes are less competent arbovirus vectors because of the small size of their basal lamina pores (Romoser et al., 2005), if the basal lamina is a potential barrier to arbovirus passage. Moreover, Chikungunya virions are mainly found in apical cavities of epithelial cells in the *Ae. albopictus* salivary glands, where they are stored to be transmitted with saliva (Vega-Rua et al., 2015). Viruses must first invade and then escape the salivary glands in order to be transmitted, which is conceptually called the salivary gland infection barrier and salivary gland escape barrier. This latter explains the high susceptibility of *Ae. hendersoni* to La Crosse virus with a low rate of oral transmission (Paulson et al., 1989).

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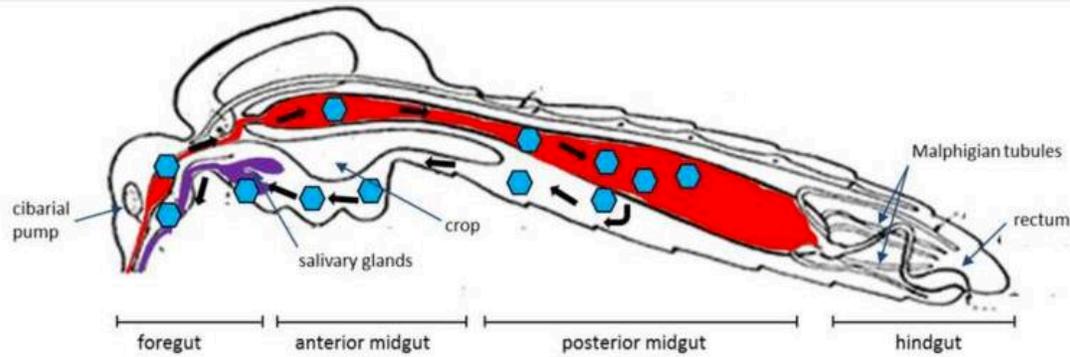


Figure 7: Tissues infected by arboviruses (Franz et al., 2015). Virus particles are represented in blue and the midgut in red. Arbovirus replication is modulated by the different tissue barriers including the salivary glands, the midgut and others.

1.3.2 Cellular responses

Cellular responses such as encapsulation, phagocytosis and apoptosis of pathogenic organisms in insects are mediated largely by hemocytes. Recognition of the pathogen by pattern recognition proteins (PRPs) triggers either humoral or cellular responses. PRPs bind pathogens on conserved domains called pathogen-associated molecular patterns (PAMPs) and induce activation of encapsulation, phagocytosis, proteolytic reactions, and synthesis of antimicrobial peptides (AMPs).

1.3.2.1 Encapsulation and phagocytosis

Encapsulation is defined as the ability of insects to form a tight, multilayered hemocytic capsule around pathogenic microorganisms causing the death of those microorganisms or the restriction of their growth and movement. Encapsulated microorganisms die by asphyxiation, accumulation of wastes, and intoxication by quinones (Ratner and Vinson, 1983). These latter are produced during the melanization process which is also involved in wound healing. Melanization causes the activation of a cascade of serine proteases leading to the conversion of the prophenoloxidase (PPO) into active phenoloxidase (PO) that is the rate-limiting enzyme. Oenocytoids are a hemocyte type involved in the production of PO in mosquitoes. On the basis of microscopic, functional and comparative studies, at least two other types of hemocytes exist in mosquitoes, the granulocytes

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and pro-hemocytes, which are hemocyte precursors. Granulocytes display acid phosphatase activity and are phagocytic cells (Hillyer and Strand, 2014). Phagocytosis involves the recognition, engulfment and intracellular destruction of infected cells. Thioester-containing protein1 (TEP1) binds to the surface of *Escherichia coli* and *Staphylococcus aureus*, leading to their phagocytosis in *An. gambiae* (Blandin and Levashina, 2007). Regulators of bacterial phagocytosis in mosquitoes include TEP1, TEP3, TEP4 and LRIM1, which are also involved in anti-*Plasmodium* responses, but their implication in antiviral responses is unknown (Blandin and Levashina, 2007). In mosquitoes, hemocytes are found near trachea and other tissues in the hemocoel (Parikh et al., 2009). The diversity and abundance of mosquito hemocytes can be modified by a bloodmeal (Ramirez et al., 2014). Granulocytes are the cells the most highly infected by Sindbis virus (SINV) in *Ae. aegypti*, and hemocytes may also be infected by arboviruses (Parikh et al., 2009). The role, if any, of hemocytes during viral infections in *Anopheles* is not clear.

1.3.2.2 Apoptosis

Apoptosis is a mechanism of programmed cell death that eliminates infected cells, cells with damaged DNA, and cancerous cells. For instance, cells infected by cricket paralysis virus in *Drosophila* induce apoptosis, leading to their elimination by hemocytes (Lamiable et al., 2016). Apoptosis is mainly initiated by the activation of cysteine aspartate-specific proteinases called caspases. Caspase zymogens are inactive forms and are activated during a proteolytic reaction. Mosquito viruses that lack apoptosis inhibitors are less virulent, but some viruses use apoptosis or caspases to aid their replication (Clem, 2016). In this way, apoptosis can be antiviral or proviral according to the viruses or host organisms. As an antiviral response, apoptosis is characterized by cytopathic effects in the midgut and the salivary glands of infected *Ae. aegypti* by Semliki Forest virus (SFV, *Togaviridae*, *Alphavirus*) (Clem, 2016). Recombinant SINV expressing the proapoptotic gene *reaper* from *Drosophila* in *Ae. aegypti* display reduced infection in the midgut and the salivary glands (O'Neill et al., 2015). In addition, SINV infection that triggers apoptosis reduces life span and increases mortality in mosquitoes (O'Neill et al., 2015). Regarding apoptosis in a proviral role, eastern equine encephalitis virus induces sloughing of midgut epithelial cells and loss of integrity of the basal lamina in *Culiseta melanura*, leading to virus dissemination (Weaver et al., 1988).

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1.3.3 Humoral responses

In insects, phenoloxidase, cell signaling pathways and antimicrobial peptides are involved in humoral responses. Mosquito antimicrobial peptides (AMPs) were initially described during bacterial infections, and are mainly synthesized in the fat body, in hemocytes and in the midgut (Vizioli et al., 2001). In *Anopheles*, defensin, cecropin, attacin, and gambicin are AMPs that are predominantly expressed in the midgut, thorax, and abdomen (Herrera-Ortiz et al., 2011; Vizioli et al., 2000). Attacin is induced by *P. berghei* infection in *An. albimanus* (Herrera-Ortiz et al., 2011). *Anopheles* defensin displays antibacterial and antifungal functions and is induced during *Plasmodium* infection. Bacterial and *P. berghei* infections induce *An. gambiae* cecropin, and its synthetic form displays activity against bacteria (Gram-positive and Gram-negative), yeast and fungi. The cecropin gene is localized on division 1C of the X chromosome of *An. gambiae* (Vizioli et al., 2000). *An. gambiae* gambicin inhibits the replication of bacteria and protects against infection by *P. berghei*. The gene is localized on chromosome 3R and is highly expressed in pupae and adults (Vizioli et al., 2001). Knowledge of the activity and implications of *Anopheles* AMPs against viruses is limited and should be investigated. The AMP HS-1 isolated from the frog, *Hypsiboas semilineatus* displays an antiviral activity against dengue-2 (*Flaviviridae*, *Flavivirus*) and dengue-3 viruses in an *in vitro* assay(s) (Monteiro et al., 2018).

Hemocytes and fat body probably contribute to the synthesis of TEP1, leucine-rich repeat immune protein 1 (LRIM1) and *Anopheles Plasmodium* responsive leucine-rich repeat 1 (APL1) that are found in the hemolymph and involved in humoral responses (Castillo et al., 2017). TEP1, LRIM1 and APL1 are important in the anti-*Plasmodium* response, but of these only APL1 plays an antiviral role during the midgut infection of ONNV in *An. coluzzii* (Blandin et al., 2004; Carissimo et al., 2015b; Riehle et al., 2006). Silencing of TEP1 suppresses the melanization of malaria parasites in *An. gambiae* (Blandin et al., 2004). Melanization depends on tyrosine metabolism which is activated by phenoloxidase, and silencing of phenoloxidase increases mortality in the shrimp *Penaeus monodon* infected with white spot syndrome virus (*Nimaviridae*, *Whispovirus*) (Sutthangkul et al., 2015). Semliki Forest virus activates the infected cascade in the U4.4 cell line of *Ae. albopictus*, and inhibition of phenoloxidase allows higher replication of *SFV* in *Ae. aegypti* (Rodriguez-Andres et al., 2012).

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There are multiple interactions between cellular and humoral responses, regulated by 4 main antiviral pathways (Fig. 8) in mosquitoes and other insects: Toll, IMD, JAK/STAT and RNAi pathways. In addition to these pathways, small RNAs such as piRNA, miRNA, and siRNA may be involved in the control of viruses in insects.

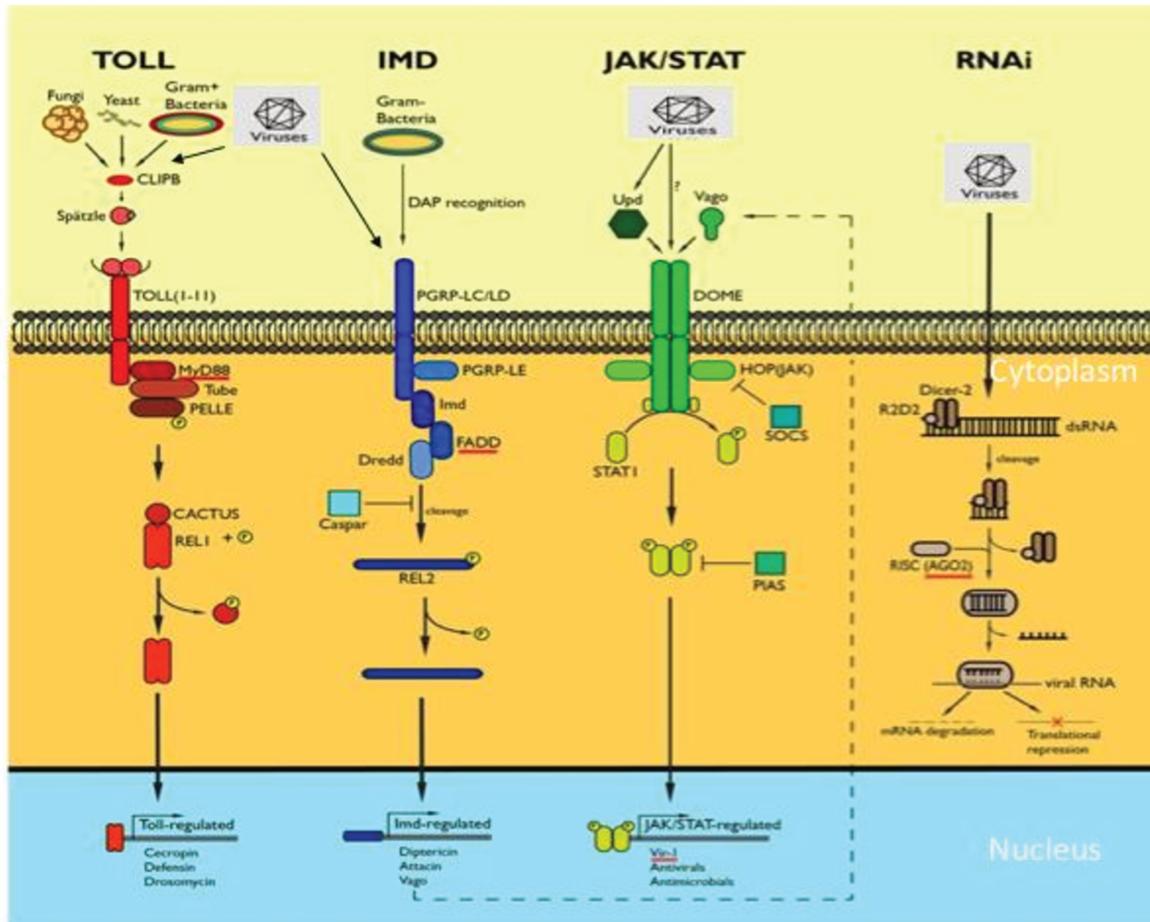


Figure 8: The main antiviral pathways in mosquitoes include Toll, Immune Deficiency (Imd), Janus Kinase-Signal Transducer Activator of Transcription (JAK/STAT) and the exogenous siRNA or RNAi pathways (Terradas et al., 2017).

1.3.3.1 Toll pathway

Toll-like receptors (TLRs) are associated with immune responses in mammals and insects. Indeed, the Toll pathway was first characterized in *Drosophila* from studies on embryogenesis and immunity (Hashimoto et al., 1988; Lemaitre et al., 1996).

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Bacterial, fungal, malaria parasite, and viral infections can lead to the production of a cytokine-like molecule Spätzle (Arnot et al., 2010). Binding of the cleaved form of Spätzle activates the Toll receptor. The cleavage of Spätzle is induced by secreted PRRs, and is mediated by a clip-domain serine protease. Subsequent activation of Toll receptor causes an intracellular recruitment of proteins such as MYD88, Tube and Pelle, and leads to the degradation of Cactus by Pelle. The signal from the Toll-like receptor is transmitted to the NF- κ B transcription factor Rel1 that has opposite effect to Cactus (Ferreira et al., 2014). Toll activation induces the transcription of antimicrobial peptides (AMPs) genes such as drosomycin, cecropin and defensin in the nucleus, as determined in *Drosophila*. *Drosophila* Toll mutants such as Pelle loss-of-function display higher viral loads of DCV and higher mortality after oral infection than the wild type, but there is no difference after injection of virus (Ferreira et al., 2014). Toll mutants (KO *dif*) infected with *Drosophila X* virus (*Birnaviridae*, *Entomobirnavirus*) die faster than wild type flies (Zambon et al., 2005).

Silencing of Cactus in *Ae. aegypti* infected by DENV-2 leads to translocation of Rel1 to the nucleus, inducing AMPs such as defensin, and cecropin, which results in 4-fold reduction of DENV in the midgut. In addition, silencing of MYD88, a Toll pathway negative regulator, increases by 2.7 times the DENV load, indicating that the Toll pathway plays an anti-dengue role in *Ae. aegypti* (Xi et al., 2008).

However, silencing of the transcription factor Rel1 in *An. coluzzii* has no effect on ONNV primary infection of the midgut (Carissimo et al., 2015a; Carissimo et al., 2015b).

1.3.3.2 IMD pathway

The immune deficiency (IMD) pathway is stimulated by the interaction between meso-diaminopimelic acid peptidoglycan (DAP) of bacteria and the PGRP-LC transmembrane receptor in *Drosophila*. IMD activation recruits dFADD and the caspase DREDD, which leads to the degradation of its negative regulator Caspar. DREDD activates the transcription factor Relish in *Drosophila*, or REL2 in *Anopheles* mosquitoes by a phosphorylation reaction. The activated transcription factor then translocates to the nucleus in order to initiate the expression of effectors and AMPs (Kleino and Silverman, 2014). In *Anopheles*, REL2 exists in two forms: a long Rel2 (Rel2-F) with an ankyrin domain and a short Rel2 (Rel2-S) isoform that lacks the ankyrin domain. Rel2-S controls the anti-*P. falciparum* effector APL1A, whereas the long isoform has no impact

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on *P. falciparum* infection in *An. gambiae* (Mitri et al., 2009). The silencing of both isoforms during the primary infection of ONNV in *An. coluzzii* increases the viral loads, indicating that Rel2-S is antiviral, while it displays no activity against *P. falciparum* (Carissimo et al., 2015b). In *Ae. aegypti*, Sindbis virus (SINV) (*Togaviridae*, *Alphavirus*) induces Rel2 in the midgut, and depletion of Caspar increases the SINV load, while decreasing the microbiota level (Barletta et al., 2017). The AMP dipteracin B (dptB) controlled by Relish is upregulated upon SINV infection in *Drosophila*, and dptB mutants display higher viral loads as compared to wild types (Huang et al., 2013).

1.3.3.3 JAK/STAT pathway

Cytokines activate the Janus kinase-signal transducer and activator of transcription (JAK/STAT) in vertebrates (Hollidge et al., 2011). In *Drosophila*, JAK/STAT activation is initiated by the recognition of *Domeless* (*Dome*) receptor by its *unpaired* (*Upd*) ligand leading to conformation modification and activation by auto-phosphorylation of JAK and *Hopscotch* (*Hop*), which are associated with *Dome*. Activation of the tyrosine kinase Hop causes phosphorylation of *Dome*, causing dimerization of STATs such as STAT92E in *Drosophila* or STAT-A in mosquitoes, and activated STAT translocates to the nucleus and activates the transcription of response genes (Souza-Neto et al., 2009). The secreted peptide *vago* reduces WNV infection by 30-fold in the *Cx quinquefasciatus* cell line Hsu by activating the JAK/STAT pathway (Paradkar et al., 2012). The infection of Hsu cells with WNV induces *Vago* by an RNAi-dependent pathway, while inactivated virus has no effect on *Vago* (Paradkar et al., 2012). The role of *Vago* in DENV infection of *Aedes* mosquitoes points to a communication between the RNAi and JAK/STAT pathways. Protein inhibitor of activated STAT (PIAS) is a negative regulator of the JAK/STAT pathway (Betz et al., 2001). PIAS depletion in *Ae. aegypti* decreases DENV-2 titer 5-fold at seven days after an infective blood meal, whereas *Dome* silencing increases the viral load by 3-fold (Souza-Neto et al., 2009). Suppressor of cytokine signaling (SOCS) proteins are other negative regulators of the JAK/STAT pathway, which are also involved in the modulation of DENV-2 infection in mosquitoes (Souza-Neto et al., 2009). In *An. coluzzii*, depletion of STAT-A increases the ONNV load during the primary infection in the midgut (Carissimo et al., 2015b).

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1.3.3.4 RNA interference (RNAi) pathway

The RNA interference pathway controls viral infections in insects by using two small interfering RNA classes (siRNA) depending on the virus type (Galiana-Arnoux et al., 2006; Keene et al., 2004). The endogenous siRNA pathway is used to silence various viruses in mosquitoes, whereas the exogenous siRNA pathway is more involved in the defense against arboviruses. However, endogenous and exogenous siRNAs share components. When a virus enters the host cell, it produces a double stranded RNA (dsRNA) replication intermediate, which is recognized by the host Dicer protein, an endoribonuclease of the RNaseIII family. In *Anopheles* species, Dicer2 (Dcr2) digests dsRNA into siRNA of 21 nucleotides which are loaded into the multi-protein RNA induced silencing complex (RISC). Argonaute 2 (Ago2), a subunit of the RISC complex with RNase activity guided by the complementary RNA, identifies and degrades the target viral mRNA (Saldana et al., 2017). Most viruses generate dsRNA during their replication in mosquitoes and the 21-nucleotide products generated by Dcr2, the dicing profile, are a marker of active virus replication (Carissimo et al., 2016). Ago2 and Dcr2 are the key factors in the RNAi pathway. Dcr-2 mutant *Drosophila* are more susceptible to the nodavirus Flock House virus (FHV) and die more rapidly than wild type flies (Galiana-Arnoux et al., 2006). In *Ae. aegypti* cell line Aag2, the depletion of Ago2 enhances Rift valley fever virus replication (Dietrich et al., 2017). In *An. coluzzii*, silencing of Ago2 during the primary midgut infection with ONNV has no effect on viral load, while the RNAi pathway does control the systemic ONNV infection after dissemination from the midgut (Carissimo et al., 2015b; Keene et al., 2004).

Because of the central role of siRNA during viral infections, many viruses have evolved suppressors of RNAi. The dsRNA-binding domain at the N-terminus of ORF 1 of DCV (DCV-1A) suppresses RNAi pathway activity by inhibiting dcr2 activity in *Drosophila* (van Rij et al., 2006). The viral suppressors of RNAi DCV-1A, and B2 from FHV, suppress the antiviral activity of the RNAi pathway by binding and masking viral dsRNA (Berry et al., 2009). WNV subgenomic flavivirus RNA (sfRNA), a noncoding RNA of positive polarity, suppresses RNAi in Vero and *Aedes* cells by inhibiting dicer (Schnettler et al., 2012).

In addition to small interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi interacting RNA (piRNA) are two other small RNA pathways involved in mosquito antiviral response.

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1.3.3.5 Piwi interacting RNA

Piwi (P element induced wimpy testis) interacting RNAs (piRNA) or repeat-associated small interfering RNAs (rasiRNAs) are endogenous small noncoding RNAs that ensure genome stability by protecting it from invasive transposable elements such as retrotransposons and repetitive or selfish sequences (Rizzo et al., 2014). There is enrichment of many long terminal repeats (LTRs) around endogenous viral elements (EVEs) in *Ae. aegypti* cells (Whitfield et al., 2017). piRNAs mainly act in the germline but their activity was detected against transposons located in the *flamenco* locus in somatic follicle cells of *Drosophila* (Li et al., 2009). Primary piRNAs of 24-29 nucleotides are the products of long precursor single stranded RNA. After cleavage, the primary piRNAs yield secondary piRNA molecules that have an A10 bias. The secondary piRNA is bound to Ago-3 to scan for the complementary RNA, which produces again primary piRNAs of antisense with U1 bias. A ping-pong mechanism thus characterizes the piRNA pathway because of generation of the sense secondary molecules with A10 bias from an antisense primary molecule with U1 bias and reciprocally. Primary piRNAs from the ping pong mechanism have mostly U1 bias and form complexes with Aubergine (Aub) and PIWI proteins (Theron et al., 2018). There are fewer piRNA clusters in *D. melanogaster* as compared to *An. gambiae* where these piRNA clusters are mainly uni-directionally transcribed and are found outside pericentromeric heterochromatin (George et al., 2015). In *An. gambiae* ovaries, 187 piRNA clusters are known, and are mainly found in euchromatic regions of chromosomes (George et al., 2015).

In *An. coluzzii*, the peak of piRNAs is seen at 27 nt, and piRNA sequences map to different types of transposable elements such as SINE, LINE, Gypsy-Like, and BEL-like transposons (George et al., 2015).

piRNAs are also involved in the persistence of viral infections resulting in the production of viral DNA forms or endogenization of non-retroviruses (Goic et al., 2013). Acute viral infections are transient and typically limited either by the death of the host or elimination of virus whereas in persistent viral infections, the infection is tolerated by the host without significant mortality. Persistent infection can be the result of an acute primary infection that is not eliminated by the host. To be persistent, the virus must escape or resist to immune responses either by genetic variation, by developing defective interfering particles, or by generation of DNA forms (Goic et al., 2013; Poirier et al., 2018).

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DNA forms can exist in cells or can be integrated into host genome as endogenous viral elements (EVEs) (Whitfield et al., 2017). DNA forms are generated by endogenous reverse-transcriptase activity (Goic et al., 2013). *Drosophila* S2 cells infected with FHV that survive lytic infection develop a persistent infection. Persistent FHV infection is not associated with fitness cost or mortality in S2 cells, although low levels of viral DNA forms are present. The production of DNA forms can be inhibited by azidothymidine (AZT), a nucleoside reverse-transcriptase inhibitor (Goic et al., 2013). DNA forms are precursors of EVE that are templates for the production of piRNAs. In fact, during SINV infection in *Ae. aegypti* Aag2 cells, EVE loci produce more anti-sense piRNAs, and piRNA clusters that contain EVEs have more piRNAs (Whitfield et al., 2017). Silencing of Piwi4 increases Phasi Charoen-like virus (PCLV) load in persistently infected Aag2 cells. Long-read sequencing shows that EVE sequences of *Ae. aegypti* and *Ae. albopictus* are different and belong to 8 virus families, *Bromoviridae*, *Closteroviridae*, *Bunyaviridae*, *Reoviridae*, *Virgaviridae*, *Chuviridae*, *Flaviviridae*, *Rhabdoviridae* (Whitfield et al., 2017). In Aag2 and U4.4 cells infected with Rift Valley fever virus, piRNAs increase during the late phase of acute infection and persistent infection (Leger et al., 2013).

1.3.3.6 microRNA (miRNA)

In insects, miRNA can be produced by canonical or non-canonical pathways. The canonical pathway is involved in the biogenesis of most miRNAs, in which they are produced from cellular transcripts called pri-miRNA, containing a short internal stem-loop structure, mediated by Drosha or Pasha in the nucleus (Asgari, 2014; Berry et al., 2009). The pre-miRNAs are exported by exportin 5 to the cytoplasm where they are cleaved by dicer 1 (Dcr-1) or loquacious (Loqs) to produce miRNA of 22 nucleotides (Saldana et al., 2017). One strand of the miRNA duplex is loaded in the RISC and it is guided to target mRNAs (Berry et al., 2009; Schnettler et al., 2012). MicroRNA can recognize the 3' UTR region of the target to modify its expression through inhibition or degradation by using AGO1.

Non-canonical pathways can also produce miRNAs, starting with pre-miRNAs made from hairpin introns during mRNA maturation, splicing, with no implication of Drosha, resulting in so-called mirtrons. Small nucleolar RNAs (snoRNA) found in the nucleolus can also produce miRNAs. The

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biogenesis of miRNA from non-canonical pathways is not well understood but Dicer 1 seems to be necessary for digestion in the cytoplasm (Asgari, 2014).

The alphavirus Zika (*Flaviviridae*, *Flavivirus*) causes differential abundance of 17 miRNAs in *Ae. aegypti* (Saldana et al., 2017).

In *An. gambiae*, the expression of miRNAs may differ by tissue. For instance, about two days after a bloodmeal, miR-275-3p, miR-276-5p, and miR-305-5p are induced while miR-989-3p is downregulated in the fat body. Blood ingestion by *An. coluzzii* also increases the level of the anti-*Plasmodium* miR-305 in the ovaries (Dennison et al., 2015; Lampe and Levashina, 2018).

CURRENT KNOWLEDGE GAPS AND OBJECTIVES

2. CURRENT KNOWLEDGE GAPS AND OBJECTIVES

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2.1 Current knowledge gaps in virus-*Anopheles* studies

The *Anopheles* virome is understudied and the number of viruses associated with *Anopheles* is unknown. Most of viruses are identified in *Culicinae* mosquitoes because they are main vectors of arboviruses (Table 1) (Liang et al., 2015). Malaria vectors are the main vectors of the arbovirus ONNV and there is no clear evidence to explain why *Anophelinae* mosquitoes are not competent to other known arboviruses, except ONNV. Artificial infections show that *Ae. aegypti* can simultaneously be infected with DENV and CHIKV or ZIKV and CHIKV and transmit them (Fig. 9) (Ruckert et al., 2017). While people are co-infected with malaria parasites and arboviruses in co-endemic areas, co-infection studies in *Anopheles* are scarce probably because *Anopheles* viruses are neglected in public health (Sow et al., 2016). In addition, *Anopheles* known viruses are probably misdiagnosed as compared to diseases such as malaria, flu and others which have fever as symptoms (Schultz et al., 2018). Similarly, interactions between ISVs and arboviruses in *Anopheles* are unknown too. Moreover, ISVs have the properties of paratransgenic candidates against malaria but paratransgenic works in *Anopheles* were done with bacteria (Capone et al., 2013; Favia et al., 2007; Wang et al., 2012).

Insect specific viruses in *Drosophila* species allowed advances in studies of antiviral responses in insects, because *Drosophila* is a developed genetic model, and its viruses are non-pathogenic to humans (Lemaitre et al., 1996). Except for several studies with the pathogenic ONNV, antiviral responses of *Anopheles* mosquitoes are under-studied and lack experimental models (Carissimo et al., 2015a; Carissimo et al., 2016; Carissimo et al., 2018; Keene et al., 2004). In France, ONNV is classified as a P3 agent, which obviously limits the research on this arbovirus. A more accessible model for studying interactions between viruses and *Anopheles* would be non-pathogenic to humans like ISVs, and could be manipulated on the bench in standard laboratories. This putative model should be present and able to replicate in wild type and laboratory *Anopheles*, and should be experimentally transmissible to uninfected *Anopheles*.

Recently several ISVs have been reported in *Anopheles*, but there no studies of their biology: their transmission routes, how they are maintained and controlled by the mosquito host, and how they could interact with each other in the virome (Colmant et al., 2017b; Fauver et al., 2016; Nanfack Minkeu and Vernick, 2018). These ISVs in *Anopheles* are mostly RNA viruses, as are most

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arboviruses (Table 1) with a high evolution rate, but data are lacking on genomic variation between the different hosts (Colmant et al., 2017b; Fauver et al., 2016; Galindo and Alonso, 2017; Nanfack Minkeu and Vernick, 2018). Moreover, the commensal relation between ISVs and their hosts sometimes leads to the integration of their genomes into host genomes. This phenomenon of integration, which is probably a consequence of a co-evolution between hosts and ISVs, is poorly understood and the underlying mechanisms involved are understudied (Goic et al., 2016; Lequime and Lambrechts, 2017; Suzuki et al., 2017). It was proposed that the high evolution rate of ISVs may allow them to evolve into arboviruses, but there is little evidence supporting this assumption (Junglen et al., 2017).

This thesis is mainly focused on the biological characterization of two ISVs, *Anopheles* cypovirus (AnCPV) and *Anopheles* C virus (AnCV). These two viruses were recently discovered by sequencing and bio-informatic analyses in a laboratory mosquito strain, *An. coluzzii* (Ngousso strain) and wild type mosquitoes (Carissimo et al., 2016). Both viruses display a profile of 21-nucleotide small RNA in *An. coluzzii*, characteristic of processing of double-stranded RNA replication intermediates into viral RNAs (viRNAs) by the RNAi pathway. This is a hallmark suggesting the viruses are actively replicating in this mosquito, but as yet there is no evidence of their replication in vertebrates. AnCV and AnCPV were classified as ISVs (Carissimo et al., 2016). RT-PCR was used to confirm the presence of AnCV and AnCPV in laboratory and wild type *Anopheles* mosquitoes (Carissimo et al., 2016).

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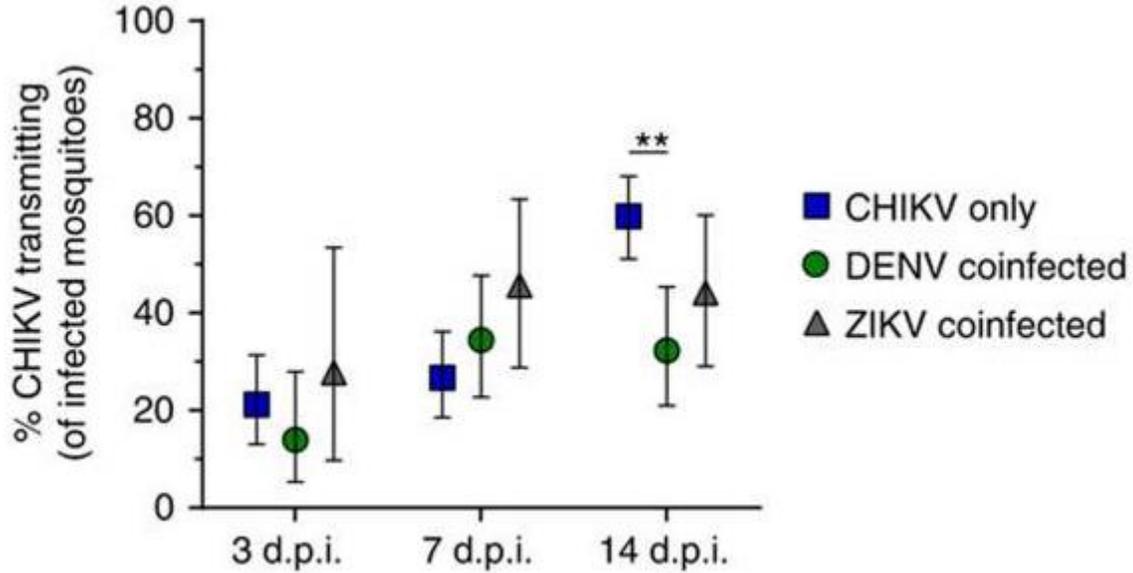


Figure 9: Simultaneous transmission of arboviruses by *Ae. aegypti*. dpi: day post infection. ** indicate a p value <0.01 (Ruckert et al., 2017).

Tableau 1: The main arboviruses with genome type and their main vectors.

Except African swine fever virus, all arboviruses have RNA genomes (Beckham and Tyler, 2015; Galindo and Alonso, 2017; Mitchell et al., 1992; Nanfack Minkeu and Vernick, 2018).

Arboviruses	Family	Genome type	Main vectors
African swine fever virus	<i>Asfarviridae</i>	DNA	<i>Ornithodoros moubata</i>
O'nyong nyong virus	<i>Togaviridae</i>	RNA	<i>Anopheles gambiae</i> and <i>An. funestus</i>
Chikungunya virus	<i>Togaviridae</i>	RNA	<i>Ae. aegypti</i> and <i>Ae. albopictus</i>
Eastern equine encephalitis virus	<i>Togaviridae</i>	RNA	<i>Culiseta melanura</i>
Dengue	<i>Flaviviridae</i>	RNA	<i>Aedes aegypti</i>
Zika virus	<i>Flaviviridae</i>	RNA	<i>Aedes</i> spp
Yellow fever virus	<i>Flaviviridae</i>	RNA	<i>Aedes</i> spp
West Nile virus	<i>Flaviviridae</i>	RNA	<i>Culex</i> spp
Japanese encephalitis virus	<i>Flaviviridae</i>	RNA	<i>Aedes</i> spp and <i>Culex</i> spp
St Louis encephalitis virus	<i>Flaviviridae</i>	RNA	<i>Culex pipiens</i>

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La Crosse virus	<i>Bunyaviridae</i>	RNA	<i>Aedes triseriatus</i>
Bluetongue virus	<i>Reoviridae</i>	RNA	<i>Culicoides imicola</i>
Rift Valley fever virus	<i>Phenuiviridae</i>	RNA	<i>Aedes</i> spp

2.1.1 *Anopheles C* virus (AnCV)

Anopheles C virus belongs to the order *Picornavirales*, family *Dicistroviridae* and the genus is *Cripavirus* (Carissimo et al., 2016). Dicistroviruses are single stranded RNA viruses of 8.5 to 10kb with positive polarity, thus RNA acts as a messenger RNA that is directly translated into two polyproteins (Bonning, 2009; Nakashima and Uchiumi, 2009). This RNA genome has a genome-linked virus protein (*VPg*) at the 5' end that plays an important role in replication (Fig. 10). The dicistrovirus virions are non-enveloped with a spherical form and a diameter of approximately 30 nm. The linear genome is composed of two open reading frames that are flanked by UTRs and separated by an intergenic region (IGR). The first ORF encodes the nonstructural proteins such as RNA helicase, cysteine protease and the RNA-dependent RNA polymerase (RdRp) (Valles et al., 2017). The second (ORF2) encodes 4 capsid proteins: CP1 to CP4 (Fig. 10). The capsid region codes for proteins involved in the entry of picornaviruses into host cells, whereas proteins from the nonstructural region are required for the replication that occurs into the cytoplasm of infected cell hosts. Before the ORF1 and between the 2 ORFs, there are two Internal Ribosome Entry sites (IRES) or IGR-IRES (Valles et al., 2017). These IGR-IRES allow the initiation of translation without the initiator factors (eIF2E, eIF4F, eIF4G) (Bonning, 2009).

Genome replication initiates with the synthesis of the minus strand which is in turn used as template to produce positive-strand RNA; the double stranded RNA (dsRNA) resulting is the replicative form. Dicistroviruses do not need the AUG (start codon), and this particularity allows them to avoid antiviral translation regulatory mechanisms. In addition, dicistroviruses can suppress host macromolecular synthesis leading to the shutoff phenomena. For instance, Cricket paralysis virus (CrPV) infection in the *Drosophila* S2 cells results in the dissociation of eIF4G and eIF4E and the induction of deIF2alpha phosphorylation leading to the translational shutoff (Garrey et al., 2010).

The replication cycle of dicistroviruses is poorly understood and most of the studies in this family were done on the enterovirus poliovirus, agent of paralytic poliomyelitis in vertebrates. The

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poliovirus cycle starts by the attachment of virions to the surface receptors of hosts and penetration by endocytosis. This latter step is followed by uncoating, and release of the viral genomic RNA into the cytoplasm. Then, translation and replication can start. The replication cycle ends with the releasing of new viral particles by infection mediated disintegration of the host cell after viral assembly (Hogle, 2002).

Known dicistroviruses mainly infect arthropods: *Drosophila C virus* (DCV) which naturally infect the flies (Jousset et al., 1977), aphid lethal paralysis virus purified from field-collected *R. padi* aphid colonies in South-Africa (Williamson, 1988), the triatoma virus was isolated from the triatomine bug, *Triatoma infestans* (Muscio et al., 1987), Israeli acute paralysis virus was isolated from honeybees in Israel (Maori et al., 2007), cricket paralysis virus (CrPV) isolated from Australian field crickets (Wilson et al., 2000). AnCV is phylogenetically close to CrPV and DCV (Carissimo et al., 2016). The figure 10 shows the structure of the dicistrovirus genome.

Anopheles C virus is the first dicistrovirus discovered in *An. coluzzii* and nothing is known about immune responses of *Anopheles* against this virus family. The viral interference or interaction of dicistroviruses with other virus families in *Anopheles* is also unknown.

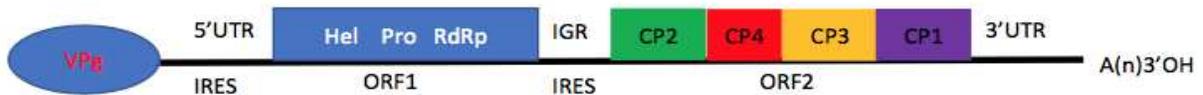


Figure 10: Genomic structure of dicistroviruses: The two non-overlapping ORFs separated by an IGR-IRES. The ORF1 encodes the non-structural proteins: RNA helicase (Hel), cysteine protease (Pro) and RNA-dependent RNA polymerase (RdRp). The 4 capsid proteins are encoded by the ORF2.

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2.1.2 *Anopheles* cypovirus (AnCPV)

Anopheles cypovirus was first detected in the *An. coluzzii* Ngousso laboratory strain (Carissimo et al., 2016). It is close to *Uranotaenia sapphirina* Cypovirus (UsCPV) based on an RNA-dependent RNA polymerase (RdRp) conserved segment. AnCPV is a non-enveloped virus classified in the family *Reoviridae*, sub-family *Spinareovirinae*, genus *Cypovirus* (Carissimo et al., 2016). Reoviruses have 9 to 12 segments and a double stranded RNA genome of approximately 25kb (Auguste et al., 2015). Segments are translated by transcriptional enzyme complexes (TECs) to specific structural or non-structural proteins (Fig. 11). The 5' end of each segment is capped and the poly-A tail is absent in the 3' end. The *Cypovirus* genome is protected by the single-shelled capsid and the icosahedral virions are resistant to alkaline treatments such as high pH, trypsin, sodium dodecyl sulfate, although they are sensitive to low pH, NP-40 detergent and urea (Zhang et al., 2002). Reoviruses penetrate into susceptible mammalian host cells by an endosomal route (Mainou and Dermody, 2012). In the cytoplasm, virus uncoating is initiated by different enzymes, resulting in the release of virus core. This latter step is followed by the formation of single stranded RNA from the double stranded RNA genome, and subsequent virus replication. The formation of occlusion or inclusion bodies that characterize cypoviruses occurs during the viral life cycle (Silverstein et al., 1976).

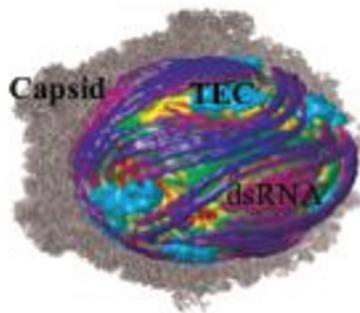


Figure 11: Overview of Cypovirus genome (Zhang et al., 2015). Capsid protect segmented dsRNA genome that is translated with the transcriptional enzyme complexes.

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Unlike dicistroviruses, reoviruses infect insects and vertebrates and the link between insect specific reoviruses and arbo-reoviruses is uncharacterized. In addition, the replication of reoviruses is quite studied in mammalian hosts but it is neglected in insects. The dual hosts of reoviruses offer opportunities to study their genomic variations and transmission dynamics between different hosts. The biology of *Anopheles* cypovirus in *Anopheles* has not been studied, and the response of *Anopheles* mosquitoes, including transcriptional regulation, upon infection with AnCPV or other ISVs has not been previously studied. In addition, the host range of AnCPV and its effects on *Anopheles* fitness are unknown.

2.2 SCOPE OF THE THESIS

The purpose of this thesis was to explore the biological interactions of two novel members of the natural virome with their host, *Anopheles* vectors of malaria, and to establish a new benchtop model system for *Anopheles*-RNA virus interactions.

The main objectives of this thesis were to:

- Identify and quantify viruses associated with *Anopheles* mosquitoes, and their implication for public health and fundamental research.
- Describe the biology of AnCPV and AnCV transmission, pathology, and abundance during mosquito development
- Develop appropriate conditions for the experimental study system and an in vitro cell system for virus production
- Generate comprehensive whole-transcriptome profiling of mosquito response to primary midgut infection, and disseminated systemic infection

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- Functional genomic dissection of host antiviral immune pathways and identification of candidate immune effectors.
- Study the experimental evolution of insect-specific viruses between different hosts, and determine the significance of potential virus genomic variation.

RESULTS

3. RESULTS

3.1 Article I: A Systematic Review of the Natural Virome of *Anopheles* Mosquitoes

HIGHLIGHTS:

Anopheles is mainly studied for its role in transmission of malaria parasites, and for this reason transmission of other pathogens is neglected. *Anopheles* have been associated with many arbovirus outbreaks, but their role as biological vectors of arboviruses is not yet established except in the case of ONNV. Moreover, high-throughput sequencing and de novo assembly has greatly increased the number of ISVs identified in malaria vectors, but it is difficult to estimate the total number of them. This study provides an estimate of the number and identity of ISVs and arboviruses which are associated to *Anopheles* mosquitoes.

We used bibliographic searches to show that *Anopheles* virome was diverse and complex and could be grouped in ISVs, potential arboviruses and arboviruses. The fact that ISVs are non-pathogenic means that they can be useful in paratransgenesis and for the study the interactions between *Anopheles* and viruses. *Anopheles* may contribute to the transmission of arboviruses but competence studies should be assessed to determine if *Anopheles* are vectors or reservoirs of arboviruses. In addition, we discuss hypotheses which might explain the difference in arbovirus competences between *Anopheles*, *Culex* and *Aedes* mosquitoes. Studies on *Anopheles* viruses should assist in preventing arbovirus outbreaks from unknown viruses and to understand the co-infection between viruses and *Plasmodium* species.

Review

A Systematic Review of the Natural Virome of *Anopheles* Mosquitoes

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Abstract: *Anopheles* mosquitoes are vectors of human malaria, but they also harbor viruses, collectively termed the virome. The *Anopheles* virome is relatively poorly studied, and the number and function of viruses are unknown. Only the o'nyong-nyong arbovirus (ONNV) is known to be consistently transmitted to vertebrates by *Anopheles* mosquitoes. A systematic literature review searched four databases: PubMed, Web of Science, Scopus, and Lissa. In addition, online and print resources were searched manually. The searches yielded 259 records. After screening for eligibility criteria, we found at least 51 viruses reported in *Anopheles*, including viruses with potential to cause febrile disease if transmitted to humans or other vertebrates. Studies to date have not provided evidence that *Anopheles* consistently transmit and maintain arboviruses other than ONNV. However, anthropophilic *Anopheles* vectors of malaria are constantly exposed to arboviruses in human bloodmeals. It is possible that in malaria-endemic zones, febrile symptoms may be commonly misdiagnosed. It is also possible that anophelines may be inherently less competent arbovirus vectors than culicines, but if true, the biological basis would warrant further study. This systematic review contributes a context to characterize the biology, knowledge gaps, and potential public health risk of *Anopheles* viruses.

Keywords: insect vectors; virome; arbovirus; insect immunity; host–pathogen interactions; malaria

1. Introduction

Anopheles mosquitoes are the vectors of human malaria, which causes at least 400,000 deaths and 200 million cases per year [1]. Approximately 90% of malaria deaths occur in sub-Saharan Africa, 7% in South-East Asia and 2% in the Eastern Mediterranean Region, with children under five years of age the most affected. More than 480 species of *Anopheles* have been described worldwide and about 70 of these are responsible for human malaria transmission, with about 40 regarded as the dominant malaria vector species [2,3].

However, the research focus on *Anopheles* as vectors of malaria has led to a relative lack of study about *Anopheles* viruses. In addition to malaria parasites, *Anopheles* mosquitoes also harbor viruses, collectively termed the virome. The *Anopheles* virome is poorly studied, and the number and function of viruses are unknown. Some of them are confirmed arthropod-borne pathogenic viruses (arboviruses), which multiply in the mosquito vector before transmission to a vertebrate host. Others are thought to be insect-specific viruses that may replicate only in the insect host [4,5].

Culicine mosquitoes such as *Aedes* and *Culex* are the main vector of arboviruses such as dengue virus (DENV; genus *Flavivirus*, family *Flaviviridae*), yellow fever virus (YFV, genus

Flavivirus, family *Flaviviridae*), chikungunya (CHIKV, genus *Alphavirus*, family *Togaviridae*), and others. Only one arbovirus is known to be consistently transmitted by *Anopheles* mosquitoes, the alphavirus o'nyong-nyong (ONNV, genus *Alphavirus*, family *Togaviridae*) [6–9], which is closely related to CHIKV [10].

RNA viruses from various families (e.g., *Flaviviridae*, *Togaviridae*, *Peribunyaviridae*, *Rhabdoviridae*, *Mesoniviridae*, *Reoviridae*, and *Dicistroviridae*) and the taxon *Negevirus* have been described in *Anopheles* mosquitoes [5,11–15]. These viruses have been discovered by isolation from cell cultures, by reverse transcriptase PCR (RT-PCR) and manual sequencing targeting regions of known viruses, or by deep sequencing of field-caught insect samples [16,17].

In addition to ONNV, other viruses with potential to cause febrile disease if transmitted to humans or other vertebrates have been isolated from *Anopheles*, including Nyando virus [18,19], Batai virus [20], Japanese encephalitis virus [21], Myxoma virus [22], and West Nile virus [23]. During a recent epidemic, Rift Valley fever virus (RVFV) was present in *Anopheles* females, males, and larvae, indicating vertical transmission [24,25].

Studies to date have not provided evidence that *Anopheles* can contribute to the transmission and maintenance of any of these arboviruses, other than ONNV. However, anthropophilic *Anopheles* vectors of malaria are also constantly exposed to arboviruses in infected human bloodmeals. It is possible that in malaria-endemic zones, febrile symptoms of malaria may mask symptoms of arbovirus infection and cause misdiagnosis. It is also possible that, for an unknown reason, anophelines are less competent arbovirus vectors than culicines, but if true, this would be biologically interesting, and would warrant further study. Mechanisms of *Anopheles* anti-viral immunity have been little examined [26–28]. The relative lack of virus transmission by *Anopheles*, if true, is puzzling because *Anopheles*, especially highly anthropophilic taxa, would seem well-placed to serve as intermediaries for virus spillover from other vertebrates to humans [29], as well as vectors for maintenance of transmission.

The characteristics of *Anopheles* viruses that comprise the natural virome flora are also poorly characterized, thereby creating little appreciation of their number and significance. Examination of the literature suggests that the number of *Anopheles* viruses is underestimated, including potentially pathogenic arboviruses. Here, we carry out a systematic literature search in order to summarize as comprehensively as possible the known viruses of *Anopheles* mosquitoes.

2. Materials and Methods

Four databases were searched in this work: PubMed, Web of Science, Scopus, and Lissa. In addition, manual searches of online and print resources were carried out.

Three combinations of keywords were used for searching the PubMed database. These were (i) (“anopheles”[Title] OR “anopheles”[All Fields]) AND (viruses[Title]) NOT (Aedes[Title] OR Culex[Title]); (ii) ((Anopheles AND viruses) NOT (Aedes OR Culex)) OR (“Anopheles/virology”[Mesh] NOT (“Culex”[Mesh])) NOT (“Aedes”[Mesh])); and (iii) ((Anopheles viruses [Title]) NOT (Aedes [Title] OR Culex [Title])).

Searching article titles proved to be useful because the search terms were compatible across bibliographic databases. Thus, search (iii) above was easy to translate into the Web of Science, Scopus, and Lissa databases, as follows. Web of Science advanced search of all databases, including Web of Science Core Collection, KCI-Korean Journal Database, MEDLINE, Russian Science Citation Index and SciElo Citation Index: TI = Anopheles AND TI = Viruses NOT TI = (Aedes OR Culex); Scopus advanced search: (TITLE (Anopheles AND Viruses) AND NOT TITLE (Aedes OR Culex)); and the French language Lissa: ‘Virus d’Anopheles.ti SAUF (Aedes OR Culex).ti’.

The above searches were carried out from 15 January to 4 May 2016. Moreover, an alert with the keyword (((Anopheles AND viruses) NOT (Aedes OR Culex))) OR (((“Anopheles/virology”[Mesh]) NOT “Culex”[Mesh]) NOT “Aedes”[Mesh]) was created and followed in PubMed from 15 January 2016 to the submission date of this manuscript. *Anopheles* virus/virus d’anophèles were also searched in Google. In addition, the key word “Anopheles” was used to identify arboviruses associated with

Anopheles species in the online ArboCat Arbovirus Catalog resource, maintained by the Centers for Disease Control and Prevention, USA (<https://www.cdc.gov/arbovat/>). In addition to these online searches, the books from the central library of the Institut Pasteur (Scientific Media and Information Center (CeRIS)) specialized in microbiology, virology, entomology, immunology, molecular biology, and biochemistry were searched.

Finally, eligibility criteria were applied for inclusion of a virus in the study: (i) virus species and name coherent with the standards of the International Committee on Taxonomy of Viruses (ICTV), (ii) reporting of diagnostic tools to permit independent detection, and (iii) some amount of nucleotide sequence. Reports of a putative virus were ineligible if they met none of these criteria, for example if the report was based only on observation of cytopathic effects on cultured cell lines, or pathology in mice.

3. Results

3.1. Bibliographic Search of Publication History on *Anopheles* Viruses

The search terms ((*Anopheles* viruses [Title]) NOT (*Aedes* [Title] OR *Culex* [Title])) yielded 36, 43 and 58 records in PubMed, Scopus, and Web of Science databases, respectively, on 4 May 2016 (PRISMA Flow Diagram, Figure S1). The 36 articles found in PubMed produced meaningful outputs, corresponding to recent reports of *Anopheles* virus. For example, in PubMed, the article number PMID: 27138938 (published 3 May 2016) was returned, but in Web of Science and Scopus the most recent articles were published in November 2015.

The alerts in PubMed using criteria (((*Anopheles* AND viruses) NOT (*Aedes* OR *Culex*))) OR (((“*Anopheles/virology*”[Mesh]) NOT “*Culex*”[Mesh]) NOT “*Aedes*”[Mesh]) yielded 14 articles between 15 January and 18 August 2016. These articles have the following numbers: PMID: 26807720, PMID: 25882523, PMID: 26821654, PMID: 25879960, PMID: 25637950, PMID: 26492074, PMID: 26416112, PMID: 26271277, PMID: 27113956, PMID: 25222233, PMID: 27138938, PMID: 26401843, PMID: 27456078, PMID: 26807720. Only the six articles in bold among these 14 correspond to *Anopheles* viruses.

The Lissa database of scientific literature written in French returned a single article using the above English search terms. However, this article in French (with English keywords) was written in 1957, and was not included in any of the three other databases. In addition, when using French language search terms, the Lissa database identified 13 additional articles (for a total of 14), all in French, that were uniquely identified by Lissa and not by the other three databases. Nevertheless, some of the search terms such as “virus” and “*Anopheles*” are spelled the same in French and English, and further work would be required to determine whether the different search results are due to search term language, or distinct database contents.

3.2. Viruses

Both DNA and RNA viruses have been reported infecting *Anopheles* species, although reports of RNA viruses are more prevalent. RNA viruses include *Alphavirus*, *Phlebovirus*, *Flavivirus*, *Orthobunyavirus*, *Dicistrovirus*, *Cypovirus*, *Mononegavirus*, *Totivirus*, and *Orbivirus* genera. DNA viruses include *Densovirus*, *Poxvirus*, *Iridovirus* (Table 1). *Anopheles* viruses have been reported on all continents except the poles (Figure 1).

Table 1. Summary of reported *Anopheles* viruses with references. (Sortable Excel table available for download from Supplementary Materials).

Virus Name	Abbreviation	Virus Genus	<i>Anopheles</i> Species	References
Anopheles A virus	ANAV	Orthobunyavirus	<i>Anopheles boliviensis</i>	[30]
Anopheles annulipes orbivirus	AAOV	Orbivirus	<i>Anopheles annulipes</i>	[31]
Anopheles associated C virus	AACV	Cripavirus	<i>Anopheles maculipennis</i>	[16]
Anopheles B virus	ANBV	Orthobunyavirus	<i>Anopheles boliviensis</i>	[30]
Anopheles C virus	AnCV	Cripavirus	<i>Anopheles gambiae</i>	[5]
Anopheles cypovirus	AnCPV	Cypovirus	<i>Anopheles gambiae</i>	[5]
Anopheles flavivirus	AnFV	Flavivirus	<i>Anopheles</i> sp.	[32]
Anopheles gambiae densovirus	AgDENV	Densovirus	<i>Anopheles gambiae</i>	[33]
Anopheles gambiae flavivirus	AngFV	Flavivirus	<i>Anopheles gambiae</i>	[34]
Anopheles hinesorum orbivirus	AHOV	Orbivirus	<i>Anopheles hinesorum</i>	[31]
Anopheles minimus virus	AMIV	Iridovirus	<i>Anopheles minimus</i>	[35]
Anopheles squamosus flavivirus	AnsFV	Flavivirus	<i>Anopheles squamosus</i>	[34]
Anopheles totivirus	AToV	Totivirus	<i>Anopheles gambiae</i>	[32]
Australian Anopheles totivirus	AATV	Totivirus	<i>Anopheles annulipes</i>	[31]
Australian Anopheles totivirus	AATV	Totivirus	<i>Anopheles hinesorum</i>	[31]
Bangui virus	BGIV	Orthobunyavirus	<i>Anopheles pharoensis</i>	[36]
Batai virus	BATV	Orthobunyavirus	<i>Anopheles maculipennis</i>	[20,37]
Bolahum virus	BOAV	Mononegavirus	<i>Anopheles</i> sp.	[32]
Bwamba virus	BWAV	Orthobunyavirus	<i>Anopheles funestus</i>	[38]
Cache Valley virus	CVV	Orthobunyavirus	<i>Anopheles quadrimaculatus</i>	[39]
Coot Bay virus	CBV	Almendravirus	<i>Anopheles quadrimaculatus</i>	[40]
Cypovirus	Unnamed	Cypovirus	<i>Anopheles stephensi</i>	[41]
Dairy Swamp virus	DSwV	Flavivirus	<i>Anopheles bancrofti</i>	[42]
Eliat virus	EILV	Alphavirus	<i>Anopheles coustani</i>	[13,43]
Gambiae virus	GAMV	Mononegavirus	<i>Anopheles</i> sp.	[32]
Haslams Creek virus	HaCV	Flavivirus	<i>Anopheles annulipes</i>	[42]
Ilesha virus	ILEV	Orthobunyavirus	<i>Anopheles gambiae</i>	[44]
Japanese encephalitis virus	JEV	Flavivirus	<i>Anopheles pedataeniatus</i>	[45]
Japanese encephalitis virus	JEV	Flavivirus	<i>Anopheles sinensis</i>	[46]
Kampung karu virus	KPKV	Flavivirus	<i>Anopheles tessellatus</i>	[47]
Karumba virus	KRBV	Flavivirus	<i>Anopheles meraukensis</i>	[42]
Leanyer virus	LEAV	Orthobunyavirus	<i>Anopheles meraukensis</i>	[48]
Long Pine key virus	LPKV	Flavivirus	<i>Anopheles crucians</i>	[47]
Mac Peak virus	McPV	Flavivirus	<i>Anopheles farauti</i>	[42]
Mapputta virus	MAPV	Orthobunyavirus	<i>Anopheles meraukensis</i>	[49]
Myxoma virus	MYXV	Poxvirus	<i>Anopheles maculipennis</i>	[22]
Ngari virus	NRIV	Orthobunyavirus	<i>Anopheles gambiae</i>	[36]
Nyando virus	NDV	Orthobunyavirus	<i>Anopheles funestus</i>	[18,50]
O'nyong nyong virus	ONNV	Alphavirus	<i>Anopheles gambiae</i>	[8,50]
O'nyong nyong virus	ONNV	Alphavirus	<i>Anopheles funestus</i>	[8,50]
Orungo virus	ORUV	Orbivirus	<i>Anopheles funestus</i>	[51]
Rift Valley fever virus	RVfV	Phlebovirus	<i>Anopheles squamosus</i>	[25]
Rift Valley fever virus	RVfV	Phlebovirus	<i>Anopheles coustani</i>	[25]
Semliki Forest virus	SFV	Alphavirus	<i>Anopheles funestus</i>	[52]
Semliki Forest virus	SFV	Alphavirus	<i>Anopheles coustani</i>	[52]
Sindbis virus	SINV	Alphavirus	<i>Anopheles pharoensis</i>	[53]
Sindbis virus	SINV	Alphavirus	<i>Anopheles albimanus</i>	[53]
Stratford virus	STRV	Flavivirus	<i>Anopheles annulipes</i>	[54]
Tahyna virus	TAHV	Orthobunyavirus	<i>Anopheles hyrcanus</i>	[55]
Tataguine virus	TATV	Orthobunyavirus	<i>Anopheles gambiae</i>	[56]
Tensaw virus	TENV	Orthobunyavirus	<i>Anopheles crucians</i>	[57]
Tibet orbivirus	TIBOV	Orbivirus	<i>Anopheles maculatus</i>	[58]
Tilligerry virus	TILV	Orbivirus	<i>Anopheles annulipes</i>	[59]
Venezuelan equine encephalitis virus	VEEV	Alphavirus	<i>Anopheles pseudopunctipennis</i>	[60]
Wesselsbron virus	WSLV	Flavivirus	<i>Anopheles coustani</i>	[34]
West Nile virus	WNV	Flavivirus	<i>Anopheles pauliani</i>	[23]
West Nile virus	WNV	Flavivirus	<i>Anopheles maculipennis</i>	[61,62]
Western equine encephalitis virus	WEEV	Alphavirus	<i>Anopheles albitarsis</i>	[63]

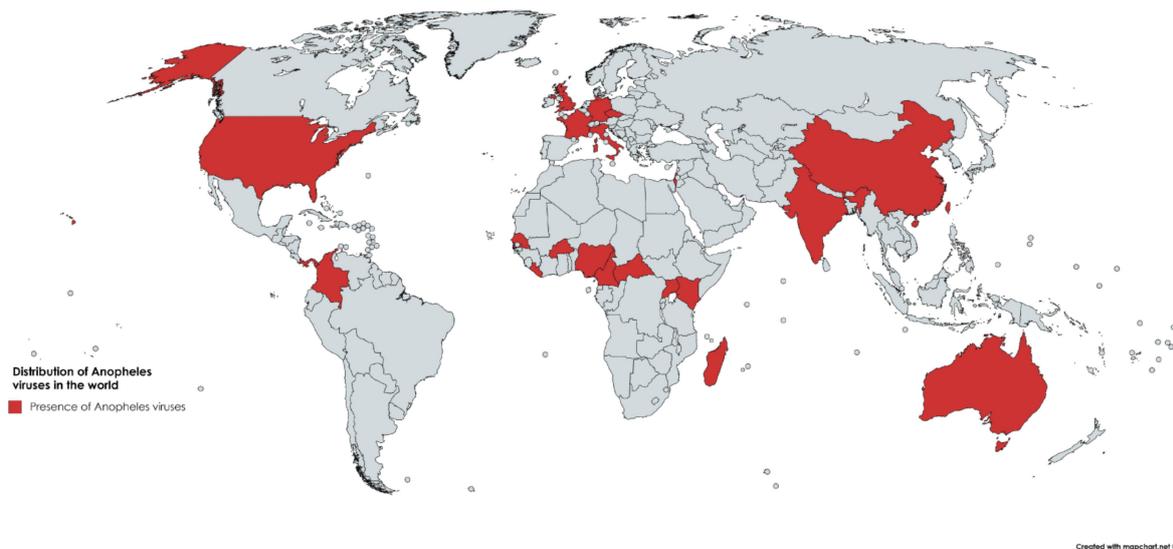


Figure 1. Global distribution of reported *Anopheles* viruses by country (red, countries with viruses in *Anopheles*).

3.3. DNA Viruses

3.3.1. Densovirus: *Anopheles gambiae* Densovirus (AgDNV)

The densoviruses belong to the *Parvoviridae* family, characterized by a non-enveloped virion containing a linear single-stranded DNA genome. AgDNV has a genome size of 4139 nt and is organized as two overlapping reading frames that encode the viral proteins (VP) of which activity is fundamental for virus infectivity and two non-structural (NS) proteins involved in the DNA replication. The NS1 portion displays 87% homology with *Aedes aegypti* densovirus (AeDNV) [33].

AgDNV was discovered in the *An. gambiae* cell line, Sua5B, during an experiment to infect the cells with *Wolbachia*. It was maintained between different generations by vertical and horizontal transmission and has no detectable effect on mortality of *An. gambiae* larvae. Virus purification was done from crude cell lysates on a density gradient, and icosahedral, non-enveloped particles of 20 nm were observed by transmission electron microscopy [33].

The use of DNVs as expression vectors was demonstrated by the transfection of *Aedes albopictus* C6/36 cell line with a plasmid containing an infectious sequence of *Ae. aegypti* DNV. This infection yielded the same quantity of the monomeric replicative form as infection with wild type virions [64]. A recombinant AgDNV carrying the enhanced green fluorescent protein (EGFP) under control of actin5C promoter has been produced [33]. EGFP transducing virions infected 50% of adults and were able to disseminate to the fat body, midgut, hindgut, malpighian tubules, and ovaries, and were vertically transmitted to subsequent generations. These results indicate that recombinant AgDNV could be a candidate for paratransgenesis, for example by carrying an anti-*Plasmodium* peptide for reducing the vector competence of *Anopheles* to *Plasmodium* spp. In addition, the AgDNV titer is higher in older *An. gambiae* adults compare to the larvae and pupae stages, suggesting that it could have potential as an adult stage bio-insecticide [65]. Tested DNVs are innocuous to mammals [64].

3.3.2. Iridovirus: *Anopheles minimus* Virus (AMIV)

Anopheles minimus virus is an iridescent virus (IIV) belonging to *Iridovirus* genus (family, *Iridoviridae*). The genera *Chloriridovirus*, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* are also included in this virus family. The icosahedral virions of AMIV are roughly 130 nm in diameter and the DNA genome is 163 kb in size [66]. The genomic DNA is associated with proteins and the internal

membrane is composed of phospholipids. Circularly permuted double-stranded DNA (dsDNA) genomes are characteristic of this family.

AMIV was isolated from wild adult *Anopheles minimus*, a major Southeast Asian malaria vector, after inoculation of C6/36 cells with mosquito extract [35]. BHK21 and Vero-E6 cells can also be infected with AMIV, with a significant cytopathic effect [66]. AMIV is the first iridovirus isolated from *Anopheles* species. An *Ae. aegypti* iridovirus (IIV-6) causes cytopathic damage leading to the reduction of body size, fecundity and longevity. Horizontal transmission by cannibalism and vertical transmission of IIV-3 were observed in *Ochlerotatus taeniorhynchus* [67]. Iridovirus infection leads to an apoptotic response in invertebrate and vertebrate cells [67].

3.3.3. Poxvirus: Myxoma Virus (MYXV)

The myxoma virus genome was detected by polymerase chain reaction (PCR) and sequencing in wild caught *An. maculipennis* that fed on wild rabbits [22]. The *An. maculipennis* group includes major historic vectors of human malaria in North African and Europe [68]. Myxoma virus is in the *Leporipoxvirus* genus and *Poxviridae* family. This family includes the subfamilies *Chordopoxvirinae* and *Entomopoxvirinae*. The latter infects insects and comprises *Alphaentomopoxvirus*, *Betaentomopoxvirus*, and *Gammaentomopoxvirus* genera. Myxoma virus can be mechanically transmitted by mosquitoes and fleas but does not replicate in them, and thus is not an arbovirus. The *Poxviridae* genome is comprised of linear dsDNA, and is enveloped. Poxviruses share features with other DNA viruses such as the asfarviruses, iridoviruses, and phycodnaviruses [69].

3.4. RNA Viruses

3.4.1. Alphavirus: O'nyong Nyong Virus (ONNV), Venezuelan Equine Encephalitis Virus (VEEV), Western Equine Encephalitis Virus (WEEV), Sindbis Virus (SINV), Semliki Forest Virus (SFV), and Eilat Virus (EILV)

The *Alphavirus* genus in the *Togaviridae* family is frequently associated with *Anopheles* mosquitoes. O'nyong nyong virus (ONNV) is generally regarded as the only arbovirus transmitted by *Anopheles* mosquitoes. Alphaviruses are enveloped viruses with a single-stranded RNA of positive sense and a genome size of around 10,000 nucleotides. The 5' end is capped and the 3' end is polyadenylated. The linear RNA genome encodes nonstructural proteins (nsP1 to nsP-4), and structural proteins, although only the nsPs are translated from the genome, while the structural proteins are translated from a subgenomic RNA transcribed after infection. The nonstructural protein nsP1 is necessary for infectivity, nsP2 is necessary for replication and transcription of viral RNAs, nsP3 forms cytoplasmic complexes with different host factors, and nsP4 is a RNA-dependent RNA polymerase. A domain-swap experiment replacing part of the CHIKV nsP3 with the ONNV sequence allowed the chimeric virus to infect *An. gambiae* [70], highlighting a determinant of mosquito host specificity that requires further investigation. The capsid protein, the glycoproteins (E1 and E2) and the small peptides, E3 and 6K are structural proteins. A single mutation of E1 glycoprotein (E1-A226V) increases CHIKV transmission by the mosquito *Aedes albopictus* [71]. E2 is involved in antigenicity and viral pathogenesis [72]. The E3 peptide is necessary for protein heterodimerization, and the deletion of the 6K peptide in Ross River Virus genome reduces pathogenicity and viral titer in mice [73]. The interactions between the structural proteins are also indispensable for virion integrity and virus assembly [32,74].

The common symptoms of ONNV in humans are fever, rash, headache, polyarthritides-like illness, and back pains, but the infection is often asymptomatic. An ONNV epidemic in Uganda, Tanzania, Kenya, Malawi, Senegal, Democratic Republic of Congo infected approximately 2 million people, but no fatal cases were reported [7,18]. Another outbreak of ONNV was reported in Uganda in 1996, with morbidity rates of 45–65% in some villages. ONNV was detected by serologic test and quantitative RT-PCR in 26% of Liberian refugees tested in 2003 [8,9]. The virus was detected in a pool of wild *Anopheles funestus* and *An. gambiae* collected in Uganda and Kisumu, Kenya by inoculation of

a suspension into albino Swiss mice, and independently also from patient sera [8,18,50]. Antibodies against ONNV found in the sera of inoculated mice suggested that the virus was pathogenic.

The important African malaria vectors, *An. funestus* and *An. gambiae*, are also able to transmit ONNV to mice [75,76]. The RNA interference (RNAi) pathway is necessary for protection of *An. gambiae* against ONNV infection [27]. However, the RNAi pathway displays ONNV antiviral activity in *An. gambiae* only during the disseminated systemic infection, and not in the primary midgut infection by bloodmeal [26]. The immune pathways Janus kinases/Signal Transducer and Activator of Transcription (JAK/STAT) and Immune Deficiency (Imd) display a reciprocal effect, as ONNV antiviral mechanisms in the primary midgut infection but not against the disseminated infection in the hemocoel.

Venezuelan equine encephalitis virus (VEEV) is an arbovirus in the Americas, where numerous outbreaks have been reported with equine and human deaths [77]. A large epidemic of VEEV in Colombia in 1995 caused more than 70,000 human cases and 300 deaths [78]. VEEV was isolated from two pools of *Anopheles p. pseudopunctipennis* in Mexico in 1972, was cultured in C6/36 cells, and an infectious clone was generated [60]. Western equine encephalitis virus (WEEV) is a recombinant virus that can infect humans and other vertebrates, with a capsid protein related to Eastern equine encephalitis virus (EEEV) whereas the glycoprotein sequences are closer to Sindbis virus [79]. An epizootic occurred in Argentina in 1982, and more than 150,000 mosquitoes of different genera were collected by Centers for Disease Control (CDC) light traps between 1982 and 1983 [63]. From these mosquitoes, WEEV was isolated from a pool of *An. albipennis* inoculated on Vero cells [63].

An. albimanus mosquitoes were competent for infection with Sindbis virus (SINV) by feeding on infected rabbit blood, displaying an infection prevalence of 64%, as well as the ability to transmit the virus to baby chicks [80]. SINV was also isolated from a pool of *Anopheles* spp. collected in China in 1990 and was used to generate an infectious clone [53]. SINV was first isolated in Sindbis village in Egypt in 1952 in *Culex* spp., but *Anopheles* mosquitoes seem also able to transmit this virus [80].

Semliki Forest virus (SFV) was discovered in *Aedes abnormalis* collected in Uganda in 1942 [27,81]. Multiple *Anopheles* species (*An. stephensi*, *An. freeborni*, *An. sudaicus* and *An. labranchia*) were infected with SFV by membrane feeding [82]. In an outbreak in Bangui, Central African Republic in 1987, SFV was isolated from patient sera and from pools of *Ae. africanus*, *Ae. aegypti*, *An. coustani*, and *An. funestus* [52].

Eilat virus (EILV) was isolated from a pool of *An. coustani* mosquitoes and displays inability to replicate in the vertebrate cellular environment [13,43]. EILV was transmitted by bloodfeeding to *An. gambiae*, *C. quinquefasciatus*, and *Ae. aegypti* but not to *Ae. albopictus*, and may be vertically transmitted in the infected species, but the virus was not detected in the ovaries [13]. EILV did not display cytopathic effect in *Aedes* C6/36 and or C7/10 cells despite high replication at 12 h post infection. One proposed hypothesis is that EILV may have secondarily lost the ability to infect vertebrate cells, rather than being insect-specific as an ancestral character.

3.4.2. Flavivirus: West Nile Virus (WNV), Japanese Encephalitis Virus (JEV), Wesselsbron Virus (WSLV), *Anopheles* Flavivirus (AnFV), *Anopheles gambiae* Flavivirus (AngFV), *Anopheles squamosus* Flavivirus (AnsFV), Stratford Virus (STRV), Karumba Virus (KRBV), Haslams Creek Virus (HaCV), Dairy Swamp Virus (DSwV), Mac Peak Virus (McPV), Long Pine Key virus (LPKV), Kampung Karu Virus (KPKV)

Members of the *Flavivirus* genus in the family *Flaviviridae* are generally thought to be transmitted by culicine mosquitoes. However, a number studies have detected flaviviruses in *Anopheles* species, suggesting that anophelines could also be involved in transmission [23]. Flaviviruses are characterized by an enveloped virion carrying a single-stranded RNA genome of positive polarity. The linear genome of 10 to 11 kb is flanked by 5' and 3' untranslated regions (UTR) that encode for a single open-reading frame. Translation produces a single polyprotein cleaved in 10 proteins: three structural (C, prM, E), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins [83].

The main reported vectors of West Nile virus (WNV) are *Culex* spp. Transmission is mainly zoonotic but with significant levels of human infection, including occasional mortality [84]. A pool of

An. pauliani wild unfed females was found to be positive for WNV RNA by RT-PCR in Madagascar [23]. A pool of *An. maculipennis* collected in Italy between 2008 and 2012 was also found positive for WNV RNA by RT-PCR [61]. A pool of *An. maculipennis* collected in Serbia after the 2012 WNV outbreak was also positive for WNV RNA by RT-PCR, which suggested a potential important role for this species in transmission [62].

Japanese encephalitis virus (JEV) is an endemic causative agent of encephalitis in Asia and India. JEV infects humans and other vertebrates such as horses, dogs, and reptiles. Based on envelope protein gene sequences, JEV strains can be classified into five different genotypes, I–V, with specific geographic distributions [85]. Due to the availability of a vaccine, outbreaks of JEV are rare. JEV was detected and isolated from a pool of *Anopheles peditaeniatus* females collected from 1985–1987 in India [45]. JEV was isolated from C6/36 cells inoculated with extract from pools of *Anopheles sinensis* captured by carbon dioxide traps or sweep nets in Taiwan from 2005 to 2012, for a calculated infection rate of 2.3 per 1000 mosquitoes [21]. JEV genotype I was isolated from host-seeking *An. sinensis* collected by aspirator in Japan [46]. *An. sinensis* is a dominant human malaria vector in Asia [2,86].

Wesselsbron virus (WSLV) shares ecological niches and similar livestock symptoms with Rift Valley Fever virus and misdiagnosis is common [34,87]. WSLV RNA was detected using molecular diagnostic tools in Kenya from *An. coustani* and in humans in Senegal [34,87]. *Aedes* mosquitoes are the presumed main vector of WSLV but the involvement of *Anopheles* mosquitoes remains to be elucidated.

Anopheles flavivirus (AnFV), the first flavivirus discovered in *Anopheles*, was identified in a population virome survey in Liberia and Senegal [32]. An RT-PCR diagnostic assay confirmed the presence of AnFV RNA in wild *Anopheles*, with a prevalence of 12%. *Anopheles gambiae* flavivirus (AngFV) and *An. squamosus* flavivirus (AnsFV) were detected in *An. gambiae* and *An. squamosus* respectively by a nucleic acid melting-curve analysis from mosquitoes collected in Kenya [34]. AngFVs and AnsFV share 77% of nucleotide identity.

Stratford virus (STRV) RNA was detected in an isolate of *Anopheles annulipes* collected in Australia from 1995–2013 [54]. Sequence analysis based on part of the NS5 gene sequence displayed 95–99% homology among the different isolates of STRV, and 2% divergence was detected between the isolates collected in 1995–2013 and the first isolates of 1961. The main vector of STRV is thought to be *Aedes* spp., without important contribution by *Culex* spp. [54].

A study of *Anopheles* samples collected in Australia identified multiple insect-specific viruses that do not infect vertebrate cells, and display fine species-specific host restriction for the *Anopheles* host in which they were identified [42]. The genome of Karumba virus (KRBV) was obtained from single and pooled mosquitoes collected in two different sites in Australia. In addition, the same authors also discovered Haslams Creek virus (HaCV), Dairy Swamp virus (DSwV), and Mac Peak virus (McPV) in pools of *An. annulipes*, *An. bancrofti*, and *An. farauti*, respectively [42]. HaCV, DSwV, and McPV failed to replicate in vitro on cell lines C6/36 *Aedes albopictus*, MOS55 *An. gambiae*, ISE6 tick, or S2 *Drosophila melanogaster*. No replication of KRBV was observed on the following vertebrate cells: BSR *Mesocricetus auratus*, Vero *Cercopithecus aethiops*, or DF-1 *Gallus gallus* [42]. The complete genome sequences were assembled by deep sequencing of mosquito RNA. Viral sequences were detected in the 21-nucleotide small RNA fraction, which suggested that double stranded RNA (dsRNA) intermediates produced during viral replication were cleaved by the siRNA pathway into viral RNAs (viRNAs). Presence of viRNAs is evidence of active virus replication. The prevalence of KRBV found in *Anopheles meraukensis* was 91.7% and 100% in Wyndham and Karumba, respectively. The prevalence of KRBV in wild type mosquitoes is quite high and highlights the need to study its impact on *Anopheles* species. Specific RT-PCR assays detected HaCV and McPV RNA in wild *An. annulipes*, *An. bancrofti*, and *An. farauti*.

Long Pine key virus (LPKV) was isolated in the United States from a pool of 50 *An. crucians*, and Kampung Karu virus (KPKV) was isolated from a single *An. tessellatus* in Malaysia [47]. Both viruses were cultured on C6/36 cells and cytopathic effects were observed at 7 d post-inoculation. Inoculation of BHK-21 and Vero cells with LPKV and KPKV produced neither replication nor cytopathic effects, and these viruses did not display pathology in mice [47]. Hemagglutination-inhibition tests for KPKV

were not possible due to absence of reactive hemagglutinin. Despite being insect-specific, LPKV and KPKV reacted serologically with antibodies directed against some dual-host flaviviruses such as WNV, JEV, and Dengue virus [47].

3.4.3. Phlebovirus: Rift Valley Fever Virus (RVFV)

Rift Valley fever virus (RVFV) belongs to the *Phlebovirus* genus in the family *Phenuiviridae*. RVFV is present throughout Africa and the Middle East and causes important economic losses in livestock [6,25,88]. During an epidemic in 2012, human cases and deaths occurred in Mauritania [6]. *Culex* and *Aedes* spp. are proven vectors of this virus, but *An. arabiensis*, *An. coustani*, *An. rufipes*, *An. pharoensis*, *An. rhodesiensis*, and *An. christyi* have also been implicated in transmission during epizootics and epidemics, as well as in maintenance by vertical transmission [25].

3.4.4. Peribunyavirus: Leanyer Virus (LEAV), Ngari Virus (NRIV), Bangui Virus (BGIV), Cache Valley Virus (CVV), Mapputta Virus (MAPV), Tahyna Virus (TAHV), Tataguine Virus (TATV), Batai Virus (BATV), Nyando Virus (NDV), Ilesha Virus (ILEV), Bwamba Virus (BWAV), *Anopheles A Virus* (ANAV), *Anopheles B Virus* (ANBV), Tensaw Virus (TENV)

At least 14 species of the genus *Orthobunyavirus* in the family *Peribunyaviridae* have been detected in *Anopheles* species. The orthobunyaviruses are characterized by a single-stranded RNA genome of negative polarity composed of three segments that are large (L), medium (M), and small (S) encoding the RNA dependent RNA polymerase, the glycoproteins (Gn and Gc) and the nucleoprotein, respectively [49].

Leanyer virus (LEAV) was isolated from *An. meraukensis* pools in a suburb of Darwin, Australia in 1974, and was cultivable on BHK-2I and Vero cells [48]. Peptide sequence analysis indicated that LEAV is related to Oropouche virus with 59% similarity in the polymerase sequence. LEAV does not cross-react serologically with other orthobunyaviruses and the L and S segment peptide sequences display divergence from other orthobunyaviruses, and therefore LEAV could be considered as a new antigenic complex [89].

Batai virus (BATV) was first isolated from *Culex gelidus* in Malaysia [90]. More recently, two different entomological surveys in Germany and Italy identified and isolated BATV from *Anopheles maculipennis* complex mosquitoes [20,37]. The Italian and German strains were related to strains isolated in Slovakia [37]. BATV causes hemorrhagic disease, with fever, headache, nausea, and vomiting. BATV was also identified in Sudan from sera of febrile patients [90]. Cattle are a potential host of BATV, and in a survey in Germany, the serological prevalence in cattle was 0.55%, while a 2.1% positivity rate was detected by RT-PCR in cattle from Mongolia [91,92]. The *An. maculipennis* complex is an important historical European vector of *P. vivax*, and a current vector in Europe and Asia [93].

Recombination events between BATV and Bunyamwera virus generated Ngari virus (NRIV), which has mainly been reported in Africa. The first isolation of NRIV was done from *Ae. simpsoni* in Senegal in 1979. It was also isolated from *An. gambiae* and *An. pharoensis*, and cytopathic effects were observed in Vero cells [36]. Pools of *An. funestus* collected in Kenya between 2007 and 2012 were positive for NRIV RNA by RT-PCR and sequencing. The full genome sequence of NRIV was obtained from mosquito and human samples [94].

Bangui virus (BGIV), a probable arbovirus of this family, was first isolated from humans in 1973 in the Central African Republic, where it was also detected in *An. pharoensis* [36]. BGIV produces cytopathic effects on Vero and amphibian *Xenopus* cells, and is sensitive to ether and acid pH [95]. Serological tests are used for the detection of BGIV, but molecular tools are lacking.

Cache Valley virus (CVV), in vector competence assays, was more infectious to *An. quadrimaculatus* than to *Coquillettidia perturbans*. At 16 d post-infection, infection prevalence was greater than 90% for both species [39]. Human and other vertebrate cases of CVV have been reported, including from a woman diagnosed with aseptic meningitis [96]. CVV can be cultured on many vertebrate cells such as

Buffalo green monkey kidney (BGMK), human colon adenocarcinoma (CaCo2), human lung carcinoma (A549), and Vero cells.

Mapputta virus (MAPV) was isolated in 1960 from *An. meraukensis* in Australia. MAPV antibodies react with other viruses of Mapputta group such as Maprik virus (MPKV), Trubanaman virus (TRUV), and Gan Gan virus (GGV), indicating that a serological test is not sufficient to distinguish them [49]. There is at least 60% nucleotide identity of S and L segments between MAPV and MPKV [49]. *An. meraukensis* bites humans and other vertebrates, but it has not been incriminated as a malaria vector [97,98]. Mapputta virus can be cultured on hamster kidney BHK-21 cells, which display cytopathic effects 4 d post-inoculation [49].

Tahyna virus (TAHV) was isolated from pools of *Anopheles hyrcanus* females collected in South Moravia [55], although it has been more often found in *Aedes* spp. *An. hyrcanus* extract was inoculated intracerebrally into mice and the virus was identified by neutralization tests on Vero E6 cells and confirmation by RT-PCR. The virus was generally fatal to inoculated mice. TAHV infection causes human fever, conjunctivitis, pharyngitis, malaise, arthralgia, headache, and drowsiness, and anti-TAHV IgM antibodies were detected by IFA in asymptomatic patients in China, but no human deaths have been attributed to this virus [55,99,100]. The *An. hyrcanus* group is a widespread species group involved in the transmission of *P. vivax* and *P. falciparum* in Europe and Asia [101,102].

Tataguine virus (TATV) takes its name from the village in Senegal where it was first isolated in 1962 from *Anopheles* and *Culex* species [56]. In 1966, it was also isolated in Cameroon from *An. gambiae* and from serum of a 14-year-old boy. The patient presented with fever, exanthema, asthenia, muscle aches, and neutropenia, and TATV was confirmed by inoculation in mice [56]. TATV was widespread in African countries surveyed from 1960 to 1970, including Nigeria, South Africa, and Ethiopia [103,104].

Nyando Virus (NDV) was isolated by inoculation of mice with extract of *An. funestus* collected in Kenya during the ONNV outbreak of 1959–1960. [18,50]. Cytopathic effects of NDV were observed on Vero E6 and RE05 cells but not on C6/36. NDV displays at least 90% nucleotide and peptide identity with Bwamba virus (BWAV) and Pongola virus (PGAV) [105]. NDV causes moderate to severe febrile disease in humans, and human exposure was detected in serological surveys in Kenya, Central African Republic, and Uganda [18,19].

Ilesha virus (ILEV) was isolated from a pool of *An. gambiae* collected in the Central African Republic [44]. ILEV was recovered in 1990 from the blood of a woman who died with fever, anemia, leucopenia, and coagulative disorders. The virus was isolated after inoculation of mosquito extract into suckling mice and was cultured on Vero E6 and AP61 cells [106].

Bwamba virus (BWAV) was isolated from extract of *An. funestus* in Uganda and from a human blood sample from a refugee camp in Tanzania [38]. BWAV appears to be a widespread human infection in Africa, with short duration symptoms including fever, headache, exanthema, arthralgia, body rash, and diarrhea [44,107].

Anopheles A virus (ANAV) and *Anopheles* B virus (ANBV) were first isolated from female *An. boliviensis* in Colombia. Mice injected with ANAV and ANBV displayed central nervous system pathologies [30], although these viruses have not been isolated from naturally-infected vertebrates. *An. boliviensis* is a minor malaria vector in Colombia [108]. ANAV and ANBV are distinct from other Bunyamwera viruses because the S segment encodes only the nucleocapsid protein N, and therefore the nonstructural protein (NSs) involved in replication and pathogenesis is absent [109–111].

Tensaw virus (TENV) from Tensaw River in the southeastern United States was isolated from *An. crucians* and *An. quadrimaculatus* [57]. Humans, dogs, raccoons and cows were positive for TENV by serological tests, indicating that TENV was transmitted from mosquitoes to vertebrates. *An. quadrimaculatus* and *An. albimanus* remained infective from 2–14 d post-infection [57].

3.4.5. Dicistrovirus: *Anopheles* C Virus (AnCV) and *Anopheles* Associated C Virus (AACV)

Two *Anopheles* dicistroviruses were identified by deep sequencing and de novo assembly. *Anopheles* associated C virus (AACV) [16] and *Anopheles* C virus (AnCV) [5] belong to the genus

Cripavirus in the *Dicistroviridae* family, non-enveloped viruses with a single stranded RNA genome of positive polarity. The dicistrovirus genome is comprised of two open reading frames (ORF). ORF1 encodes the non-structural proteins necessary for virus replication, and ORF2 the viral proteins VPO to VP4. In the C-terminal region of ORF1, there is an RNA dependent RNA polymerase (RdRp) domain that is highly conserved among dicistroviruses [112]. Dicistroviruses are only known to infect insects.

AnCV is distinct from but related to Drosophila C virus, and was discovered in wild-caught human host-seeking *Anopheles* in Senegal, and in laboratory colonies of *An. coluzzii* in France and *An. dirus* in Cambodia [5]. Both horizontal and vertical modes of transmission were demonstrated in the Ngousso colony of *An. coluzzii*, while a colony of *Anopheles stephensi* maintained in the same laboratory as the *An. coluzzii* colony was not infected by AnCV [5].

AACV was discovered in *Anopheles maculipennis* collected in the Camargue region of France. AACV displays approximately 20% peptide sequence divergence from chronic bee paralysis virus (CBPV) in examined regions of the genome [16].

3.4.6. Cypovirus: *Anopheles* Cypovirus (AnCPV)

Viruses in the genus *Cypovirus*, family *Reoviridae*, are characterized by a genome of 9 to 12 segments of linear dsRNA within a single capsid shell [113]. Cypoviruses, or cytoplasmic polyhedrosis viruses, are so named because the infectious forms are occlusion bodies in crystalline polyhedral form within the cytoplasm of infected cells. The protein polyhedra protect the virus against harsh environmental conditions such as high pH. Cypoviruses are only known to infect insects. The first *Anopheles* cypovirus was detected in adult *An. stephensi* by microscopy after staining with ammonium molybdate [41]. However, culture and transmission experiments did not succeed, the virus disappeared from the colony after nine months, and was not sequenced or named. This cypovirus was observed within the cytoplasm of *Plasmodium berghei* or *P. yoelii* rodent malaria oocysts in co-infected mosquitoes, and observations suggested it might have reduced the numbers of developing oocysts.

More recently, *Anopheles* cypovirus (AnCPV) was discovered in wild-caught human host-seeking *Anopheles* in Senegal and Cambodia, and was also present in the Ngousso laboratory colony of *An. coluzzii* [5]. AnCPV infection was absent in an *An. stephensi* colony maintained in the same laboratory.

3.4.7. Orbivirus: Tibet Orbivirus (TIBOV), *Anopheles annulipes* Orbivirus (AAOV) *Anopheles hinesorum* Orbivirus (AHOV), Orungo Virus (ORUV), Tilligerry Virus (TILV)

Viruses of the *Orbivirus* genus, in the *Reoviridae* family, infect plants and vertebrates, and are characterized by a dsRNA genome of 10–12 segments. Tibet orbivirus (TIBOV) was isolated from *Anopheles maculatus* collected in China [58]. TIBOV was cultured from mosquito extract inoculated on C6/36 and BHK-21 cells. Cytopathic effects characterized by cell rounding, lysis, and floating cells were observed only in the BHK-21 cells after 3 d of infection, but viral RNA was detected by RT-PCR in both cell types. Gel electrophoresis revealed ten dsRNA genome segments, which were sequenced [58]. *An. maculatus* species are important vectors of human malaria in Asia [114].

Orbivirus sequences detected in *Anopheles annulipes* and *An. hinesorum* from Australia were named *Anopheles annulipes* orbivirus (AAOV) and *Anopheles hinesorum* orbivirus (AHOV) [31]. The viruses could not be cultured on C6/36 cells, but virus sequences were present in the mosquito 21-nucleotide viral RNA fraction, indicative of active replication and dicing of dsRNA replication intermediates.

Tilligerry virus (TILV) was isolated in 1971 from *An. annulipes* in Australia, and the complete genome sequence was determined [59]. The G + C content of the full genome of TILV is 45% and its 10 segments are visible on agarose and acrylamide gels [59,115]. TILV leads to cytopathic effects in BHK and BSR cells 2–3 d post inoculation [59]. TILV cross-reacts with bluetongue virus in complement fixation tests [115].

Orungo Virus was isolated in Uganda from *An. funestus* after inoculation on Vero and BHK-21 cells [51]. Viral replication was detected in the brains of inoculated mice and hamsters. Antibodies against ORUV were detected in human sera from Nigeria. The human symptoms of ORUV are fever, headache, myalgia, nausea, and vomiting, and ORUV also infects other vertebrates such as sheep, monkeys and cows [116]. On the basis of nucleotide G + C content and amino acid composition of the T2 protein, ORUV appears closer to *Culicoides*-borne than mosquito-borne orbiviruses [51].

3.4.8. Mononegavirus: Bolahun Virus (BOAV) and Gambiae Virus (GAMV)

The order *Mononegavirales* are non-segmented and negative-sense single stranded RNA viruses encoding 5–10 ORFs. Sequences of two mononegaviruses were detected in *Anopheles* spp. in Liberia, Senegal, and Burkina Faso [32]. The two viruses, Bolahun virus (BOAV) and Gambiae virus (GAMV), have similar genome organization with six non-overlapping ORFs.

3.4.9. Almendravirus: Coot Bay Virus (CBV)

Coot Bay virus in the *Almendravirus* genus belongs to the *Rhabdoviridae* family in *Mononegavirales* order [40]. CBV was isolated from *An. quadrimaculatus* mosquitoes collected in 2013 in Florida, USA [40]. CBV could be cultured in *Aedes* C6/36 cells, but not in mammalian BHK-21 and Vero cells [40]. CBV does not cause apparent illness or deaths in suckling mice. The virion has a diameter of about 50 nm [40].

3.4.10. Totivirus: *Anopheles* Totivirus (AToV) and Australian *Anopheles* Totivirus (AATV)

A virus of the genus *Totivirus*, family *Totiviridae*, *Anopheles* totivirus (AToV) was discovered in *An. gambiae* from Liberia, with an infection prevalence of 1.3% [32]. Another totivirus, Australian *Anopheles* totivirus (AATV), was detected in *An. annulipes* and *An. hinesorum* collected in Australia [31]. Despite the lack of AATV replication in C6/36 cells, the presence of 21-nt viral RNA sequences was regarded as diagnostic of active virus replication in mosquitoes. AATV and AToV share ~25% identity at both nucleotide and peptide sequence levels. The genus *Totivirus* also includes protozoa-infecting members such as *Trichomonasvirus*, *Victorivirus*, *Giardiavirus*, and *Leishmanivirus* [117]. This genus also commonly infects plants. A maize-associated totivirus was identified in China [118].

4. Discussion

Little is known about the *Anopheles* virome, as evidenced by the relatively small number of scientific publications on the topic. Nevertheless, when the literature summarized here is taken together, it is evident that *Anopheles* viruses, including among them pathogens of humans and other vertebrates, are abundant in nature but understudied.

We found published evidence of at least 51 viruses associated with *Anopheles*. This number is likely an underestimate, because it does not include publications in journals not indexed by the databases searched. The *Anopheles* virome appears to be dominated by RNA viruses. RNA viruses are also dominant in *Aedes* (Yellow fever virus, Zika virus, dengue virus, chikungunya virus) and *Culex* (Eastern equine encephalitis virus, Rift Valley fever virus, West Nile virus). RNA viruses evolve rapidly because of high mutation and recombination rates [119], and can potentially adapt rapidly to new hosts. There could also be an ascertainment bias favoring the detection of RNA as compared to DNA viruses, because sequencing of small viral-derived RNAs is a powerful tool to identify actively replicating RNA viruses [5,16,17].

It is likely that the main evolutionary pressure shaping mosquito antiviral mechanisms in general is their persistent exposure in nature to members of the natural virome, rather than the probably less frequent exposure to vertebrate-pathogenic arboviruses. Despite the apparently abundant presence of viruses in *Anopheles*, there is debate as to whether *Anopheles* mosquitoes serve as merely occasional hosts of pathogenic arboviruses, or to what extent they help mediate transmission as vectors. The distinction between host and vector will require evaluation of *Anopheles* vector competence in the laboratory

and *Anopheles* vectorial capacity in the field. Vector competence is the ability to acquire, maintain, disseminate, and transmit a virus, whereas vectorial capacity or vector efficiency is the rate at which a putative vector population generates new inoculations from an infectious case.

The potential of the majority of *Anopheles*-associated viruses for transmission to humans or other vertebrates is currently unknown, because few studies of host range and transmission have been done. Some viruses may have a host range restricted to only *Anopheles* and other insects. For example, *Anopheles* cypovirus and *Anopheles* C virus were found to replicate and be maintained by vertical transmission in *An. coluzzii*, but were not able to infect *Ae. aegypti* in exposure experiments [5]. Both of these viruses were able to replicate in *An. stephensi* after exposure, but *Anopheles* C virus was not stably maintained and disappeared after several generations. Thus, these two viruses may be *Anopheles*-specific, but possibly not adapted to all *Anopheles* species.

A first group of viruses display either known or potential restriction of infection to *Anopheles* or insect cells. In some cases, detailed studies have demonstrated insect host restriction, while in other cases vertebrate cell infection or transmission potential has not yet been tested. This group includes *Anopheles gambiae* densovirus, Eilat virus, *Anopheles* flavivirus, *Anopheles gambiae* flavivirus, *Anopheles squamosus* flavivirus, Karumba virus, Haslams Creek virus, Dairy Swamp virus, Mac Peak virus, *Anopheles* C virus, *Anopheles* Associated C virus, *Anopheles* cypovirus, *Anopheles annulipes* orbivirus, *Anopheles hinesorum* orbivirus, Bolahun virus, Gambiae virus, Coot Bay virus, *Anopheles* totivirus, Australian *Anopheles* totivirus, Long Pine key virus, and Kampung Karu virus.

A different group of *Anopheles* viruses possesses likely vertebrate transmission potential, because studies have detected presence in vertebrates and/or replication in vertebrate cells. Further work will be required to confirm and characterize transmission between *Anopheles* species and vertebrates and evaluate their risk. This group of potential arboviruses includes *Anopheles minimus* virus, Leanyer virus, Tilligerry virus, Stratford virus, Ngari virus, Bangui virus, Mapputta virus, Nyando virus, Ilesha virus, Bwamba virus, Orungo virus, and *Anopheles* B virus.

Finally, known pathogenic arboviruses with evidence of presence in *Anopheles* include O'nyong nyong virus (ONNV), Venezuelan equine encephalitis virus, Western equine encephalitis virus, Sindbis virus, Semliki Forest virus, Rift Valley fever virus, West Nile virus, Japanese encephalitis virus, Wesselsbron virus, Tataguine virus, Batai virus, Cache Valley virus, Tahyna virus, and Tensaw virus. Myxoma virus can be mechanically transmitted by mosquitoes and fleas but does not replicate in them, and thus is not an arbovirus. Of these, transmission by *Anopheles* has been demonstrated for ONNV, but additional work is required to determine the vector competence and capacity of *Anopheles* for the other arboviruses.

The above grouping of viruses is likely to be porous and is expected to change with the addition of data from new studies. The interesting question remains, nevertheless, whether *Anopheles* are less efficient arbovirus vectors than *Aedes* and *Culex*, or are simply under-recognized as virus vectors. If the first case were true, that is, lower vector competence of *Anopheles* for arboviruses, then understanding the biological mechanisms leading to their general resistance to virus transmission would be important and could lead to novel tools to control arbovirus transmission by *Aedes* and *Culex* vectors. As the current systematic review indicates, *Anopheles* are not inherently resistant to virus replication. Although the natural virome data do not yet exist to make a numerical comparison with *Aedes* and *Culex*, there is no evidence that rates of natural carriage of viruses are substantially different between these mosquito genera.

Genetically encoded differences between mosquito species can interact with viral factors to influence host permissiveness and restriction. The protein nsP3 of ONNV influences host specificity of this virus to *Anopheles* as compared to *Aedes*, because substitution of chikungunya virus nsP3 by ONNV nsP3 in the chikungunya backbone allows chikungunya infection of *An. gambiae* [70]. In addition to interaction between viral factors and host cell proteins, differences in the small RNA regulatory pathways such as microRNAs and piwi-RNAs between *Culex*, *Aedes*, and *Anopheles* may also play a role in restricting host range [120].

The incidence of co-infection of malaria and arboviruses is probably underestimated in endemic areas. In a recent survey in Senegal, the frequency of human co-infection by *P. falciparum* malaria and arboviruses (dengue, yellow fever, Zika, chikungunya, Rift Valley fever) was close to 50% [121]. It is unknown whether members of the natural virome can influence malaria parasite or arbovirus infection and transmission by the vector, for example by stimulating or diverting mosquito basal immunity, or otherwise either promoting or diminishing superinfection.

One conclusion of this study is that the databases PubMed, Web of Science, Scopus, and Lissa can yield different results for the same specific area of research. The four bibliographic databases are complementary. PubMed and Lissa are free to access without registration, which is not the case for Scopus and Web of Science. Web of Science yielded the most results (58 articles), because it includes articles from many countries. However, PubMed contains the most recent articles. In contrast, the articles found in Lissa are in French and include older works, which can fill gaps not covered by other databases. The Arbocat Arbovirus Catalog indexes arboviruses or potential arboviruses harbored by arthropods, but for some of these viruses, the support is unpublished or no longer available.

Finally, it should be noted that a systematic automated search profile can only reveal reports that are indexed using informative combinations of terms. A limiting factor is thus the quality of index terms in the records. The search parameters presented here could perhaps be modified with additional systematic terms. However, search terms that might appear to be more precise may in fact generate less informative results and lead to diminishing returns. For example, searching of databases with [virus name + Anopheles], can generate large numbers of often low-quality results (e.g., [West Nile virus] + Anopheles generates 140 records). Such searches require increased levels of labor-intensive manual curation to identify on-target records. Systematic automated searches can be easily re-run. Moreover, the most important biological observation is made when a virus is first reliably reported in at least one *Anopheles* species. After that, finding the virus in other *Anopheles* is useful, but hardly surprising, while studies of the biology of host range restriction and mode of transmission of *Anopheles* viruses are sorely lacking.

5. Conclusions

This study is the first, to our knowledge, to present an overview of the published literature on the *Anopheles* virome. At least 51 viruses have been reported in *Anopheles* in almost all continents. The quantification and identification of the *Anopheles* virome is important for general understanding of microbiome diversity, for surveillance and prevention of emergence of unknown viruses, to understand the phenomenon of human malaria and arbovirus co-infection, and to study the antiviral and immune responses of *Anopheles* mosquitoes. The availability of next-generation sequencing and de novo assembly will likely continue to augment knowledge of *Anopheles* viruses, and more effort will be required to characterize their biology and public health risk.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4915/10/5/222/s1>, Figure S1: Prisma Flow Diagram of search results and record filtering. Table S1, Summary of reported *Anopheles* viruses with references.

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3.2 Article II (submitted): Interaction of RNA viruses of the natural virome with the African malaria vector, *Anopheles coluzzii*

HIGHLIGHTS:

Anopheles mosquitoes transmit human malaria parasites and the arbovirus O'nyong nyong (ONNV). This virus is highly pathogenic to humans, with 2 major outbreaks having occurred in Africa. *Anopheles* also harbor several viruses, the impacts of which on biology and immunity of *Anopheles* are unknown. Except in the case of ONNV, immune responses of *Anopheles* against viruses are understudied.

Here we studied the immune responses of *Anopheles coluzzii* against two of its natural viruses *Anopheles C* virus (AnCV) and *Anopheles cypovirus* (AnCPV) by using RNAi-mediated gene silencing to query the function of key factors of the main immune signaling pathways. We described the transmission routes of these viruses and the factors involved in their persistence in this malaria vector.

Our results showed that the persistence of AnCV and AnCPV is associated with their transstadial and trans-ovarial transmissions in *An. coluzzii*. We observed a heterologous interference between the two viruses in coinfections. The prevalence of AnCPV was impacted by the silencing of Cactus and STAT-A, while AnCV infection was not influenced by the main pathways (RNAi, IMD, Toll and JAK/STAT).

Our studies show for the first time the immune responses of *An. coluzzii* infected with two insect specific viruses. This work contributes to better understand the competence of *Anopheles* to viruses *in vivo*. Viral interference has mainly been studied in cell lines, which is a weakness for predicting the outcomes in natural conditions. Here, heterologous interference is detected between AnCV and AnCPV in *in vivo* conditions. We show for the first time that insect specific viruses can be used as a laboratory model to study the interactions between viruses and malaria vectors.

1 **Interaction of RNA viruses of the natural virome with the African malaria vector,**
2 ***Anopheles coluzzii***

3
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27 **ABSTRACT**

28

29 Mosquitoes are colonized by a little-studied natural virome. Like the bacterial microbiome, the
30 virome also probably influences the biology and immunity of mosquito vector populations, but
31 tractable experimental models are lacking. We recently discovered two novel viruses in the
32 virome of wild *Anopheles* and in colonies of the malaria vector *Anopheles coluzzii*: *Anopheles*
33 *C* virus and *Anopheles* cytovirus. Here, we describe biological interactions between these two
34 viruses and *An. coluzzii* mosquitoes. One or both viruses are present in all tested laboratory
35 colonies of *An. coluzzii* and *An. gambiae*. Viral abundance varies reproducibly during mosquito
36 development. DNA forms of these viruses were not detected, and thus viral persistence is likely
37 based on vertical transmission of RNA genomes. At least *Anopheles C* virus is vertically
38 transmitted by an intraembryonic route. Relative abundance of the two viruses is inversely
39 correlated in individual mosquitoes. One possible mechanism for this could be interactions with
40 host immunity, and functional genomic analysis indicated differential influence of at least the
41 Toll and JAK/STAT immune signaling pathways upon the viruses. The nonrandom
42 distributions and interactions with host immunity suggest that these and other members of the
43 natural virome may constitute a source of unrecognized heterogeneity in malaria vector
44 populations.

45 INTRODUCTION

46

47 *Anopheles* species are the main vectors of human malaria. In addition to *Plasmodium*,
48 *Anopheles* mosquitoes transmit filarial worms and arboviruses ^{1,2}. *Anopheles* mosquitoes also
49 harbor a diverse natural virome of RNA viruses ²⁻⁵. The *Anopheles* virome is composed mainly
50 of insect specific viruses (ISVs) that multiply only in insects, but also includes relatives of
51 arboviruses that can replicate in both insects and vertebrates. However, *Anopheles*-RNA virus
52 interactions have been relatively unexamined, and it is unknown why *Anopheles* are efficient
53 vectors of human malaria but do not transmit viruses as well as *Aedes* and *Culex* mosquitoes.

54

55 The only arbovirus known to be consistently transmitted by *Anopheles* mosquitoes is O'nyong
56 nyong virus (ONNV, genus *Alphavirus*, family *Togaviridae*). ONNV is mainly transmitted by
57 *An. gambiae* and *An. funestus*, two major African malaria vectors ⁶. Millions of people were
58 infected in known ONNV epidemics ⁶⁻⁸. *Anopheles* species may also contribute to transmission
59 of other arboviruses such as West Nile virus (WNV), Japanese encephalitis virus, and
60 Wesselsbron virus ^{2,5,9,10}.

61

62 *Anopheles* antiviral immunity to ONNV has been studied ¹¹⁻¹³, but the mechanisms of
63 competence and immunity of these mosquitoes to other RNA viruses are essentially unknown.
64 RNA interference (RNAi), Toll, Imd and JAK/STAT are the main antiviral immune signaling
65 pathways described in mosquitoes. The RNA interference (RNAi) pathway controls ONNV
66 replication in *An. coluzzii* during the disseminated systemic infection ^{11,12,14}, but RNAi has no
67 detectable protective function against the primary blood-induced infection ¹². Conversely, the
68 activity of the JAK/STAT and Imd pathways are required for protection against the primary
69 midgut infection by ONNV, but play no protective role against the disseminated infection ¹².

70 Thus, distinct antiviral mechanisms function against the primary midgut infection or the
71 disseminated systemic infection.

72

73 However, besides ONNV, no studies have been reported on the functional interactions between
74 *Anopheles* mosquitoes and other RNA viruses, to our knowledge. Studies of other RNA viruses
75 in *Anopheles* would indicate the specific or general nature of the antiviral mechanisms observed
76 for ONNV. Deep sequencing has recently facilitated discovery of replicating RNA viruses in
77 insects, by identifying viral RNA (viRNA) products of siRNA cleavage of the double-stranded
78 RNA intermediates of active viral replication ^{15,16}. By this approach, two viruses were recently
79 identified in *An. coluzzii*: *Anopheles* C virus (AnCV) and *Anopheles* cypovirus (AnCPV) ¹⁶.
80 Wild *Anopheles* of diverse species collected in Senegal and Cambodia were positive for
81 AnCPV, and AnCV was present in samples from Senegal. Therefore, both viruses belong to
82 the natural virome of *Anopheles*.

83

84 A recent survey found published evidence of at least 51 viruses naturally associated with
85 *Anopheles* ⁵. Because *Anopheles* are persistently exposed in nature to members of the natural
86 virome, it is likely that these ISVs have been the main evolutionary pressure shaping *Anopheles*
87 antiviral immunity, rather than the less frequent exposure to arboviruses such as ONNV. Thus,
88 here we characterize the biology and host in the interaction of *Anopheles* with two RNA viruses
89 from the natural virome, AnCPV and AnCV. Understanding the biology underlying the
90 apparent inefficiency of arbovirus transmission by *Anopheles* may reveal potential new tools to
91 control virus transmission efficient arbovirus vectors such as *Aedes* and *Culex*.

92 **RESULTS**

93

94 **Relative composition of the virome varies during mosquito development**

95 It was previously shown that *Anopheles C* virus (AnCV) and *Anopheles* cypovirus (AnCPV)
96 are present in the natural virome of wild *Anopheles*, and also in *Anopheles* laboratory colonies,
97 including all colonies of *An. coluzzii* tested to date¹⁶. To determine the abundance of the two
98 viruses during host development, we measured their prevalence in different life stages of the
99 *An. coluzzii* Ngousso strain, which is persistently infected by the two viruses (Figure 1,
100 Supplementary Table S1). Infection prevalence of AnCV decreases between pupae and newly
101 emerged adults ($p=2.789e-07$, chi-square=41.08, df=6, 3 biological replicates), suggesting an
102 impact of metamorphosis on AnCV replication. In contrast, infection prevalence did not change
103 between larvae and pupae, or between 1 week and 2-week-old adults for both viruses. The
104 infection prevalence of the two viruses displays a consistent reciprocal pattern in the aquatic
105 larval and pupal stages, with high prevalence of AnCV, while AnCPV infection prevalence is
106 low in larval stages and newly emerged adults, increasing in adults by 1 week after emergence.

107

108 **Absence of DNA forms for both AnCV and AnCPV**

109 DNA forms of non-retroviruses have been implicated in viral persistence in *Drosophila* and
110 *Aedes* species^{17,18}. We surveyed *An. coluzzii* to detect DNA forms of AnCV and AnCPV by
111 PCR using specific primers of each virus. The template was genomic DNA extracted from *An.*
112 *coluzzii* pools, or cDNA generated from RNA of mosquito pools. Despite the high infection
113 prevalence of these viruses, no signal was detected from DNA template (Supplementary Figure
114 S1). As a positive control, PCR amplification was detected in all cDNA pools, and the larger
115 size of the control ribosomal protein S7 (rpS7) product in the cDNA reactions confirmed the
116 RNA source of the template. To further strengthen this observation, both virus sequences were

117 used to query the *An. gambiae* PEST genome assembly using blastn, and no significant hits
118 were returned. Taken together these results indicate that persistence of AnCV and AnCPV in
119 *An. coluzzii* is probably not based on transmission of DNA forms of the virus genomes.

120

121 **Transovarial intraembryonic route of AnCV persistence**

122 It was previously observed that AnCV and AnCPV are vertically transmitted in *An. gambiae*
123 colonies¹⁶, but the transmission routes were not examined. Viruses can pass to progeny within
124 the eggs by a transovarial, intraembryonic route, or by egg surface contamination by a
125 transovum route, for example in ovarian fluid. To distinguish between these routes, *An. coluzzii*
126 eggs were treated with 0.025% sodium hypochlorite (NaOCl) to inactivate viruses on the egg
127 surface, rinsed, and eggs were placed individually in a well of 24-well plates for hatching to
128 prevent horizontal transmission between larvae. Controls were treated identically, but without
129 exposure to NaOCl. Larvae (L3/L4 stages) were tested using an RT-PCR assay. Larvae from
130 untreated eggs were infected with both viruses, and larvae from the NaOCl-treated group were
131 infected with detectable levels of at least AnCV (Figure 2a).

132

133 Liquid rinsed from the surface of *An. coluzzii* eggs containing oviposition fluid was tested using
134 the duplex Taqman real-time RT-PCR assay (Taqman RT-qPCR), to simultaneously measure
135 abundance of both viruses. The oviposition fluid washed from eggs was orally administered to
136 *An. stephensi* larvae by feeding in the water, to test for virus particles competent for horizontal
137 transmission. The oviposition fluid from *An. coluzzii* was positive for RNA of AnCV and
138 AnCPV, however the exposed *An. stephensi* larvae were not infected by either of the two
139 viruses (Figure 2b). As a positive control, we previously showed that exposure of *An. stephensi*
140 larvae by the same methodology to filtered extract of homogenized *An. coluzzii* Ngousso larvae
141 caused infection of 100% of exposed *An. stephensi* larvae¹⁶. Although we cannot rule out a

142 dose effect of virus load difference between oviposition fluid and larval extract, this result may
143 also mean that viruses in oviposition fluid on the egg surface are less infectious or non-
144 infectious virus particles. Taken together, these results suggest that vertical transmission is
145 unlikely to be maintained by simple environmental contamination of oviposition sites with
146 maternal oviposition fluid. These data indicate that an intraembryonic transovarial route is
147 involved in the vertical transmission of at least AnCV, and that consistent maintenance by an
148 egg surface transovum route does not seem likely for either virus.

149

150 **Abundances of AnCV and AnCPV are inversely correlated in mosquitoes**

151 Host interaction with the virome establishes the basal physiological and immune environment
152 encountered by transmissible pathogens, which are probably not frequent components of the
153 pre-existing virome. Thus, interactions among members of the virome might have implications
154 for susceptibility of the host to exogenous viruses. For example, infection with Nhumirim virus,
155 an insect-specific flavivirus found in the insect virome, reduced by 10,000-fold the viral load
156 of West Nile virus in *Ae. albopictus* cells, and may serve as a barrier to WNV transmission by
157 *Culex*¹⁹. Cases of homologous and heterologous viral interference were described with
158 mosquito cells²⁰. Knowledge of viral interactions is lacking in *Anopheles*.

159

160 *An. coluzzii* is persistently coinfecting with both AnCV and AnCPV, and we examined the
161 relationship between abundance of the two viruses in individual mosquitoes. Infection intensity
162 and prevalence of both viruses was measured simultaneously in RNA of individual mosquitoes
163 using a duplex Taqman RT-qPCR assay. The abundance of the two viruses in individual
164 mosquitoes is not independent, but rather is inversely correlated. Mosquitoes with high AnCV
165 infection were significantly protected against infection with AnCPV, and the reciprocal (Figure

166 3a; combined $p=2.17 \times 10^{-5}$, chi-square=26.81, $df=4$; replicates shown in Supplementary Figure
167 S2). Co-infection occurs only when both viruses are present at low viral loads.

168

169 Physical injury of *Anopheles* triggers expression of a suite of wound-response genes that render
170 mosquitoes more resistant to *Plasmodium falciparum*²¹. Therefore, we also measured virus
171 abundance distributions in mosquitoes that were wounded by injection of buffer with irrelevant
172 double-stranded RNA, similar to the above cited work. The wounded mosquitoes displayed the
173 same significant inverse correlation of virus abundance as non-injected mosquitoes (Figure 3b;
174 combined $p=3.044 \times 10^{-12}$, chi-square=76, $df=10$; replicates shown in Supplementary Figure S2).
175 The p-value is more significant from the five combined replicates of wounded mosquitoes
176 (Figure 3b) as compared to the two combined non-injected replicates (Figure 3a), however p-
177 values of the individual replicates are comparable for untreated and wounded mosquitoes,
178 indicating that wounding does not alter the conditions that influence distributions of the two
179 viruses. These results indicate that infection efficiency of each virus is not independent of the
180 other, but does not shed light on the mechanism. Potential mechanisms include interference by
181 virus manipulation of the cellular environment and/or host immunity to exclude the other virus,
182 or segregating host genetic differences that are favorable to one virus or the other. We next
183 examined the interaction of the two viruses with host immunity.

184

185 **The Toll signaling pathway influences AnCPV abundance in mosquitoes**

186 Maintenance of ISVs in a non-pathogenic or commensal state probably requires active policing
187 by basal host immunity, similar to the continual dialog between host immunity and the bacterial
188 microbiome²². AnCV and AnCPV are widespread members of the natural *Anopheles* virome,
189 and it is likely that the mosquito host deploys immunity to limit viral replication and potential

190 pathogenesis. Here, we used RNAi-mediated gene silencing assays to query the influence of
191 key immune signaling pathways on AnCV and AnCPV abundance.

192

193 The Toll pathway controls rodent malaria parasite infection in *Anopheles*^{23,24} and limits
194 replication in *Aedes* of dengue (DENV, genus Flavivirus, family Flaviviridae) and Semliki
195 forest viruses (SFV, genus Alphavirus, family Togaviridae)^{25,26}. In *Anopheles*, the Toll
196 pathway influence on viruses has only been studied for ONNV¹². ONNV strongly inhibits
197 activation of Toll in *Anopheles*, possibly as an adaptive mechanism because when ectopically
198 activated, Toll significantly inhibits ONNV replication. DENV and SFV are also able to inhibit
199 signaling of Toll in *Aedes*^{27,28}.

200

201 We examined the role of the Toll pathway in the control of AnCV and AnCPV in *An. coluzzii*
202 by silencing Cactus, a negative regulator of the Toll pathway, to activate Toll signaling.
203 Mosquitoes were injected with double-stranded RNA targeting Cactus transcript (dsCactus),
204 and virus abundance was measured by the duplex Taqman RT-qPCR assay. Aggregate analysis
205 of all mosquitoes after silencing Cactus displayed a non-significant tendency of lower AnCV
206 infection intensity (Supplementary Figure S3) and inconsistent results for AnCPV infection
207 intensity (Supplementary Figure S3).

208

209 However, considering the inverse correlation of abundance between the two viruses (Figure 3),
210 we then analyzed the effect of Cactus silencing by infection level category rather than in all
211 categories confounded. In the group with low or no AnCV load (equivalent to NI and Low
212 infection categories in Figure 3), analysis revealed that Toll activation by dsCactus treatment
213 significantly reduced AnCPV infection prevalence as compared with the dsGFP controls
214 (Figure 4; combined $p=0.001315$, $\chi^2=21.8$, $df=6$). These results support the conclusion

215 that AnCPV is sensitive to antiviral activity mediated by the Toll pathway, while AnCV is
216 insensitive to the effects of the Toll pathway. The simplest hypothesis is that AnCV inhibits
217 Toll pathway activation, similar to ONNV in *Anopheles*¹². AnCV would thereby protect itself
218 from suppression by Toll-dependent immunity, but would also protect the AnCPV present in
219 the high AnCV infection background. High AnCV infection also exhibits interference against
220 AnCPV, which suggests the complexity of the trade-offs that structure the ecology of the
221 virome.

222

223 **JAK/STAT signaling activity promotes AnCPV infection in *An. coluzzii***

224 The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is
225 implicated in antiviral protection in *Anopheles* against ONNV¹², and in *Aedes* against DENV
226 and SFV^{25,26}, as well as in mice and *Drosophila*^{29,30}. We silenced STAT-A, a positive regulator
227 of the JAK/STAT pathway, in *An. coluzzii* mosquitoes to inhibit activation of the pathway
228 (silencing validation shown in Supplementary Figure S4), and measured virus infection by the
229 duplex Taqman RT-qPCR assay. Inhibition of the JAK/STAT pathway by silencing of STAT-
230 A decreased AnCPV infection prevalence of mosquitoes (Figure 5, combined $p=0.02$, chi-
231 square=11.4, $df=4$, for 2 replicates). This indicates that the JAK/STAT immune pathway plays
232 a significant role in promoting infection by this cypovirus member of the virome. Interestingly,
233 unlike for the Toll pathway, the effect of STAT-A silencing was detected by the aggregate
234 analysis of all mosquitoes after silencing STAT-A, and did not require analysis of only the low-
235 AnCV fraction of mosquitoes for power to detect an effect. This suggests that, unlike for Toll,
236 AnCV does not appear to influence activation of the JAK/STAT pathway.

237

238 **RNA interference, Imd, and Pastrel do not significantly influence AnCV and AnCPV**
239 **levels in *An. coluzzii***

240 The RNA interference (RNAi) pathway is a major antiviral mechanism in *Drosophila* and
241 mosquitoes³¹⁻³³, including in *Anopheles* against ONNV^{11,12,14}. Argonaute-2 (Ago2) is a key
242 factor of the RNAi pathway, and an antagonist of ONNV in *Anopheles*. To assess the influence
243 of the RNAi pathway for the control of AnCV and AnCPV in *An. coluzzii*, Ago2 was depleted
244 by silencing, and virus levels were measured by the duplex Taqman RT-qPCR assay. There
245 was no consistent effect of inhibition of RNAi activity by Ago2 silencing upon the abundance
246 of AnCV and AnCPV, and none of the replicates was individually significant (Supplementary
247 Figure S5).

248
249 The immune deficiency (Imd) pathway plays an antiviral role against ONNV during the primary
250 midgut infection of *An. coluzzii*¹², but has no effect during the secondary disseminated systemic
251 infection¹⁴. There are two isoforms of the Imd positive activator, Rel2: the longer Rel2-F and
252 shorter Rel2-S. Silencing of both isoforms by targeting a shared region, and specifically
253 silencing only Rel2-F, both caused increased ONNV infection in the midgut, and thus Imd-
254 mediated protection against ONNV in the midgut requires at least the long Rel2-F isoform.
255 Here, we targeted both isoforms by injecting double-stranded Rel2 (dsRel2) directed against
256 the common region. Depletion of Rel2 led to a consistent but non-significant tendency in three
257 replicates towards lower infection prevalence for AnCPV (Supplementary Figure S6). Because
258 Imd influences the bacterial microbiota³⁴, as for JAK/STAT also, this result could suggest a
259 weak effect of the microbiome on AnCPV levels.

260
261 Pastrel (pst) has been reported in *Drosophila* as an antiviral factor against picorna-like viruses
262 and some dicistroviruses, such as *Drosophila* C-virus, a dicistrovirus related to AnCV³⁵⁻³⁷.

263 There is currently no pst ortholog annotated in the *An. gambiae* PEST genome assembly. By
264 searching for reciprocal best hits, we identified *An. gambiae* AGAP011771 as a candidate for
265 *Anopheles* pst. We designed a double-stranded RNA construct to silence the gene, and
266 measured viral loads by the duplex Taqman RT-qPCR assay. A non-significant increase of
267 AnCPV-infected mosquitoes was observed after depletion of pst candidate AGAP011771 in
268 four biological replicates (Supplementary Figure S7).

269 **DISCUSSION**

270

271 The *Anopheles* virome comprises multiple viruses, among them ISVs⁵. These ISVs are
272 maintained in mosquitoes by vertical transmission but their biology has not been examined.
273 The goal of this study was to characterize the biological interaction of both *Anopheles* C virus
274 (AnCV) and *Anopheles* cypovirus (AnCPV) with *An. coluzzii*, the mosquito where they were
275 discovered by deep sequencing of RNA and de novo assembly¹⁶. They have also both been
276 identified in wild-caught *Anopheles*.

277

278 We studied interaction and immune response to AnCV and AnCPV in co-infected *An. coluzzii*.
279 In order to quantify the two viruses simultaneously, we developed a duplex Taqman real-time
280 RT-PCR assay that is specific, sensitive, and rapid. We detected a consistent pattern of virus
281 abundance corresponding to stages of host development. We showed that AnCV uses a
282 transovarial, intraembryonic route of transmission. The mechanism of AnCPV persistence is
283 less clear because we did not detect transovarial transmission by AnCPV, despite the previous
284 demonstration that infected larval extract is infectious when administered in larval water to
285 uninfected mosquitoes. The discrepancy could be explained if the AnCPV viral particle has low
286 environmental stability. Transovarial transmission was demonstrated for DENV and Zika virus
287 in *Aedes* mosquitoes^{38,39}, but data are lacking on transmission modes of ONNV in *Anopheles*.

288

289 We identified a significant dependence of the abundance distributions of the two viruses in
290 individual mosquitoes. The inversely correlated abundance profiles could be a consequence of
291 cellular interference, manipulation of host immunity, or segregating genetic variation in the
292 host. In order to survey potential host immune effects, we queried the most important immune
293 signaling pathways for influence on the two virome members. We identified a role of the Toll

294 pathway in limiting AnCPV abundance. The effect was only detectable in the background of
295 low infection with AnCV, which could be explained if AnCV inhibits Toll pathway activation.

296

297 We also identified a role of the JAK/STAT pathway in promoting infection of *An. coluzzii* by
298 AnCPV. Silencing of the JAK/STAT positive regulator STAT-A to inhibit pathway activity
299 reduces AnCPV infection prevalence. JAK/STAT is involved in maintaining homeostasis of
300 the bacterial microbiome, and this result might suggest an implication of the bacterial
301 microbiome in regulating virome composition. In contrast to the positive effect of JAK/STAT
302 activity on AnCPV levels, the pathway is antagonistic to ONNV during the primary midgut
303 infection in *An. coluzzii*¹², and has no effect on the disseminated systemic ONNV infection
304 after intrathoracic injection¹⁴. In *Drosophila*, the loss of *hop*, which regulates the single STAT
305 factor, STAT92E increases the viral loads of *Drosophila C* virus, and the mutant flies die faster
306²⁹. In contrast, depletion of STAT-A in *An. gambiae* reduces the number of *Plasmodium* oocysts
307 in the midgut but increases their survival⁴⁰. These results from different pathogens indicate a
308 diversity of effects of the JAK/STAT pathway in *Anopheles* depending on pathogen type and
309 other factors.

310

311 Finally, we identified and functionally assayed a candidate for *Anopheles* pastrel. Mosquitoes
312 depleted of transcript for this candidate displayed a non-significant tendency to increased
313 infection by AnCPV. In order to further characterize the pastrel candidate, as well as a weak
314 potential effect of the Imd pathway, it may be necessary to eliminate or control for the effects
315 of the coinfection of AnCV and AnCPV, which are clearly not independent.

316

317 Work still remaining includes examining the influence of the virome upon pathogen
318 susceptibility and transmission by these vectors of malaria and ONNV. Our initial attempts to

319 test the influence of the two viruses upon *P. falciparum* infection were inconclusive, because
320 the persistent infection of *An. coluzzii* colonies with both viruses demands large sample sizes
321 for statistical power of correlation. *Anopheles* line free of AnCV and/or AnCPV would permit
322 controlled reinfection studies to develop a detailed understanding of the physiological and
323 immune effects of these two natural *Anopheles* specific viruses. There are interesting examples
324 from other mosquitoes of virome interaction with transmissible pathogens. The insect specific
325 flavivirus of *Aedes*, Cell fusing agent virus, enhances DENV replication and the reciprocal in
326 *Aedes* cells ⁴¹. Palm Creek virus, another insect specific flavivirus, causes reduced replication
327 of the West Nile virus and Murray Valley encephalitis arboviruses in *Aedes* cells ⁴². However,
328 these studies were done in cultured cells, and studies on interactions between ISVs and
329 transmissible pathogens such as arboviruses and *Plasmodium in vivo* in mosquitoes, closer to
330 the natural conditions, remain to be done.

331 **METHODS**

332 **Mosquitoes**

333 The *Anopheles coluzzii* Ngousso strain was initiated in Cameroon in 2006⁴³ and was obtained
334 by the Institut Pasteur CEPIA facility in 2008. The *Anopheles stephensi* strain SDA500 was
335 initiated in Pakistan in 1982⁴⁴ and was obtained by the Institut Pasteur CEPIA facility between
336 2000-2004. Larvae were grown in distilled water supplemented with 0.01% of mineral salt, and
337 fed on Friskies cat food. Adults were reared at 28°C ±1, at 80% ±5 humidity on a 12 h light–
338 dark cycle. Adults were fed on sterile filtered and autoclaved 10% sucrose solution and females
339 were blood-fed on anaesthetized rabbits for colony maintenance.

340

341 **Molecular detection and quantification of virus RNA**

342 Template RNA was extracted from individual or pooled mosquitoes using Direct-zol RNA
343 MiniPrep reagents with DNase I treatment (Zymo Research). cDNA was synthesized from the
344 RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) and random
345 hexamer primers (Roche).

346

347 Virus detection of AnCV and AnCPV by simple RT-PCR followed by agarose gel analysis was
348 carried out as described¹⁶. A duplex Taqman real-time RT-PCR assay (Taqman RT-qPCR) was
349 developed (Supplementary Table S2) to detect the two viruses simultaneously. Taqman primers
350 and probes were designed using Primer Express Software v2.0 (Applied Biosystems). Probe
351 and primer sequences are listed in Supplementary Table S3. All have a minor groove binder/
352 non-fluorescent quencher (MGB-NFQ) at the 3' end and the 6-carboxyfluorescein (6-FAM),
353 VIC and NED fluorophores at the 5' end for AnCV, AnCPV and The *An. coluzzii* rpS7 gene
354 respectively. Taqman RT-qPCR was performed with Taqman Universal Master Mix II, with
355 UNG on a QuantStudio 12K Flex instrument (Applied Biosystems). Primer concentration was

356 0.9 μ M and probe concentration 0.25 μ M. The cycling protocol was a hold step 50°C for 2 min
357 and 95°C for 10 min, followed by the PCR stage of 40 cycles: 95°C for 15 secs and 60°C for 1
358 min. Negative controls were run with each test. A pool of 10 mosquitoes harboring both viruses
359 was used to make the standard curve. Ten-fold serial dilutions were made between the five
360 dilutions (dil1 to dil5) of the standard curve to test efficiency, specificity and sensitivity. All
361 reactions were performed in triplicate.

362

363 No amplification was observed in negative controls with no template. Sensitivity was approved
364 with amplification in the five dilutions of the standard sample (3000 ng for dil1 to 0.03ng for
365 dil5), and Ct values were inversely proportional to the expected amount of target nucleic acid
366 in each dilution. Efficiency was over 90% in all assays. The *An. coluzzii* rpS7 gene was
367 amplified in the same plate as the endogenous control gene for relative quantification.

368

369 **Egg treatment to determine virus transmission route**

370 Eggs were treated with sodium hypochlorite (NaOCl) to eliminate viruses on the egg surface.
371 In a previous study, eggs of the beet armyworm (*Spodoptera exigua*) were decontaminated of
372 nucleopolyhedroviruses (genus *Alphabaculovirus*, family *Baculoviridae*) by treatment with
373 0.25ppm (0.000025%) NaOCl⁴⁵. We determined that *Anopheles* eggs were more resistant to
374 NaOCl, possibly because of the impermeable egg chorion, and that eggs treated with 0.025%
375 were still viable. Consequently, 0.025% NaOCl was used in the current study.

376

377 A batch of freshly laid *An. coluzzii* Ngousso strain eggs was divided into 3 batches. The
378 experimental batch was treated with 0.025% NaOCl for 10 min, rinsed with distilled water, and
379 placed as individual eggs in a well of a 24-well plastic culture plate for hatching and growth
380 until L3/L4 larval stages. The control batch was mock-treated by only rinsing with distilled

381 water (with no NaOCl treatment) and placed as individual eggs in a well of a 24-well plastic
382 culture plate for hatching and growth until L3/L4 larval stages. L3/L4 larval stages from each
383 batch were collected and tested using an RT-PCR assay for presence and abundance of AnCV
384 and AnCPV virus genomes.

385

386 An additional egg batch was washed with 300 μ l of distilled water, which was then collected
387 and filtered with the 5 μ m and 0.2 μ m filters to remove bacteria. The filtered solution containing
388 oviposition fluid was tested using the duplex Taqman RT-qPCR assay for presence and
389 abundance of AnCV and AnCPV virus genomes. The liquid rinsed from the surface of eggs
390 containing oviposition fluid was fed in the larval water to L2 larvae of *Anopheles stephensi*.

391

392 **Gene silencing assays**

393 Gene-specific fragments of Ago2, Cactus, the pastrel candidate AGAP011771, Rel2, STAT-A,
394 and GFP control were generated by PCR using primers tagged at their 5' end with T7 promoter
395 sequences. All T7 primer sequences are listed in Supplementary Table S3. The PCR products
396 were used as template for in vitro dsRNAs synthesis using the MEGAscript RNAi Kit
397 (Ambion). The targeted gene was silenced by injecting 500 ng of dsRNA into the thorax of ice-
398 anesthetized 1-2 days old post-emergence of *An. coluzzii* females using a nanoinjector
399 (Nanoject II; Drummond Scientific) and glass capillary needle as previously described⁴⁶. Four
400 days after the dsRNA treatment, silencing of the target gene was verified using total RNA from
401 a pool of five mosquitoes by SYBR Green RT-qPCR. RNA was extracted using Direct-zol
402 RNA MiniPrep reagents with DNase I treatment (Zymo Research). cDNA synthesis was done
403 using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen) and random
404 hexamer primer (Roche). The RT-qPCR primers used for gene expression are given in the
405 Supplementary Table S3.

406

407 Total RNA extracted from individual mosquitoes 4 days post-dsRNA injection was used to
408 assess virus infection intensity and prevalence by duplex Taqman real-time RT-PCR assay
409 using relative quantification. The rpS7 gene was used as a housekeeping calibrator for
410 normalization of nucleic acid quantity. Analysis of the expression of transcript relative to rpS7
411 was performed according to the $2^{-\Delta\Delta C_t}$ method ⁴⁷. Intensity is the log10 of relative expression
412 of virus to rpS7.

413

414 **Statistical analysis**

415 All statistical details are presented in Supplementary Table S4. For comparisons of virus
416 infection prevalence of different mosquito developmental stages, the chi-square test was
417 applied, and p-values with a null distribution were estimated by the Monte-Carlo method with
418 10,000 permutations. Multiple testing correction was done by the Bonferroni method. Box and
419 bar plots were made using the beeswarm package in R ⁴⁸. For viral load (infection intensity),
420 statistical significance was determined using the Wilcoxon–Mann–Whitney rank-sum test, and
421 p-values were assessed with a null distribution of the statistical test approximated using Monte-
422 Carlo resampling with 1,000,000 permutations. The p-values from independent tests of
423 significance were combined using the meta-analytical approach of Fisher ⁴⁹.

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558

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566 decision to publish, or preparation of the manuscript.

567

568 **Author contributions statement**

569 Conceived and designed the experiments: FNM, KDV, EB (Belda)

570 Performed the experiments: FNM, CM, IC

571 Analysed the data: FNM, CM, EB (Bischoff), KDV

572 Wrote the manuscript: FNM, CM, EB (Bischoff), KDV

573 All authors read and approved the final manuscript.

574

575 **Data availability**

576 All data are available in the article.

577

578 **Competing interests statement**

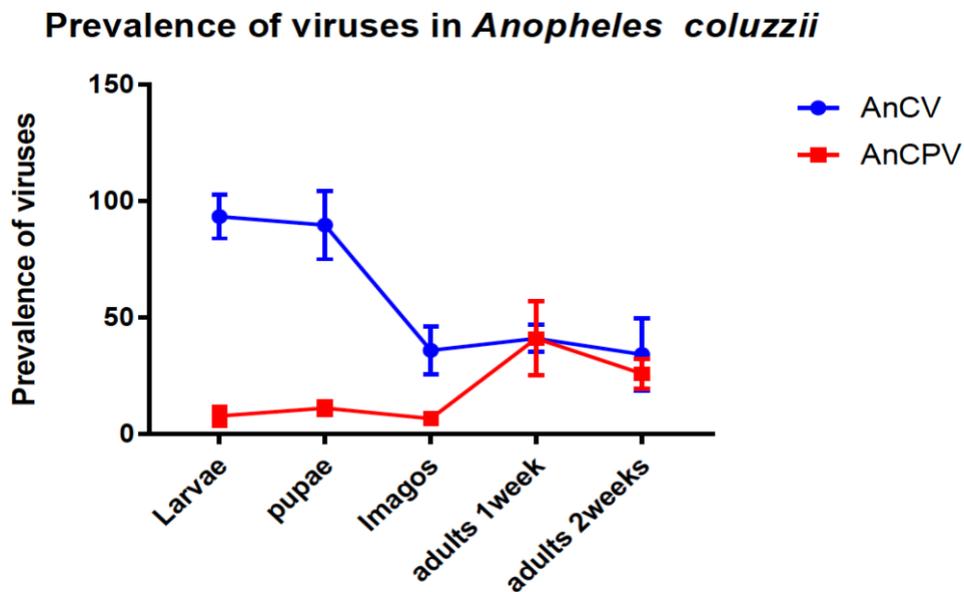
579 The authors declare no competing interests.

580 **FIGURE LEGENDS**

581

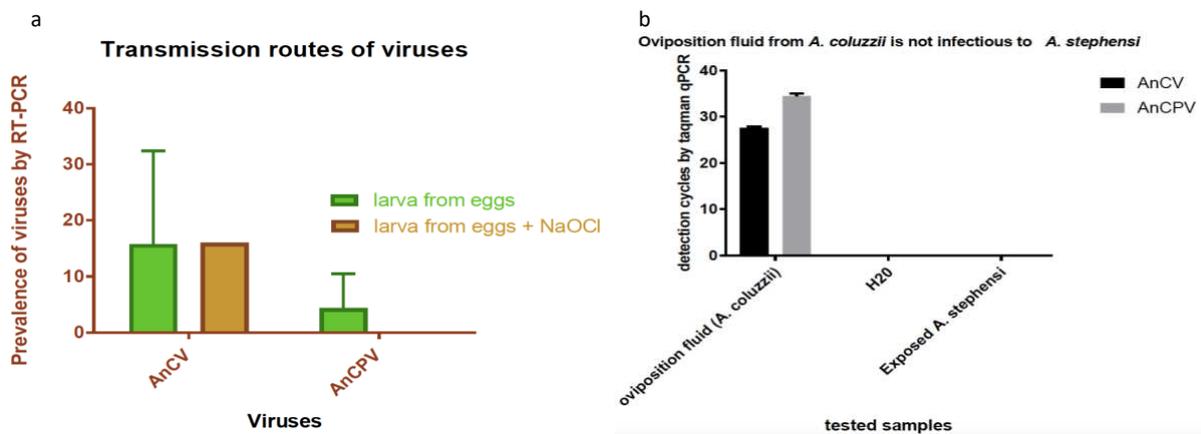
582 **Figure 1. Infection prevalence of viruses varies consistently during development of**
583 *Anopheles coluzzii*. Each line represents average infection prevalence for *Anopheles C* virus
584 (AnCV, blue line) and *Anopheles cypovirus* (AnCPV, red line). Error bars symbolize standard
585 deviation from 3 biological replicates from each *Anopheles* stage: larvae (n= 90), pupae (n=88),
586 Imagos (n=89), adults 1 week (n=90), adults 2 weeks (n=88). Detailed statistics in
587 Supplementary Table S1.

588



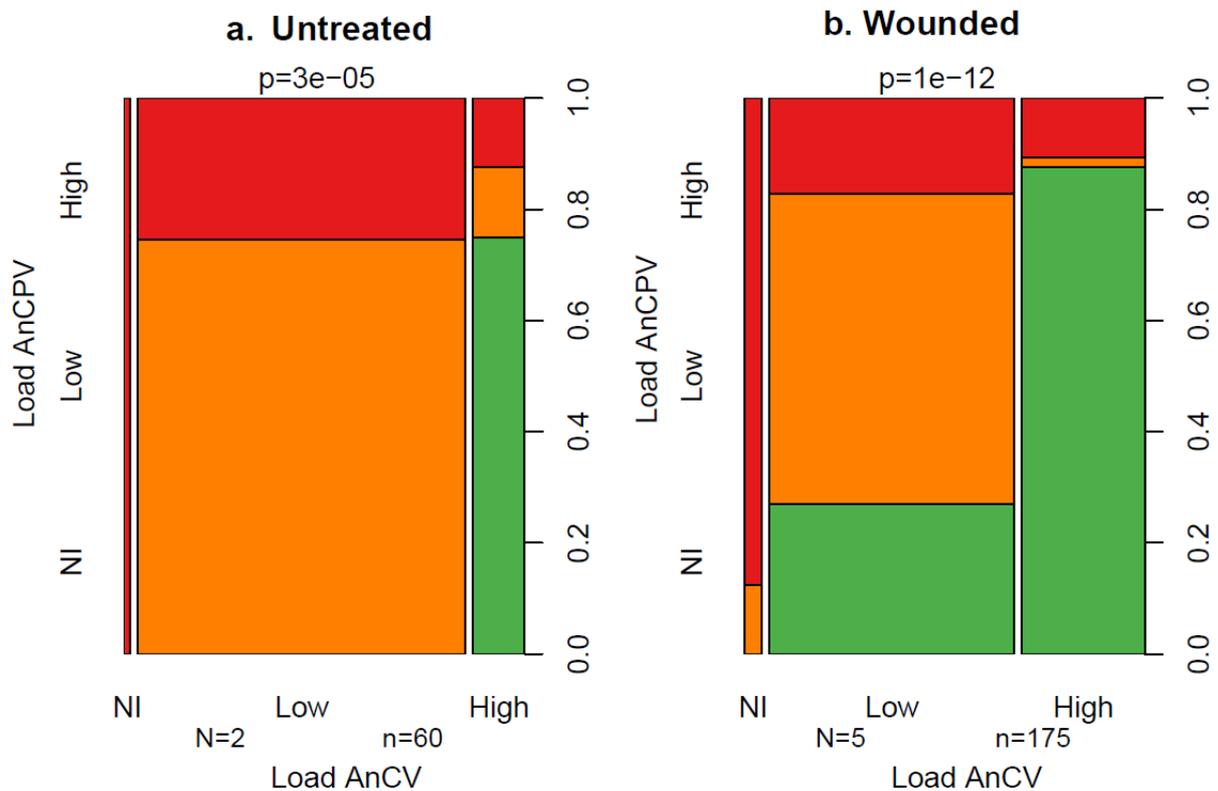
589

590 **Figure 2. Vertical transmission of Anopheles C virus is intraembryonic. A.** Evaluation of
 591 transovarial and transovum routes of AnCV and AnCPV vertical transmission using an RT-
 592 PCR to detect both viral genomes in *An. coluzzii* larvae hatched in individual wells. Control
 593 larvae (green bars) were hatched from eggs mock-treated without NaOCl, and carry both
 594 viruses. Larvae grown from eggs treated with NaOCl (orange bars) still carry AnCV, while
 595 AnCPV was not detectable. **B.** Both viruses were present in oviposition fluid washed from the
 596 surface of *An. coluzzii* eggs (oviposition fluid) and tested using a duplex Taqman RT-qPCR
 597 assay. Viruses were absent from the pre-rinse water (H₂O). The virus-positive oviposition fluid
 598 was fed to uninfected *An. stephensi* in larval water, and the exposed larvae did not become
 599 infected (exposed *A. stephensi*).
 600



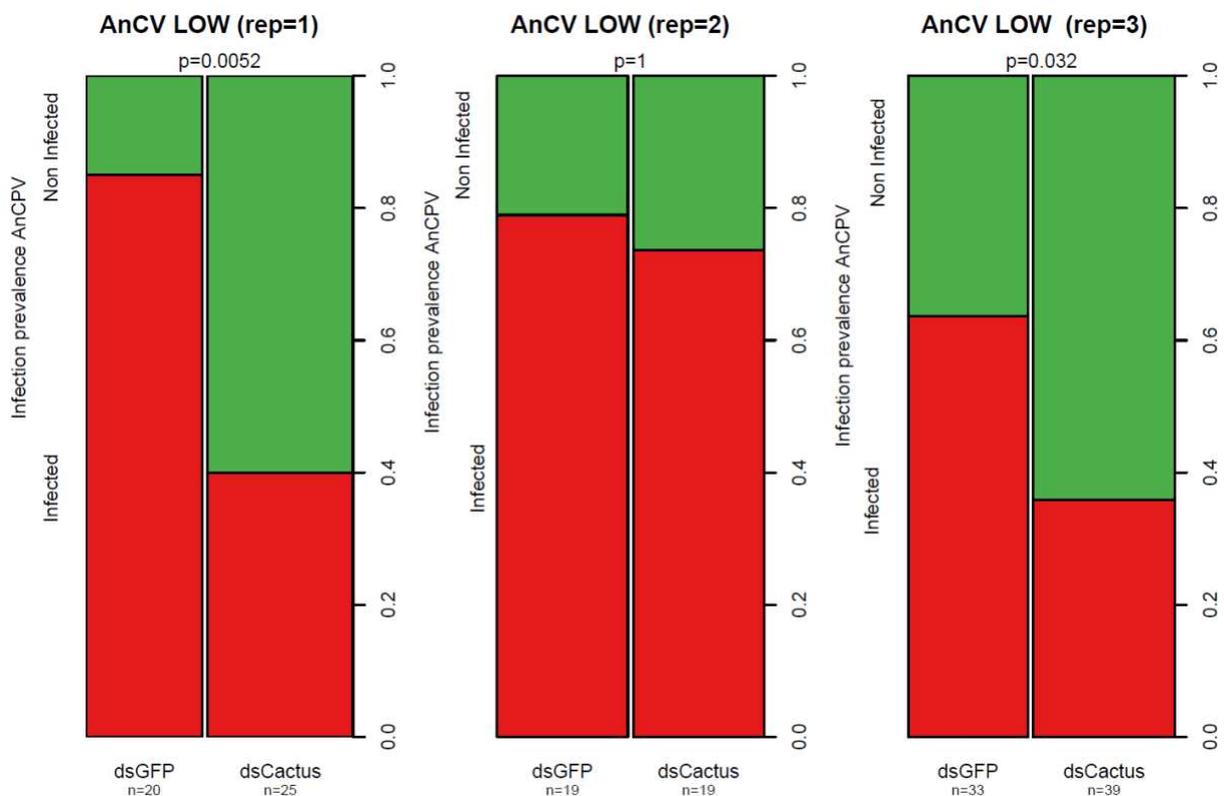
601

602 **Figure 3. Inverse correlation between AnCV and AnCPV infection levels in *An. coluzzii*.**
603 Graphs indicate abundance of each virus in individual mosquitoes. Mosquito viral loads were
604 categorized as non-infected (NI, green), Low infected (orange), and High infected (red) for each
605 virus. Low and high infection levels were defined as virus signal (relative to rpS7) smaller or
606 greater than $\log_{10}=0$, respectively. Infection category of AnCV is indicated by the three
607 histogram bars on the x-axis, and AnCPV by the three bars on the left y-axis. **A.** Virus infection
608 levels measured in untreated mosquitoes. **B.** Virus infection levels measured in mosquitoes
609 wounded by injection. (N) indicates the number of biological replicates and (n) the total number
610 of mosquitoes for all replicates. Statistical differences were first tested independently within
611 replicates by the Wilcoxon-Mann-Whitney test using 100,000 permutations to assess the p-
612 value. Individual replicates displayed a consistent direction of change (Supplementary Figure
613 S2), and consequently individual p-values were combined using the method of Fisher to
614 generate a combined p-value, shown above each plot. Detailed statistics in Supplementary
615 Table S4.



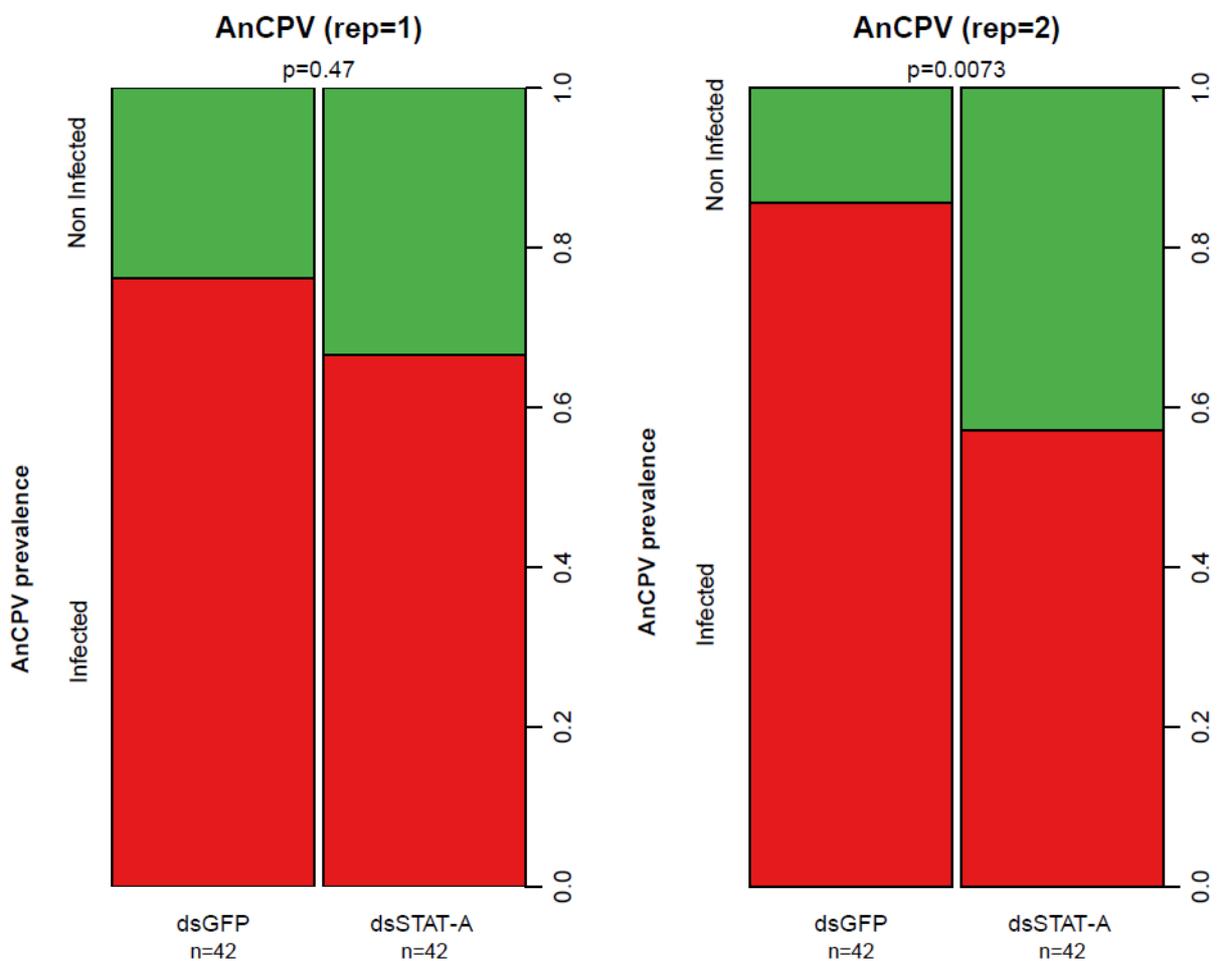
616

617 **Figure 4. Toll pathway activity limits levels of AnCPV in *An. coluzzii*.** Silencing the Toll
618 negative regulator Cactus activates Toll signaling, and decreases the prevalence of AnCPV
619 when analyzed in the AnCV low-infected group of mosquitoes. dsCactus indicates mosquitoes
620 injected with double-stranded RNA directed against Cactus transcript, and dsGFP is the control
621 group injected with irrelevant dsRNA. Silencing efficiency was verified by RT-PCR. X-axis,
622 dsRNA treatment, y-axis, AnCPV infection prevalence. Low and high infection levels were
623 defined as virus signal (relative to rpS7) smaller or greater than $\log_{10}=0$, respectively. (n)
624 indicates the total number of mosquitoes for each of 3 biological replicates. Statistical
625 differences were first tested independently within replicates by the Wilcoxon-Mann-Whitney
626 test using 100,000 permutations to assess the p-value (given above each plot). Individual
627 replicates displayed a consistent direction of change, and consequently individual p-values were
628 combined using the method of Fisher to generate a combined p-value (combined $p=0.001315$,
629 $\chi^2=21.8$, $df=6$). Detailed statistics in Supplementary Table S4.



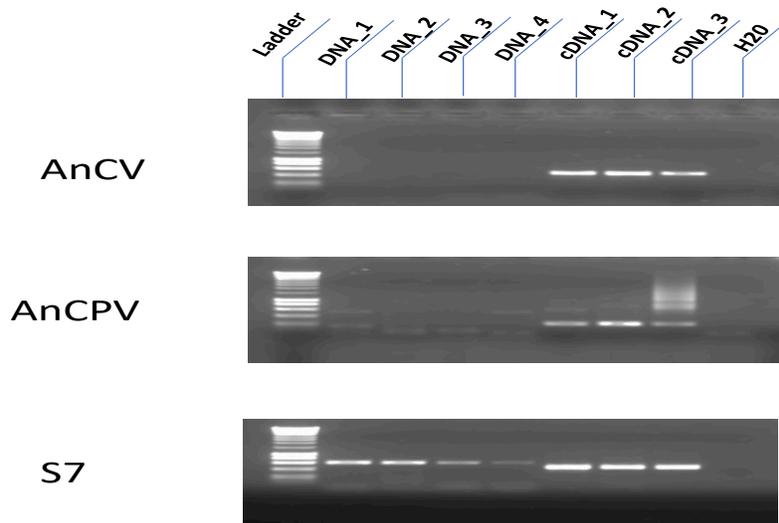
630

631 **Figure 5. JAK/STAT pathway activity promotes AnCPV infection in *An. coluzzii*.**
 632 DsSTAT-A indicates mosquitoes injected with double-stranded RNA directed against STAT-
 633 A transcript, and dsGFP is the control group injected with irrelevant dsRNA. X-axis, dsRNA
 634 treatment, y- axis, AnCPV infection prevalence. (n) indicates the total number of mosquitoes
 635 for each of the 2 replicates. Statistical differences were first tested independently within
 636 replicates by the Wilcoxon-Mann-Whitney test using 100,000 permutations to assess the p-
 637 value (given above each plot). Individual replicates displayed a consistent direction of change,
 638 and consequently individual p-values were combined using the method of Fisher to generate a
 639 combined p-value (combined p=0.02, chi-square=11.4, df=4). Detailed statistics in
 640 Supplementary Table S4.
 641

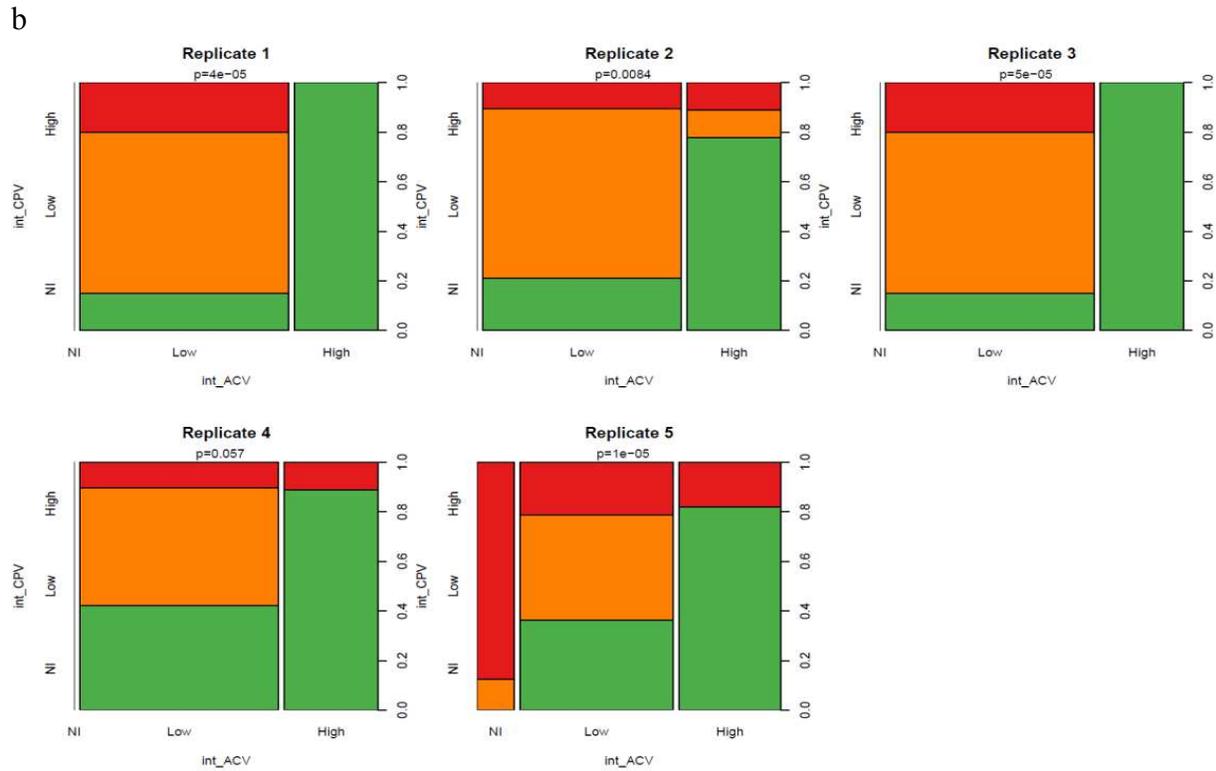
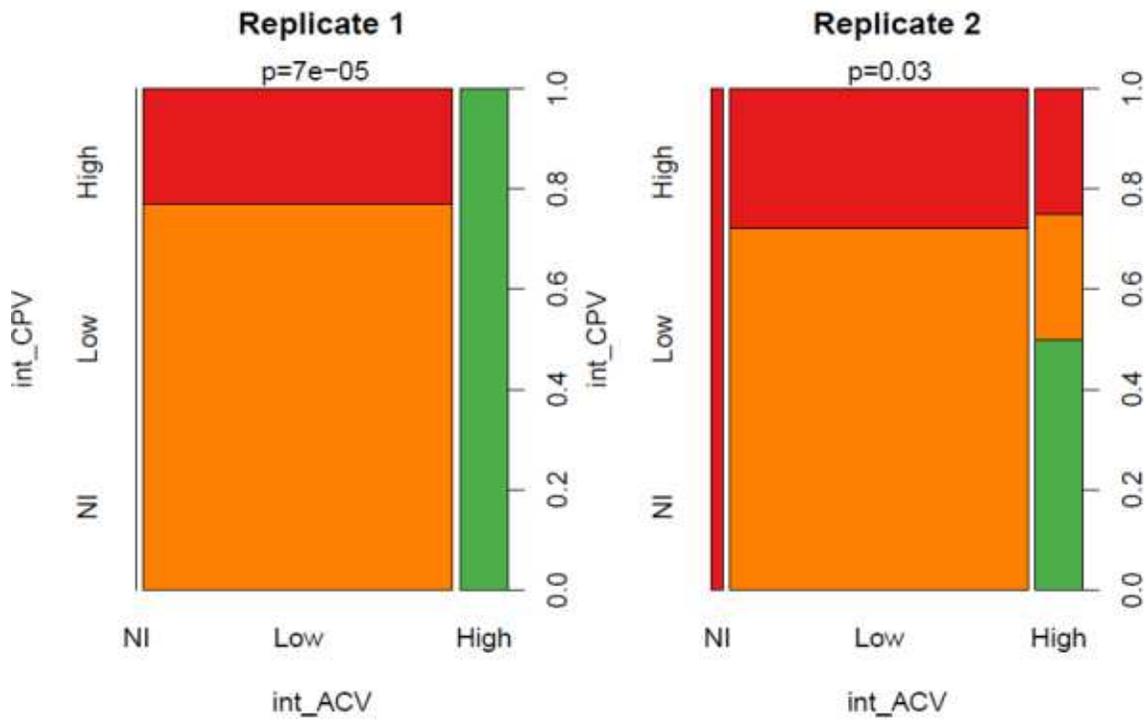


642

Supplementary Figures

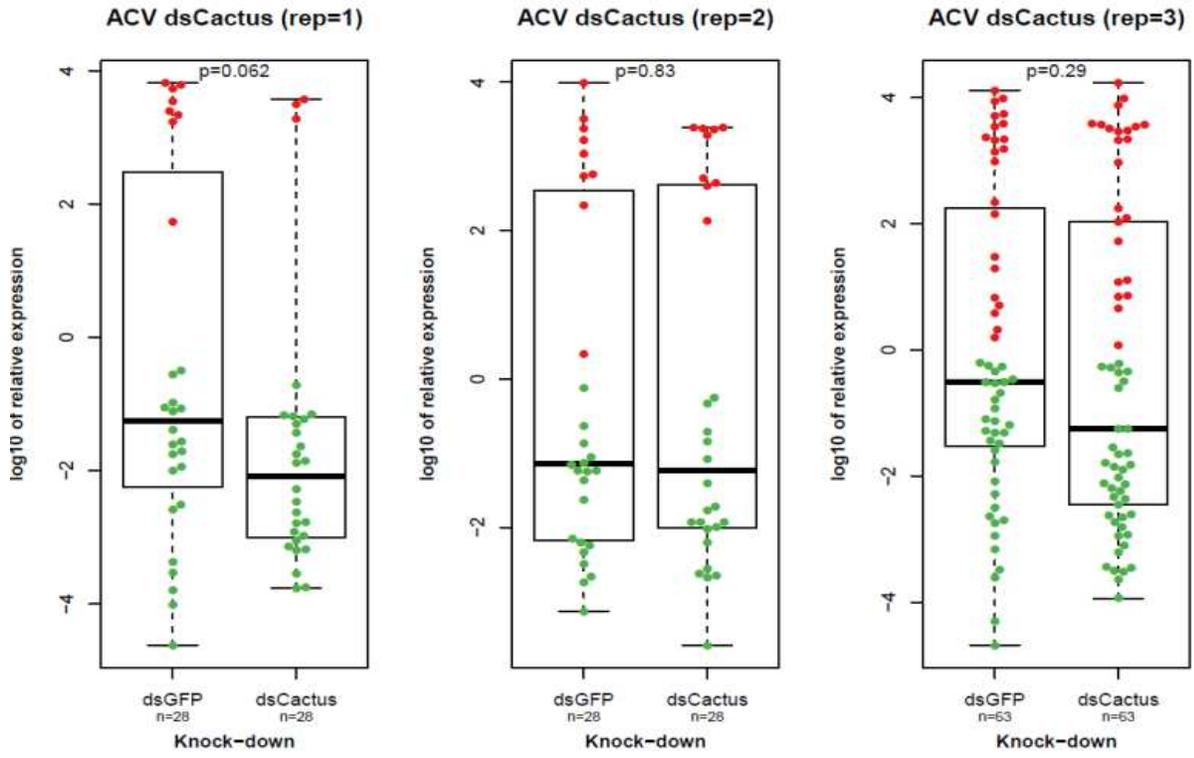


Supplementary Figure S1. Absence of detectable DNA forms of viral genomes of AnCV and AnCPV in *An. coluzzii* Ngouso strain. PCR primers targeted the RNA-dependent RNA polymerase genes of each virus. DNA template was extracted from pools of 5 to 10 mosquitoes each using DNAzol, and cDNA was generated from RNA of pools 5 to 10 mosquitoes each using Trizol. PCR reactions of DNA and cDNA templates were run simultaneously. PCR of the ribosomal protein S7 spanned an intron so that product sizes confirm the difference between cDNA and DNA template. Water was used as negative control. The amplification of AnCV and AnCPV was obtained only from cDNA, indicating the absence of DNA forms of these RNA viruses.

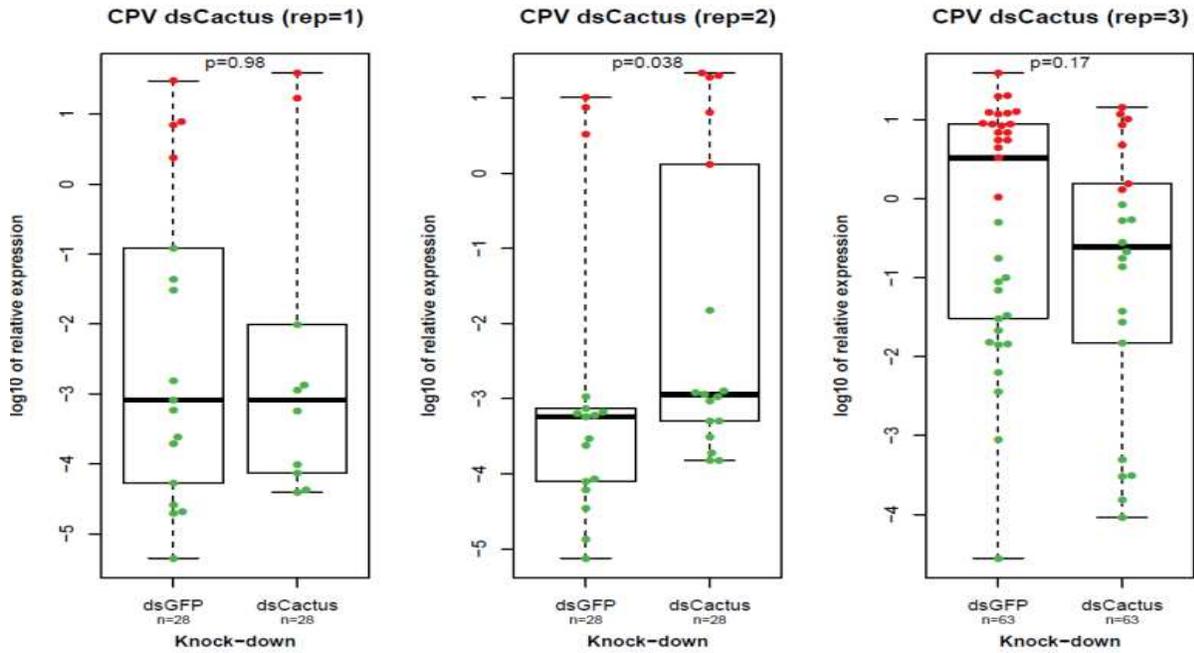


Supplementary Figure S2. Individual replicate tests and p-values of virus abundance correlation. A. Two replicates for Figure 2a. B. Five replicates for Figure 2b.

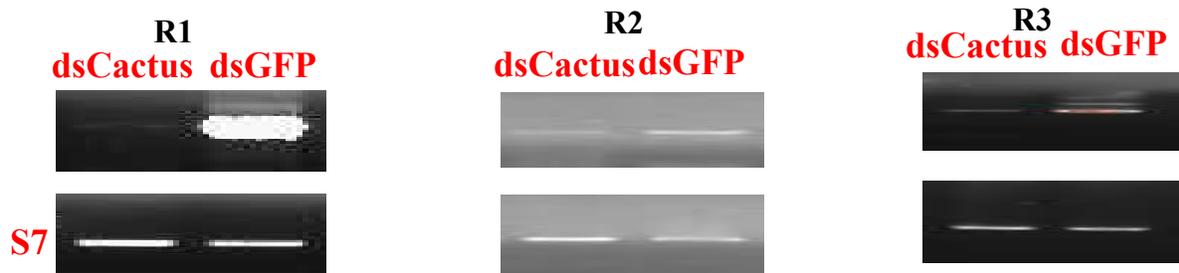
a



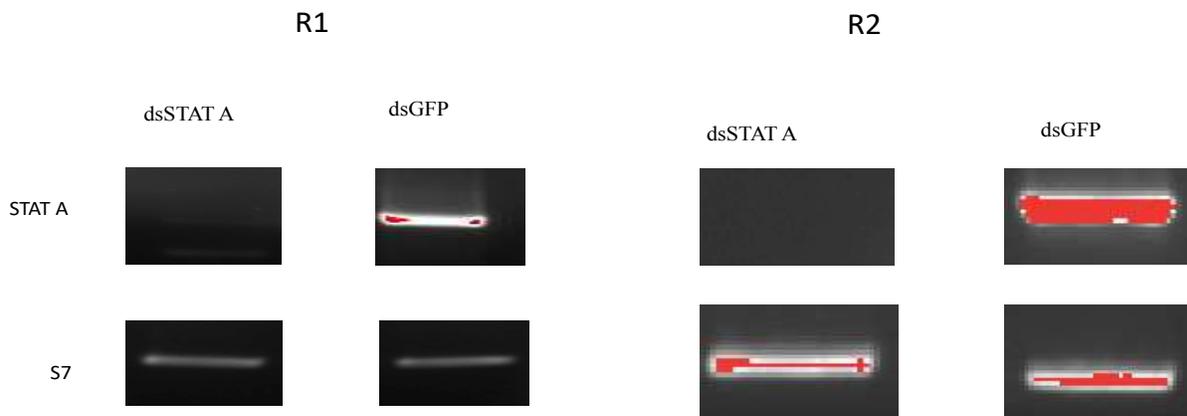
b



c

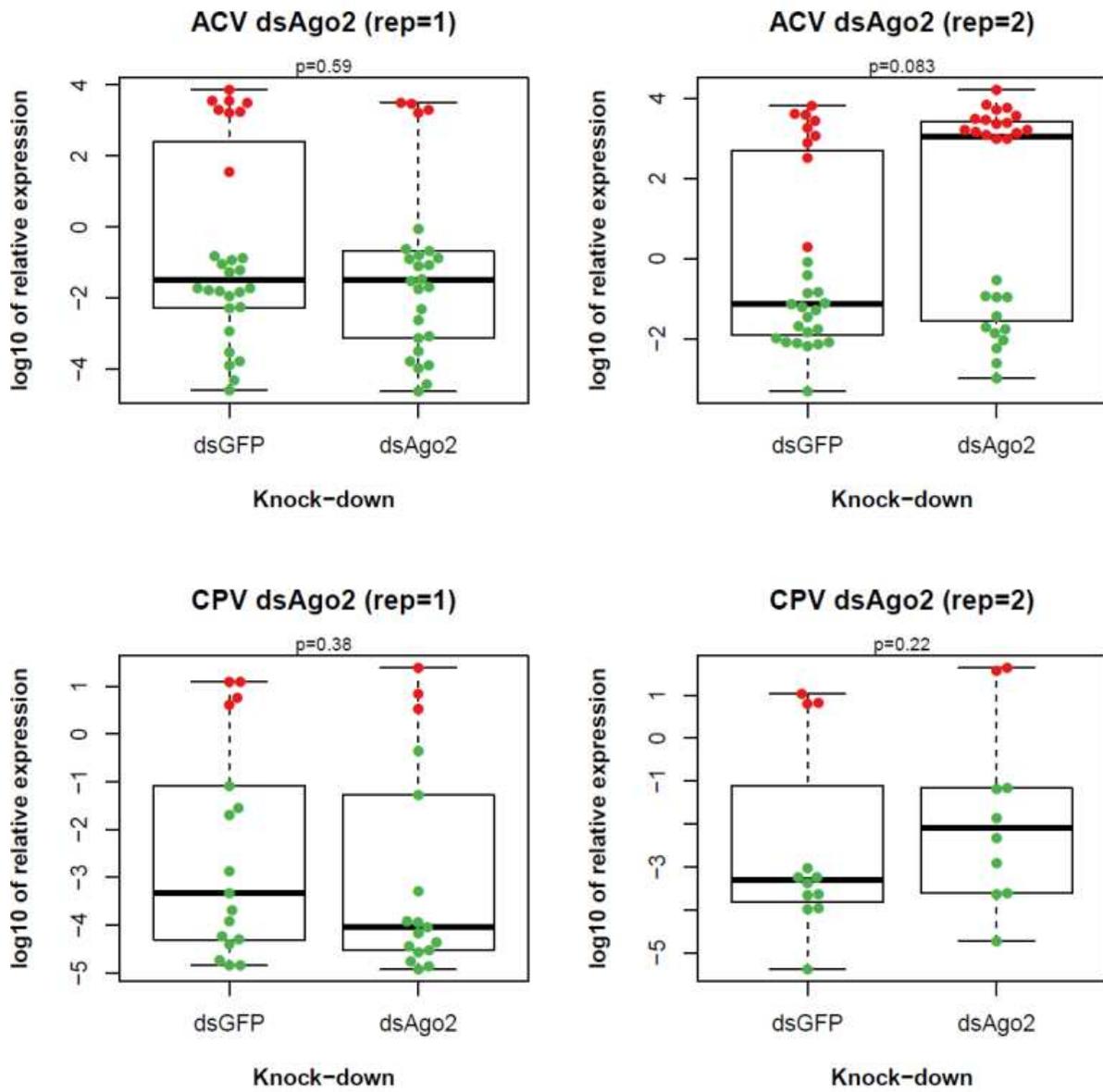


Supplementary Figure S3. Effect of Toll activation on the aggregate mosquito sample. A. Toll activation produces a non-significant tendency towards lower infection intensity by AnCV in 3 independent replicates. dsCactus indicates mosquitoes injected with ds Cactus and dsGFP is the control group injected with irrelevant dsRNA. Silencing efficiency was verified by RT-PCR (Supplementary Figure S3C). x-axis, dsRNA treatment, y-axis represents AnCV expression relative to ribosomal protein S7 in log10. Red, highly infected mosquitoes for indicated virus (\log_{10} of relative expression > 0). Green, low infected mosquitoes for indicated virus (\log_{10} of relative expression < 0). (n) indicates the total number of mosquitoes dissected for each of the biological replicates. Statistical differences were first tested independently within replicates with the Wilcoxon-Mann-Whitney test using 100,000 permutations for assessing the p-value. Then if individual replicates showed consistent direction of change, individual p-values were combined using the meta-analytical approach of Fisher. **B.** Toll activation produces inconsistent effects on AnCPV infection intensity. Details as in part A. **C.** Validation of Cactus silencing after injection of dsCactus, 3 independent replicates detected by RT-PCR followed by agarose gel.

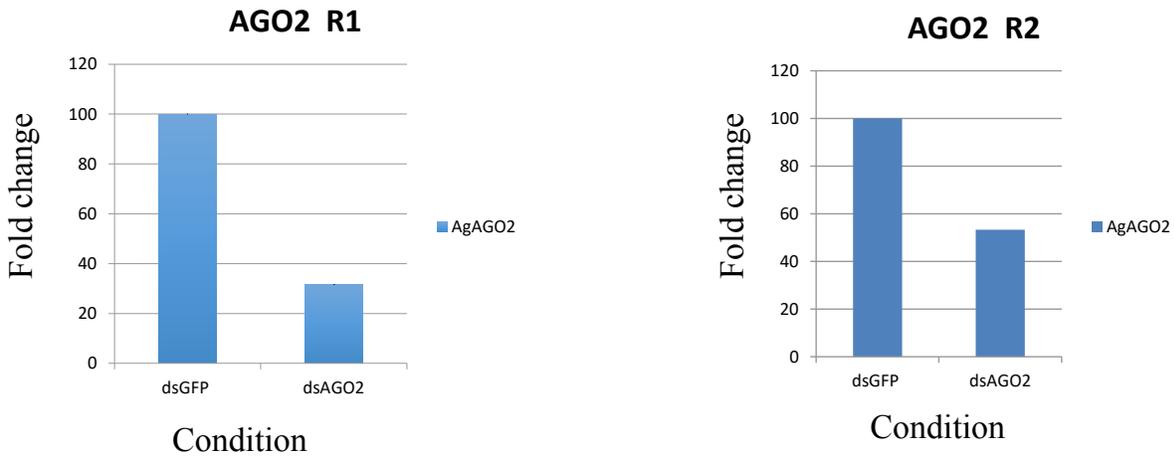


Supplementary Figure S4. Validation of STAT-A silencing. Two independent replicates, detected by RT-PCR followed by agarose gel.

a

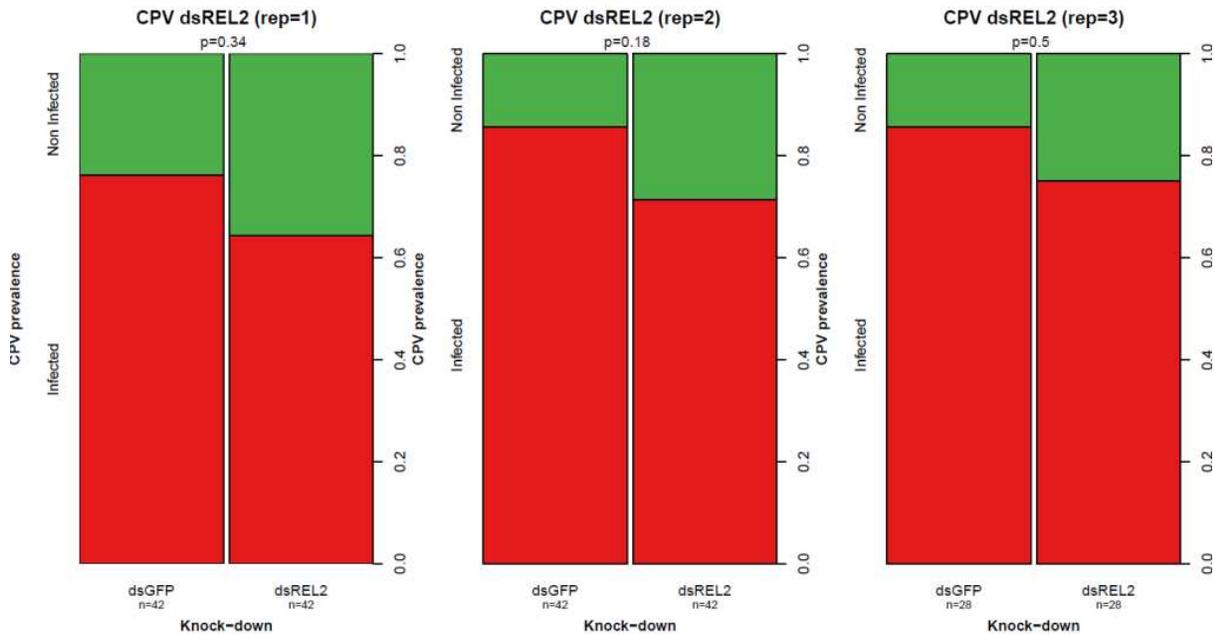


b

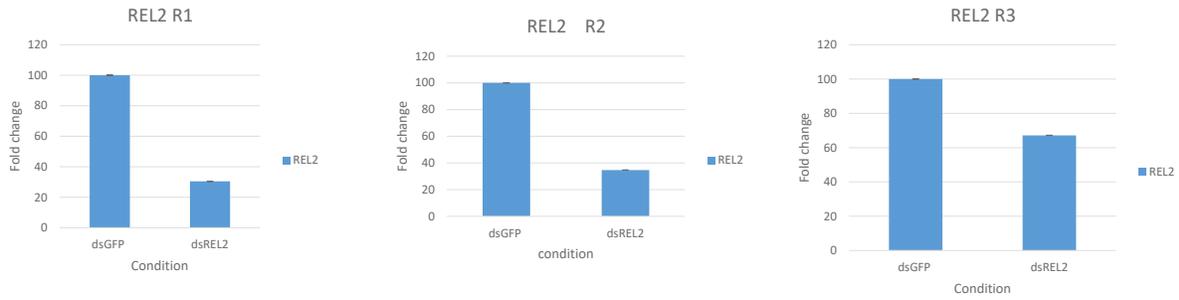


Supplementary Figure S5. RNA interference activity does not significantly influence AnCV and AnCPV infection. A. Details as in Supplementary Figure S3a legend, except DsAgo2 indicates the mosquitoes injected with double stranded RNA directed against Ago2. B. Validation of Ago2 silencing. Two independent replicates, detected by SYBR Green RT-qPCR. Fold-change indicates Ago2 expression after normalization with the ribosomal protein S7 gene, taking expression in dsGFP controls as 100%. Ago2 expression was reduced by at least 60% in replicate 1 and at least 40% in replicate 2.

a

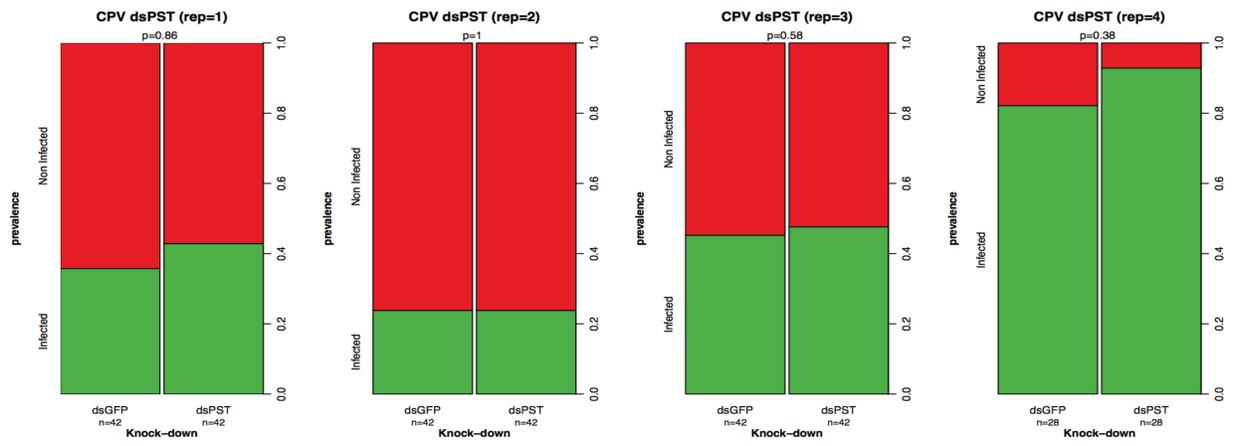


b

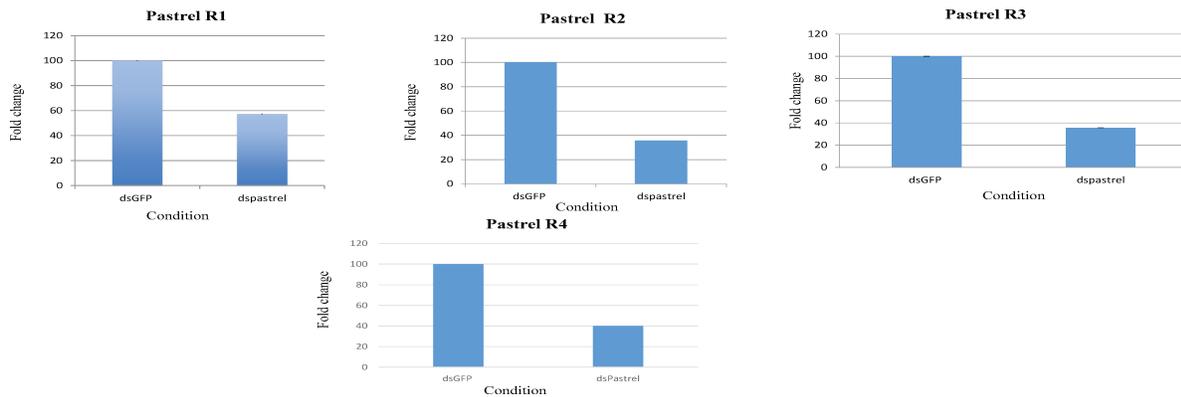


Supplementary Figure S6. Imd pathway activity displays a non-significant positive effect for AnCPV infection. **A.** Details as in Supplementary Figure S3a legend, except dsRel2 indicates the mosquitoes injected with double stranded RNA directed against Rel2. Statistical differences were first tested independently within replicates with the Wilcoxon-Mann-Whitney test using 100,000 permutations for assessing the p-value (given above each plot). Then since individual replicates showed consistent direction of change, individual p-values were combined using the meta-analytical approach of Fisher (combined p-value=0.326, chi-square=6.94, df=6,). **B.** Validation of Rel2 silencing. Details as in Supplementary Figure S5b legend. Rel2 expression was reduced by at least 60% in replicates 1 and 2 and 35% in replicate 3.

a



b



Supplementary Figure S7. Candidate pastrel ortholog PEST AGAP011771 displays a non-significant protective tendency against AnCPV infection. A. Details as in Supplementary Figure S3a legend, except DsPst indicates the mosquitoes injected with double stranded RNA directed against pst candidate, AGAP011771. **B.** Validation of pst candidate AGAP011771 silencing. Details as in Supplementary Figure S5b legend. Rel2 expression was reduced by at least 60% in replicates 2, 3 and 4 and at least 40% in replicate 1.

Supplementary Table S1. Variation of infection prevalence in *An. coluzzii* developmental stages for Anopheles C Virus (AnCV) and Anopheles Cypovirus (AnCPV).

Average prevalence and 95% confidence interval are given for each virus and developmental stage. N=30 per biological replicate. Three biological replicates were done for each stage.

Viruses	Larvae (L3/L4)	Pupae	New Adults	Adults, 1 week	Adults, 2 weeks
AnCV	93.33 [80.27 -106.40]	89.65 [69.38-109.93]	35.90 [21.86 - 49.94]	41.11 [33.26 - 48.96]	34.21 [12.87 - 55.54]
AnCPV	7.78 [2.02 - 13.54]	11.19 [6.98 - 15.39]	6.74 [2.97 - 10.5]	41.11 [19.01- 63.21]	25.95 [17.17 - 34.74]

Supplementary Table S2. Summary of settings for duplex Taqman real-time RT-PCR assay (Taqman RT-qPCR).

Efficiency (Eff) = $10^{-(1/\text{slope})} - 1$, R, reproducibility, Dil 1, dilution 1 of standard sample.

Assays	R²	Slope	Eff (%)	Ct Mean Dil1	Ct Mean
ACV simplex	0.996	-3.431	95.63	12.169	27.339
ACV duplex	0.998	-3.352	98.78	13.008	28.657
CPV simplex	0.989	-3.313	100.40	19.663	19.095
CPV duplex	0.997	-3.186	105.99	20.709	18.924

Supplementary Table S3. List of primers and probe sequences. F, forward and R, reverse, q, quantitative, taq, Taqman.

Virus/Genes	Primers	Sequences
RT-PCR		
AnCV	AnCV-F	CAAGGAGGCTTTTTGAGTGC
	AnCV-R	GCTTTGGGTAAGCTGTGCGAG
AnCPV	AnCPV-F	TGAGCGAATCGTGCACCATG
	AnCPV-R	GGTTTTCCGACTAGCCTTCC
rpS7	S7-F	AGGCGATCATCATCTACGTGC
	S7-R	GTAGCTGCTGCAAACCTTCGG
RT-qPCR SYBR Green		
rpS7	S7-qF	AGAACCAGCAGACCACCATC
	S7-qR	GCTGCAAACCTTCGGCTATTC
AnCV	AnCV-qF	ATCGCGTAATTAGGGCTCCA
	AnCV-qR	TTGAGACACAGGACAGCGAT
AnCPV	AnCPV-qF	TCGACAGATGCAGCTCAAGG
	AnCPV-qR	AACCACGCGTCACTTCAAGA
qPCR Taqman		
AnCV	AnCV-Taq-F	ACCAGGAAAGAACGACGTAGACA
	AnCV-Taq-R	CGCACCCCTTAACAGCTTTGG
	AnCV-Probe (FAM)	TTTCATGCGCAGGCTCGACAGC
AnCPV	AnCPV-Taq-F	CAGCTCAAGGTAAACAGGTTGGT
	AnCPV-Taq-R	CGTCTGAGTTGTCGCGAATAAC
	AnCPV-Probe (VIC)	ATTCAATGGACCTTCAAGAC
rpS7	Ag-S7- Taq-F	CAAGCGTATCCGCGTCAAG
	Ag-S7- Taq-R	GGTGGTCTGCTGGTTCTTATCC
	Ag-S7- Probe (NED)	TCGACGGATCCCAGCTGATAAAGGTG
RT-PCR : double stranded RNA synthesis		
GFP	T7-GFP-F	GAATTGTAATACGACTCACTATAGGGCATGGTGAGCAAGGGCGAG
	T7-GFP-R	GAATTGTAATACGACTCACTATAGGGCTTACTTGTACAGCTCGTC
AGO2	T7-Ago2-F	TAATACGACTCACTATAGGGGTTTCGCGCCCATACCTAAA
	T7-Ago2-R	TAATACGACTCACTATAGGTTGTTTTGTTTCAGCGCCTG
Cactus	T7-Cactus-F	TAATACGACTCACTATAGGTGGTTCGTCGATTGCTGG
	T7-Cactus-R	TAATACGACTCACTATAGGCTTTCGTTCAAGTTCTGTGC
REL2	T7-REL2-F	TAATACGACTCACTATAGGGCAACAGCAGCAACAACATC
	T7-REL2-R	TAATACGACTCACTATAGGGCACAGGCACACCTGATTGAG
STAT A	T7-STATA-F	TAATACGACTCACTATAGGCCGGAGAGCAACTTCACGAT
	T7-STATA-R	TAATACGACTCACTATAGGGATGAACGTGTTGTAATGAGC
AGAP011771 (Pastrel)	T7- AGAP011771- F	TAATACGACTCACTATAGGACGAGGATGATGGTAGGCTG
	T7- AGAP011771- R	TAATACGACTCACTATAGG AGTAATTCGGATCCTGGCGG
Sybr green to verify gene expression after silencing		
Ago2	Ago2-qF	CCAAGCCGACCAAGTACG
	Ago2-qR	GCAAACAGGTGGCACAGATT
AGAP011771 (pastrel)	AGAP011771- qF	GCAACAACAAGTACCCGTGCG

	AGAP011771-qR	TACCGCCCGACAGAAAGATG
rpS7	S7-qF	AGAACCAGCAGACCACCATC
	S7-qR	GCTGCAAACCTTCGGCTATTC
REL2	REL2-qF	CTCAATCAGGTGTGCCTGTGC
	REL2-qR	GGTGGTGCTGAGCCGGCAGATC

Supplementary Table S4. Detailed statistical test results. Cond, condition. #, number. WMW, Wilcoxon Mann Whitney test, ds, double stranded.

Figure 3. Inverse correlation between AnCV and AnCPV infection levels in An. coluzzii.											
	samples			WMW test for each replicate				Fisher's combined probability			
	cond	repl icate	# samp les	chi2	p- valu e			Chi-2	d f	p- val ue	Virus es
Fi g. 3a	Non injected	1	30	30	7,00 E-05			26.1	4	2.9 8e- 05	ACV/ CPV
Fi g. 3a	Non injected	2	30	16.6	0.03 03			—	—	—	ACV/ CPV
Fi g. 3b	dsGFP	1	28	17.3	4,00 E-05			78.4	1 0	1.0 4e- 12	ACV/ CPV
Fi g. 3b	dsGFP	2	28	9.02	0.00 842			—	—	—	ACV/ CPV
Fi g. 3b	dsGFP	3	28	17.3	5,00 E-05			—	—	—	ACV/ CPV
Fi g. 3b	dsGFP	4	28	6.6	0.05 69			—	—	—	ACV/ CPV
Fi g. 3b	dsGFP	5	63	31.7	1,00 E-05			—	—	—	ACV/ CPV
Supplementary Figure S3a. Supplementary Figure S3. Effect of Toll activation on the aggregate mosquito sample											
Fi g. S3 a	dsGFP/ dsCactu s	1	28		0.06 204			8.42	6	0.2 09	ACV
Fi g. S3 a	dsGFP/ dsCactu s	2	28		0.82 829						ACV

Fig. S3a	dsGFP/ dsCactus	3	63		0.28 872							ACV
Supplementary Figure S3b. Supplementary Figure S3. Effect of Toll activation on the aggregate mosquito sample												
Fig. S3b	dsGFP/ dsCactus	1	28		0.98 18			NA	NA	NA		CPV
Fig. S3b	dsGFP/ dsCactus	2	28		0.03 78			—	—	—		CPV
Fig. S3b	dsGFP/ dsCactus	3	63		0.17 38			—	—	—		CPV
Figure 4. Toll pathway activity limits levels of AnCPV in <i>An. coluzzii</i>.												
Fig. 4	dsGFP	1	20		0.00 5			17.5	6	0.0 07 53		CPV
Fig. 4	dsGFP	2	19		1			—	—	—		CPV
Fig. 4	dsGFP	3	41		0.03			—	—	—		CPV
Fig. 4	dsCactus	1	25		0.00 5			—	—	—		CPV
Fig. 4	dsCactus	2	19		1			—	—	—		CPV
Fig. 4	dsCactus	3	40		0.03			—	—	—		CPV
Figure 5. JAK/STAT pathway activity promotes AnCPV infection in <i>An. coluzzii</i>.												
Fig. 5	dsGFP/ dsSTAT_A	1	42	0.933	0.46 7			11.4	4	0,0 22 8		CPV
Fig. 5	dsGFP/ dsCactus	2	42	8.4	0.00 732			—	—	—		CPV
Supplementary Figure S5. RNA interference activity does not significantly influence AnCV and AnCPV infection.												
Fig. S5	dsGFP/ dsAgo2	1	28		0.58 845			NA	NA	NA		ACV
Fig. S5	dsGFP/ dsAgo2	2	28		0.08 34			—	—	—		ACV

Fi g. S5	dsGFP/ dsAgo2	1	28		0.37 776			—	—	—	CPV
Fi g. S5	dsGFP/ dsAgo2	2	28		0.22 433			—	—	—	CPV
Supplementary Figure S6. Imd pathway activity displays a non-significant positive effect for AnCPV infection.											
Fi g. S6	dsGFP/ dsREL2	1	42	1.42	0.34 1			6.94	6	0.3 26	CPV
Fi g. S6	dsGFP/ dsREL3	2	42	2.55	0.18 2			—	—	—	CPV
Fi g. S6	dsGFP/ dsREL4	3	42	1.02	0.50 1			—	—	—	CPV
Supplementary Figure S7. Candidate pastrel ortholog PEST AGAP011771 displays a non-significant protective tendency against AnCPV infection.											
Fi g. S7	dsGFP	1	15	0.0379	1			3.32	8	0.9 13	CPV
Fi g. S7	dsGFP	2	10	0	1			—	—	—	CPV
Fi g. S7	dsGFP	3	19	1.24	0.33 3			—	—	—	CPV
Fi g. S7	dsGFP	4	23	0.477	0.57 2			—	—	—	CPV
Fi g. S7	dsPST	1	18	0.0379	1			—	—	—	CPV
Fi g. S7	dsPST	2	10	0	1			—	—	—	CPV
Fi g. S7	dsPST	3	20	1.24	0.33 3			—	—	—	CPV
Fi g. S7	dsPST	4	26	0.477	0.57 2			—	—	—	CPV

RESULTS

3.3 Article III (In preparation): An insect specific virus can be transmitted like an arbovirus

3.3 Article III (In preparation): An insect specific virus can be transmitted like an arbovirus

ABSTRACT:

The *Anopheles* virome is mainly made up of ISVs and arboviruses. Metagenomic studies have increased the numbers of ISVs but data are lacking in biological characterization. ISVs can be used to study interaction between *Anopheles* and viruses. Mosquito competence to ISVs and their evolutionary effect in shaping antiviral responses to arboviruses are under analyzed.

We created and implemented an infective bloodmeal model with two natural members of the *Anopheles* virome, the RNA viruses *Anopheles* C virus (AnCV) and *Anopheles* cypovirus (AnCPV), in order to assess their pathogenicity, to characterize the *Anopheles* transcriptional response to infection, and to shed light on the potential pathway of evolution from ISV into arbovirus.

AnCPV was found to be transmitted to rabbits by infected *An. coluzzii* mosquitoes, and subsequently the infected rabbits transferred it to uninfected *An. stephensi* mosquitoes. As far as we know, this is the first time an ISV has been reported to be transmitted like an arbovirus, and highlights the importance to characterize other ISVs. Analyses of AnCPV genomes within *An. stephensi* and *An. coluzzii* revealed genomic variation between both the source and recipient hosts, confirming the high mutation rate of RNA viruses. In addition, *An. stephensi* infected with AnCPV died faster as compared to uninfected ones, suggesting a fitness cost to both host and virus in the new infection that could potentially drive evolution towards lower virus virulence and/or greater host resistance and tolerance to create a lower-cost interaction. Moreover, primary infection of *An. stephensi* with AnCPV modulates expression of genes belonging to antiviral pathways such as Toll, RNAi, JAK/STAT while the disseminated infection with AnCPV modifies expression of immune genes as CLIP, LRIM15, and prophenoloxidase. These results will lead to functional genomics studies to understand the immune response of *Anopheles* mosquitoes after ISV infections.

Keywords: Insect specific virus, arbovirus, malaria, *Anopheles*, immune responses, genomics

RESULTS

INTRODUCTION

Anopheles mosquitoes are vectors of human *Plasmodium* species, which are responsible for transmission of malaria. With more than 400,000 deaths per year malaria is one of the deadliest diseases in the world. Sub-Saharan Africa and South-East Asia pay the heaviest burden with 90% and 7% of mortality respectively (WHO, 2017).

Recently, many viruses that replicate only in insects (insect specific viruses, ISVs) have been reported in *Anopheles* in different geographic locations. Those ISVs associated specifically to *Anopheles* could be considered *Anopheles* specific viruses (ASVs) and include *Anopheles gambiae* densovirus (AgDENV, *Parvoviridae* family), Australian *Anopheles totivirus* (AATV, *Totiviridae*), *Anopheles flavivirus* (AnFV, *Flaviviridae*), *Anopheles C virus* (AnCV, *Dicistroviridae*), *Anopheles cypovirus* (AnCPV, *Reoviridae*), *Anopheles annulipes orbivirus* (AAOV, *Reoviridae*) and others (Colmant et al., 2017a; Colmant et al., 2017b; Fauver et al., 2016; Nanfack Minkeu and Vernick, 2018). Except for AgDENV, ASVs are RNA viruses, which have high mutation rates due to the high replication errors of their polymerase. RNA viruses evolve quickly in different mosquito species but little is known about their genetic variability. Moreover, mosquito pathogenicity and transcriptomic studies of infected *Anopheles* mosquitoes by ASVs are lacking. In addition to harboring ISVs, *Anopheles* species transmit the arbovirus O'nyong nyong (ONNV, *Togaviridae*, *Alphavirus*), which has been involved in multiple known epidemic outbreaks (Nanfack Minkeu and Vernick, 2018; Williams et al., 1965). The biggest epidemic of ONNV occurred in Africa in 1959-1960 with about two million reported cases (Williams et al., 1965). In 1996 another outbreak of ONNV caused up to 80% of morbidity in some Ugandan villages (Rwaguma et al., 1997; Sanders et al., 1999). ONN-1996 and ONN-1959 have 78 non-synonymous mutations (with amino acids altered), and new stop codons are present in ONN-1996 (Lanciotti et al., 1998).

The replication of ISVs occurs exclusively in insects or insect cell lines, and ISVs are unable to infect vertebrate tissues or cells, while arthropod-borne viruses (arboviruses) can replicate in both (Roundy et al., 2017). Studies on *Anopheles* competence to other arboviruses are absent and the potential evolution of ASVs to be arboviruses is unknown.

AnCPV and AnCV were discovered in the *An. coluzzii* Ngousso strain by metagenomic analysis (Carissimo et al., 2016). Wild *Anopheles* collected in Africa and Asia were also found to be infected by AnCV and AnCPV, however these viruses were absent in the *An. stephensi* SDA500 strain available at *Institut Pasteur-Paris* (Carissimo et al., 2016). In the current study, we

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exploited this uninfected status of *An. stephensi* to develop an experimental infection model for AnCV and AnCPV transmission from an infected strain (*An. coluzzii* Ngousso) to the uninfected *An. stephensi* strain through a vertebrate host.

We found that AnCPV, but not AnCV, could be transmitted like an arbovirus from the bite of infected *An. coluzzii* Ngousso mosquitoes to an uninfected vertebrate host rabbit and then to previously uninfected *An. stephensi* mosquitoes. We also performed a transcriptomic analysis of *An. stephensi* infected with AnCPV and identified potential candidates for studying the interaction between *Anopheles* and AnCPV.

We analyzed the genetic diversity of both AnCV and AnCPV in *An. coluzzii* Ngousso. AnCV displays little genetic diversity. In contrast, there was high diversity of AnCPV in *An. coluzzii*. Only a subset of genetic variants were maintained in newly infected *An. stephensi* mosquitoes, suggesting that selection pressure in the new host may have led to virus adaptation.

RESULTS

Insect specific virus AnCPV can be transmitted through a vertebrate host

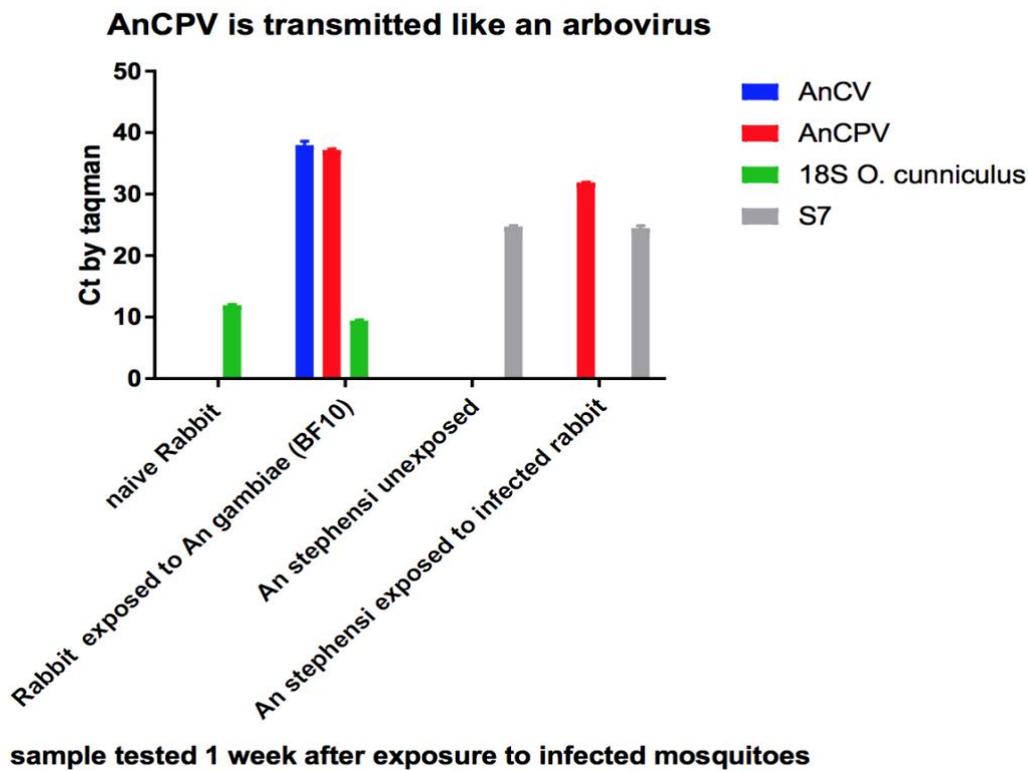
We established an experimental model to test the conditions of transmission of AnCV and AnCPV. In particular, we wished to determine whether the viruses had the capacity, or could evolve the capacity, for transmission through an uninfected intermediate vertebrate host to uninfected mosquitoes.

Rabbits are used as the blood source to maintain the two mosquito colonies. We used a new rabbit to feed the *An. coluzzii* Ngousso colony weekly. The rabbit was confirmed to be negative for both viruses by testing a blood sample. Blood samples were collected from the rabbit one week after each blood feeding, to allow clearance of passively circulating virus in the rabbit. Blood samples were tested for the presence of both AnCV and AnCPV in the rabbit. After the fourth week of rabbit exposure to the *An. coluzzii* Ngousso colony, the blood one week post-feeding was positive for both AnCV and AnCPV (Fig 12A). We pursued the survey until the 10th week of exposure and still detected both viruses. After ten weeks, we used the virus-positive rabbit to feed the uninfected *An. stephensi* colony. At 7 d after feeding on the virus-positive rabbit, the *An. stephensi* colony was tested. The *An. stephensi* mosquitoes exposed to the virus-positive rabbit were positive for AnCPV but not AnCV (Fig. 12A). To control for a possible route of virus transmission, we tested other rabbits that had been exposed to the infected *An. coluzzii* Ngousso colony mosquitoes. All rabbits exposed to the infected colony

RESULTS

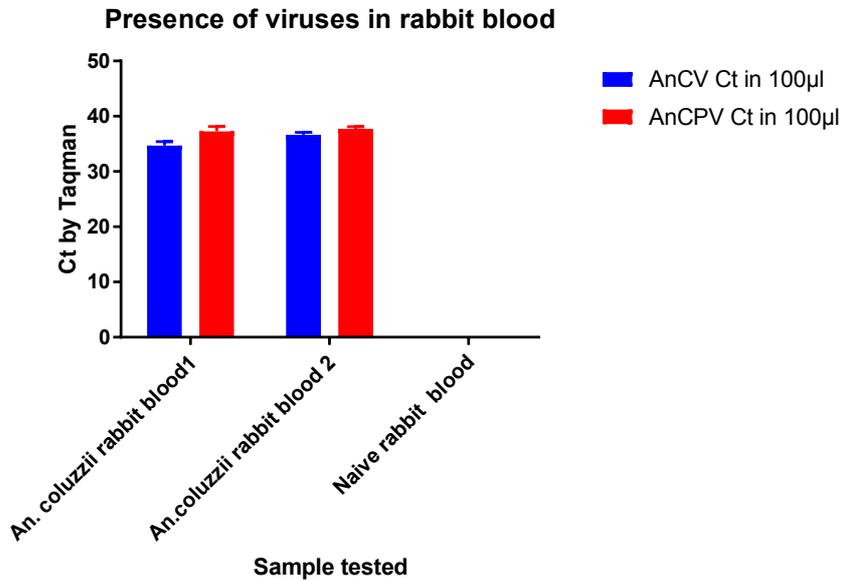
were positive for both viruses (Fig. 12B). Hence, we concluded that AnCPV, but not AnCV, could be transmitted by a mechanism like an arbovirus, involving conversion of the vertebrate host from virus-negative to positive status, followed by blood-feeding dependent infection from the positive vertebrate host to uninfected mosquitoes. In order to characterize the potential interaction of the viruses with vertebrate cells, as a model for the rabbit host, added virus-infected extract from the *An. coluzzii* Ngousso colony to a culture of Vero cells, African green monkey kidney epithelial cells used to cultivate arboviruses. AnCV appeared to replicate in Vero cells, while AnCPV did not replicate (Fig. 12C).

a



RESULTS

b



c

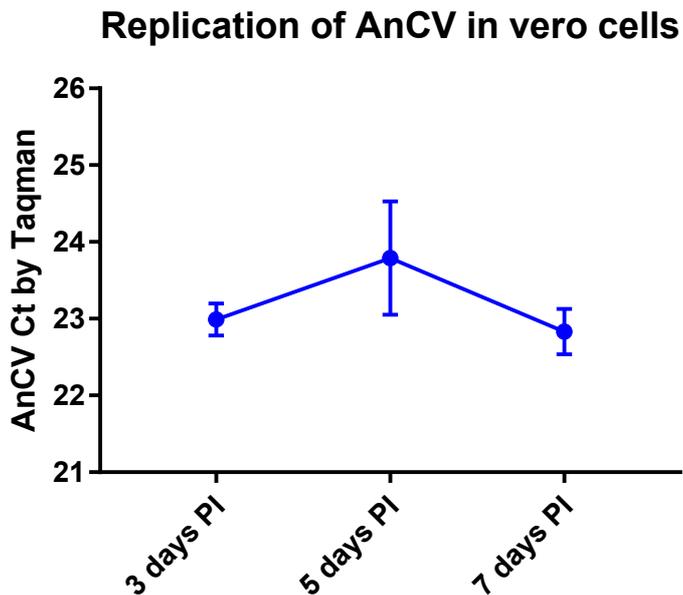


Figure 12: *Anopheles* cyrovirus AnCPV can be transmitted like arbovirus.

A. Rabbit was exposed to viruses AnCPV and AnCV by bloodfeeding of infected *An. coluzzii* Ngouso colony mosquitoes and was subsequently able to transmit AnCPV to uninfected mosquitoes (*An. stephensi* Lille colony, 2 biological replicates). Y-axis represents Ct by duplex Taqman RT-qPCR indicating virus presence. X-axis represents the tested samples. Naïve rabbit

RESULTS

blood indicates a new rabbit with no previous contact with infected mosquitoes. 18S and S7 are control housekeeping genes for rabbit blood and *Anopheles* respectively. B: presence of viruses in rabbit blood. Naïve rabbit is a new rabbit with no contact with mosquitoes. C: AnCV is replicating in Vero cell line. X-axis represents the time; the supernatants were collected on days 3, 5 and 7 post inoculation of cells with Ngousso extract. Medium was changed on days 1, 3 and 5.

Using a non-infected *Anopheles* line as a model for studying *Anopheles* specific virus interaction

We observed that *An. stephensi* mosquitoes free of AnCV and AnCPV can be infected through an infectious blood meal from an infected rabbit (Fig. 12A). Therefore, we established an experimental infection system, by feeding uninfected *An. stephensi* mosquitoes on an artificial membrane feeder with blood supplemented with extract of *An. coluzzii* Ngousso colony larvae that carried both AnCV and AnCPV. The infected larval extract was filtered first with a 5 µm filter to remove particulate matter, followed by a 0.2 µm filter to remove bacteria and other microbes. Naïve blood was fed to negative control mosquitoes. Mosquitoes were collected on day 3 and day 7 post-blood meal and their infection status was assessed.

All mosquitoes that received naïve blood were negative for AnCV and AnCPV while those that received infective blood were positive for both viruses on day 3 (Fig. 13A). Seven days after the infectious blood meal, AnCV was not detectable while AnCPV reached 100% of infection prevalence (Fig. 13A).

We then assessed the systemic dissemination of both viruses on days 3 and 7 after the infective blood meal using RNA extracted from mosquito legs. Only AnCPV was detectable in mosquito legs at both time points (Fig. 13B).

Because the AnCPV infection disseminated beyond the midgut, we assessed the vertical transmission potential of AnCPV from the infected F0 *An. stephensi*. Seven days after the infective blood meal, the same batch of females were fed a normal blood meal for egg development. Eggs were collected, hatched, and L3-L4 larval stages were collected and tested by pools for virus presence. All tested pools were negative for both AnCV and AnCPV (Table 2), indicating the absence of vertical transmission when mothers are infected by blood ingestion.

RESULTS

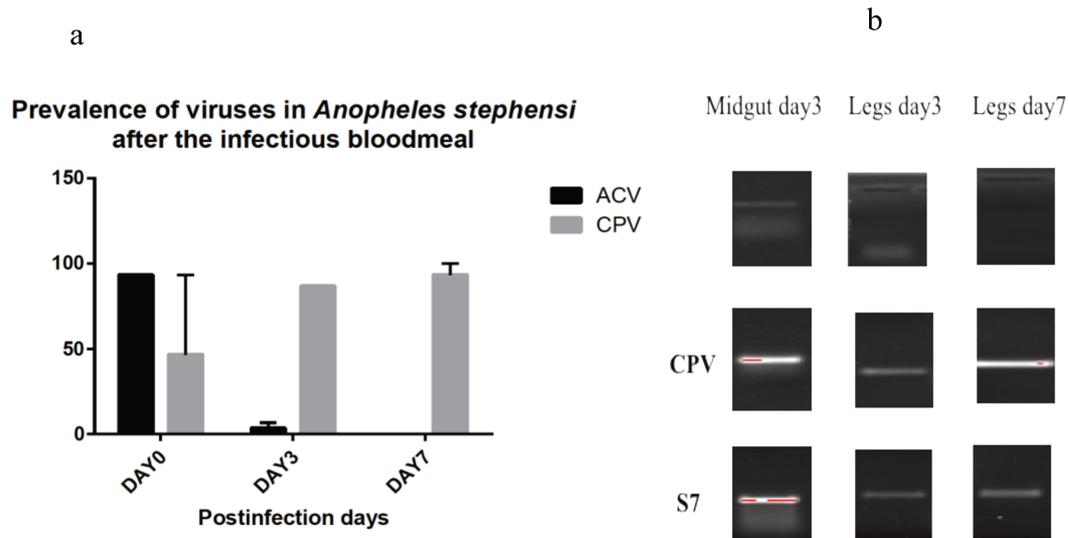


Figure 13: An experimental infection model to study interactions between viruses and malaria vectors. A: Uninfected *An. stephensi* received an infective blood meal containing AnCV and AnCPV (3 biological replicates, n=45). Y-axis indicates virus prevalence as detected by RT-PCR, and the X-axis is time after the infective blood. AnCV is unable to establish a midgut infection or escape the midgut barrier, whereas AnCPV replicates and disseminates. B: dissemination of AnCPV 7 d after infective blood meal. No virus was found in *An. stephensi* controls receiving a normal blood meal.

Tableau 2: Summary of results from infective blood meal with *An. stephensi*. Human blood was mixed with infected *An. coluzzii* larval filtered extract. RT-PCR was used to detect AnCV and AnCPV in adults (F0) and their larvae (F1), 10 pools of 5 larvae were tested per replicate.

Anopheles stages/Status	Infective blood meal	
	AnCV	AnCPV
Adults (F0)	Infected on day 3	Infected on days 3 and 7
Larvae (F1)	Uninfected	uninfected

Anopheles cytovirus displays a pathogenic pattern for *An. stephensi*

Pathogenicity of an ISV or ASV could be considered as one criterion for a potential biological control agent for mosquitoes (Ren et al., 2014; Rwegoshora and Kittayapong, 2004). As AnCPV was detected on day 7 after infectious blood meal (Fig. 13A), we assessed its effect on *An. stephensi* longevity. The daily mortality rate was recorded for AnCPV infected versus non-infected groups. We observed that *Anopheles* mosquitoes infected with AnCPV displayed higher mortality as compared to the control group fed a normal bloodmeal (Fig. 14), indicating a potential pathogenic effect of AnCPV on *An. stephensi* adults.

RESULTS

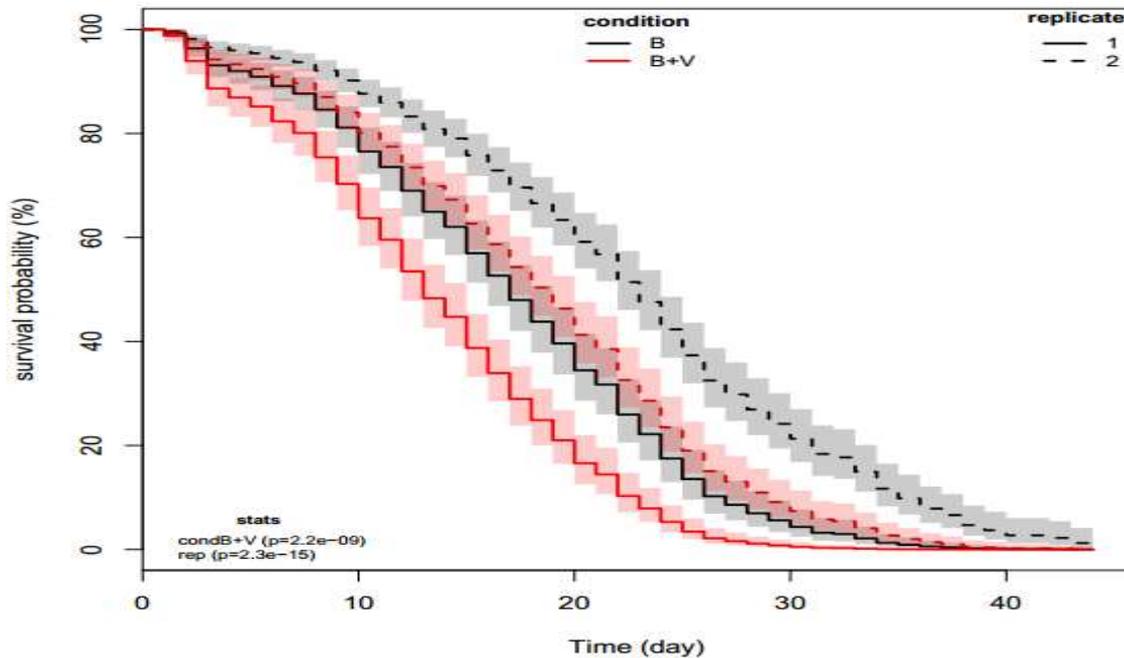


Figure 14: Survival of AnCPV infected and noninfected adult *An. stephensi*. A difference in mortality between control and experimental groups is observed around 10 days after the bloodmeal (x-axis). Two independent replicates were performed and illustrated as replicate 1 (full line) and replicate 2 (dotted line). *An. stephensi* was tested before and during experiment to verify the absence of virus in the colony and control group. Key: B, mosquitoes fed a normal bloodmeal in black, B+V, mosquitoes fed blood with virus in red.

Transcriptomic analysis of *An. stephensi* during primary and disseminated infection with AnCPV

Gene expression profiles of *Anopheles* after infection with ISVs has not been previously examined, thus limiting the understanding of *Anopheles* competence to ISVs. The experimental infection model developed above was used to perform a transcriptomic analysis of *An. stephensi* during the primary infection with AnCPV on day 3 and the disseminated infection with AnCPV on 7d post infective bloodmeal. *An. stephensi* fed on normal blood was used as a negative control group for the analysis. Although viral infections are largely limited to the midgut on 3 d post-infective bloodmeal (Fig. 13B), RNA from whole mosquitoes was purified on day 3 in order to also detect any eventual disseminated signal prior to virus escape from the midgut. A duplex Taqman RT-qPCR assay was used to confirm that all pools from the uninfected groups were free of both viruses and pools with infected ones had AnCV and AnCPV on day 3 and only AnCPV on day 7. On day 3, there is almost 80% of reduction of AnCV (Fig. 13A) while

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AnCPV increases two-fold. In addition, only AnCPV is present on day 7, therefore we take the sample at day 3 as the primary infection with AnCPV, and on day 7 the disseminated infection with AnCPV.

During the primary infection with AnCPV, the expression of 261 genes was significantly modulated (Fig. 17). Some of those genes are part of immune pathways in mosquitoes such as caspase and Toll related genes (Table 3). Others are involved in cellular immune responses like apoptosis.

On day 7, during the disseminated infection with AnCPV, 936 candidate genes were significantly modulated. Among them are genes involved in immune response such as CLIP domain protein genes, LRIM15, prophenoloxidase 9 and others (Table 3). In addition to immune genes, genes involved in metabolism were also regulated upon primary and disseminated infections with AnCPV in *An. stephensi*, suggesting possible metabolic cost during viral infections. The regulated genes associated with metabolic processes include alanyl-tRNA synthetase, chondroitin 4-sulfotransferase, UDP-glucuronosyltransferase, chitin synthase, choline kinase and others (Table 3). Moreover, cytochrome P450 and glutathione S-transferase enzymes, which are involved in detoxification processes, were modulated on day 3 (Fig. 15) and day 7 (Fig. 16). ADP-ribosylation factor-like 6 (Arl) was also upregulated during primary and disseminated infection with AnCPV.

A set of genes with p-value at least 10^{-10} modulated by primary infection with AnCPV on day 3 and disseminated infection with AnCPV on day 7 post-bloodmeal are presented in Figure 15 and Figure 16 respectively. 171 genes were found commonly modulated on day 3 and day 7 post-bloodmeal (Fig. 17).

RESULTS

Day 3 – primary infection with AnCPV

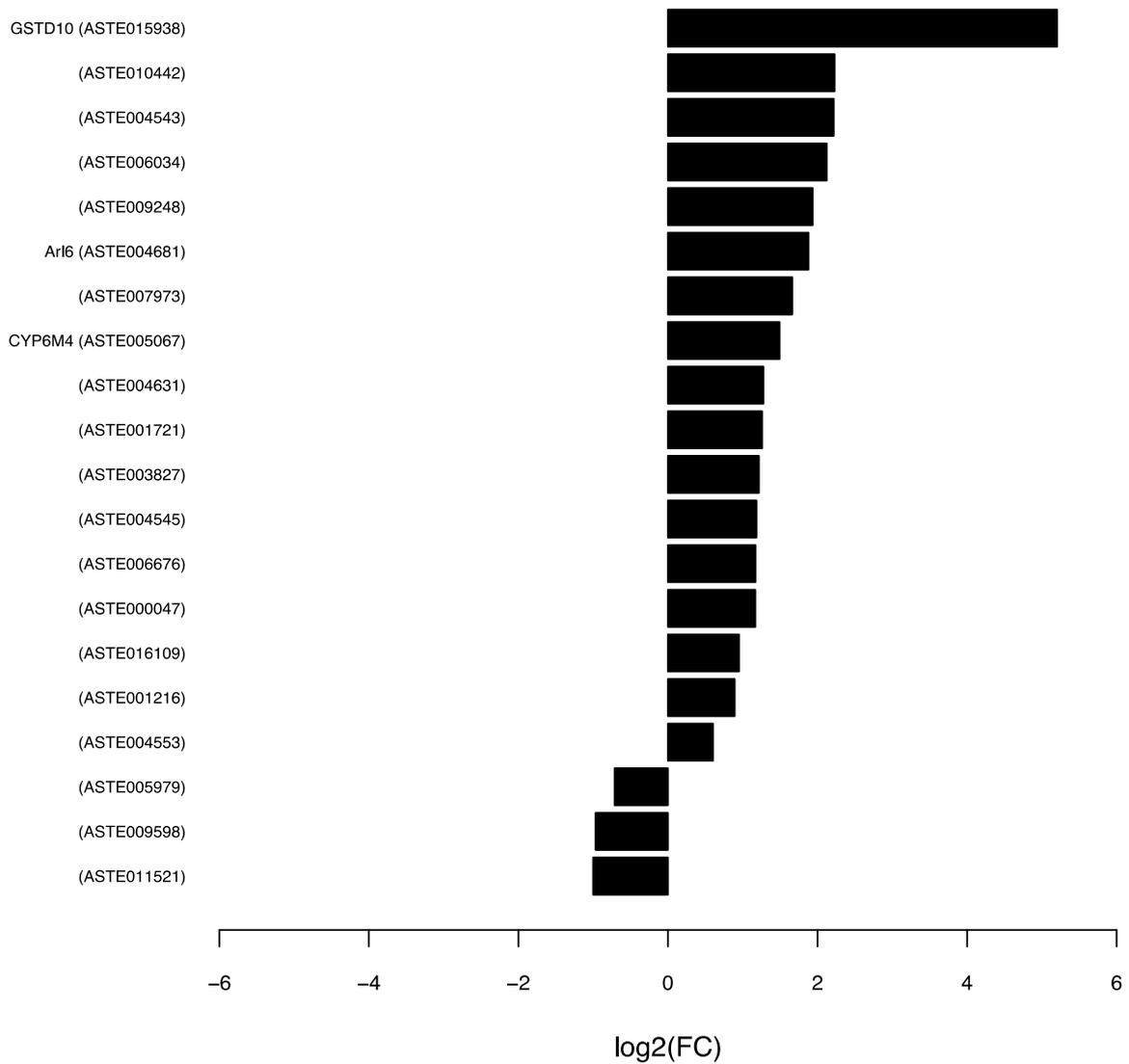


Figure 15: The most significant genes regulated during primary infection with AnCPV 3 days post-bloodmeal. Genes with adjusted p-value at least 10^{-10} are presented. Downregulated genes have a negative log₂Fold change (FC) whereas upregulated have a positive log₂FC.

RESULTS

Day 7 – disseminated infection with AnCPV

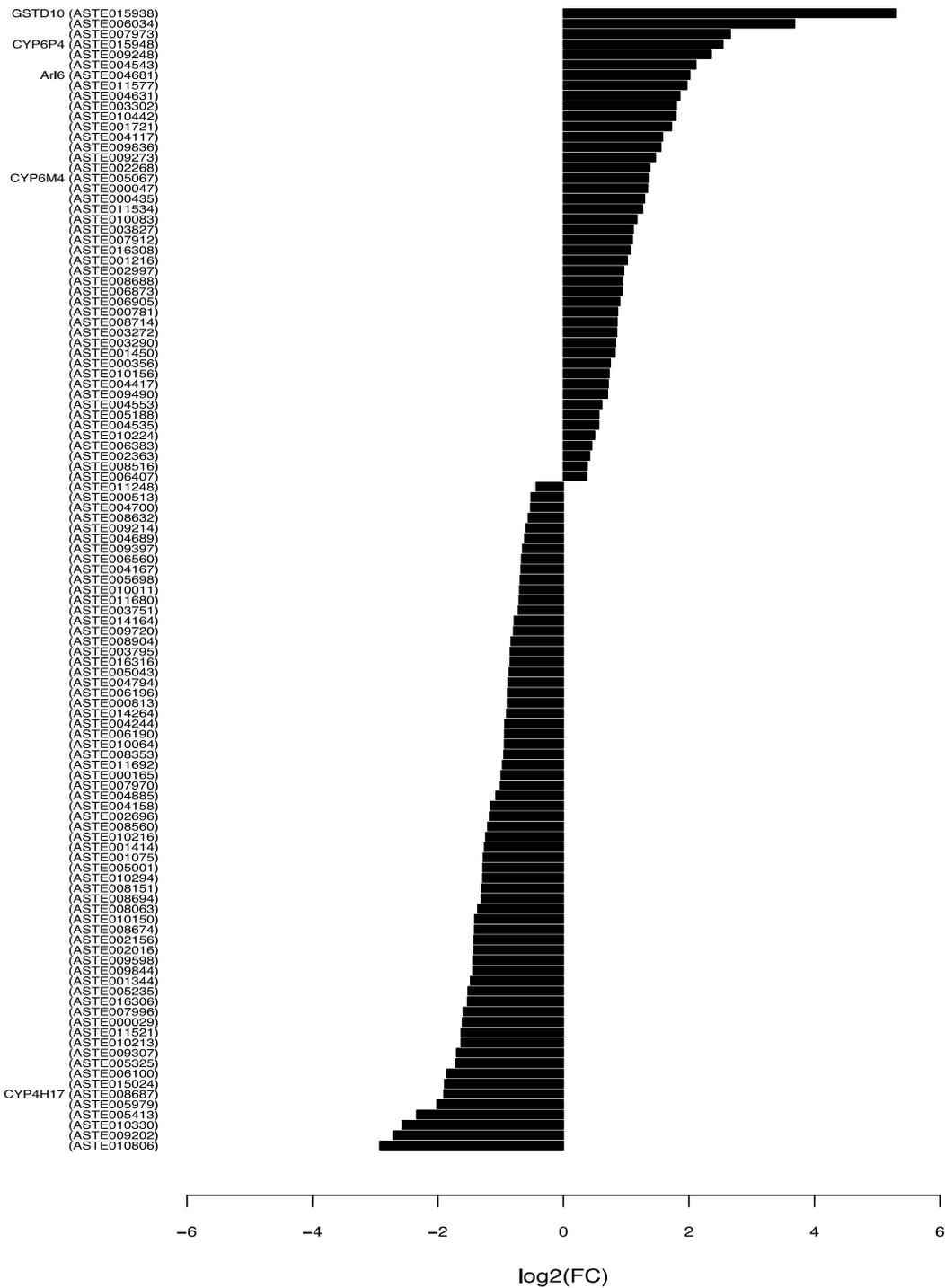


Figure 16: The most significant genes regulated by the disseminated infection of AnCPV 7 days post-bloodmeal. Genes with adjusted p-value at least 10^{-10} are presented. Downregulated genes have a negative log₂Fold change (FC) whereas upregulated have a positive log₂FC.

RESULTS

Venn diagram with all DE genes

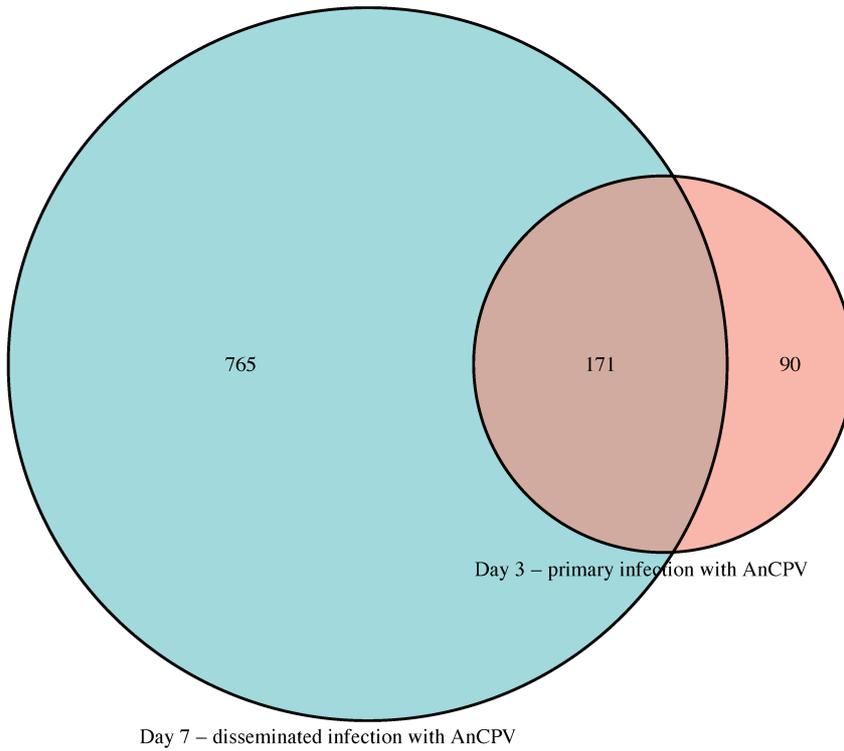


Figure 17: Venn diagram of genes differentially expressed after AnCPV infection. 261 genes are regulated upon primary infection with AnCPV in the midgut, 936 genes during systemic disseminated infection with AnCPV and 171 genes are differentially regulated at both time points. Genes with adjusted p-value at least 0.05 are presented.

Tableau 3: Partial list of immune and metabolic genes significantly regulated on day 3 and 7 post-infection. Partial list of immune and metabolic genes significantly regulated on day 3 and 7 post-infection.

Gene ID	Immune/metabolic genes	Fold change	Adjusted p-value
Primary infection with AnCPV			
ASTE011577	Toll receptor	1.47741341341	0.000000322
ASTE010191	short caspase	0.612771511	0.00087946
ASTE016316	juvenile hormone-inducible protein	-0.53173875	0.002336757
ASTE007572	alanyl-tRNA synthetase	0.412971829	0.006953555
ASTE007038	chondroitin 4-sulfotransferase	0.47095188	0.000192414
ASTE011680	UDP-glucuronosyltransferase	-0.383378543	0.002274824
ASTE006362	choline kinase	0.194985569	0.01267555

RESULTS

Disseminated infection with AnCPV			
ASTE001116	spaetzle-like cytokine	0.28	0.001
ASTE001475	Serpin	0.6	0.000001
ASTE006017	chitin synthase	-0.5	0.00003
ASTE008345	LRIM15	-0.5	0.006
ASTE016300	PPO9	-1.35	0.0007
ASTE000120	autophagy	-0.33	0.008
ASTE016282	CLIP	-0.4	0.01

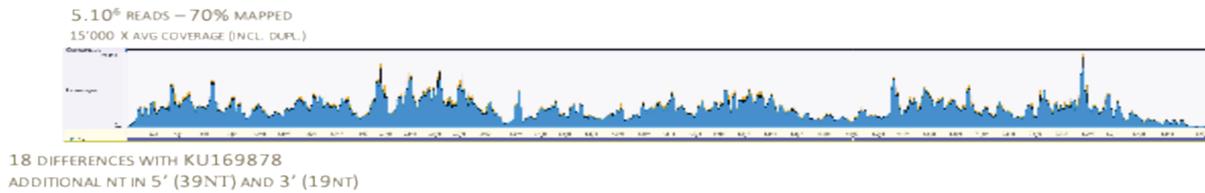
Genomic variation of AnCPV in malaria vectors

We examined AnCPV virus sequence from the source host *An. coluzzii* Ngousso colony and the recipient *An. stephensi* to identify virus genome sequence variation between the two hosts. RNA was extracted from individual mosquitoes and viral genomes were obtained by RNAseq followed by de novo assembly. The almost complete sequence of AnCV genome is available as accession number ([KU169878](#)). We previously determined the sequences of polyhedrin ([KU169880](#)) and RNA-dependent RNA polymerase (RdRp) ([KU169879](#)) segments of AnCPV (Carissimo et al., 2016). The viral load of rabbit blood was too low to obtain viral genome sequences.

Virus genome sequences from *An. coluzzii* Ngousso and experimentally infected *An. stephensi* indicated that the AnCV genome contained low levels of polymorphism (Fig. 18A). Non-synonymous and synonymous mutations are detectable between AnCPV sequences generated from different wild AnCPV isolates, suggesting a high level of genetic diversity of this virus (Fig. 18B). Interestingly, genomic sequence variation of AnCPV was detected between *An. coluzzii* Ngousso source and *An. stephensi* recipient infections, including the apparent fixation of some variable sites in the AnCPV found in the *An. stephensi* recipient mosquitoes (Fig. 19). This finding is suggestive of virus adaptation to a new host environment, and will require further analysis once an AnCPV genome assembly is available.

RESULTS

a



POS	ID	REF	ALT	FREQ	PVAL
169	-	G	T	5.69%	2.0479E-55
372	-	A	C	2.89%	5.3377E-50
1048	-	T	A	1.56%	1.22E-24
2526	-	C	G	5.57%	1.3597E-112
8841	-	T	G	1.07%	3.1035E-3

non coding
non coding
S to T
non coding
non coding

b

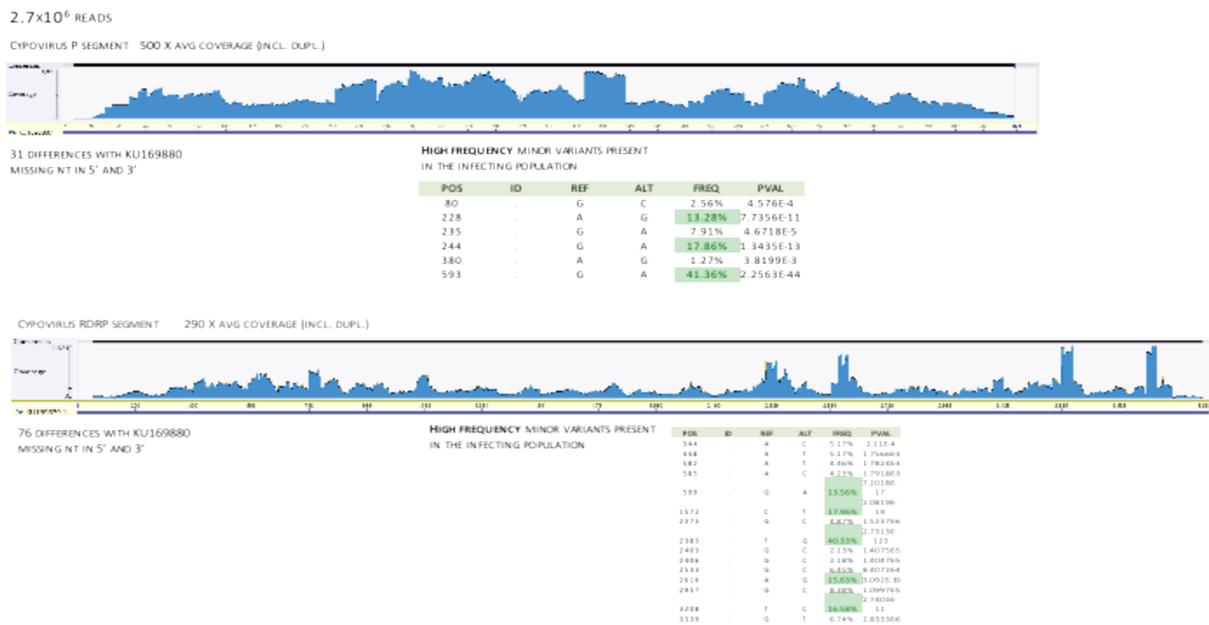


Figure 18: Polymorphism of AnCV and AnCPV. A: AnCV displays a low level of genetic diversity as low frequency variants. B: Polymorphism of AnCPV based on the polyhedrin (P) and RdRp segments. Reference sequences were published by Carissimo and collaborators (Carissimo et al., 2016).

RESULTS

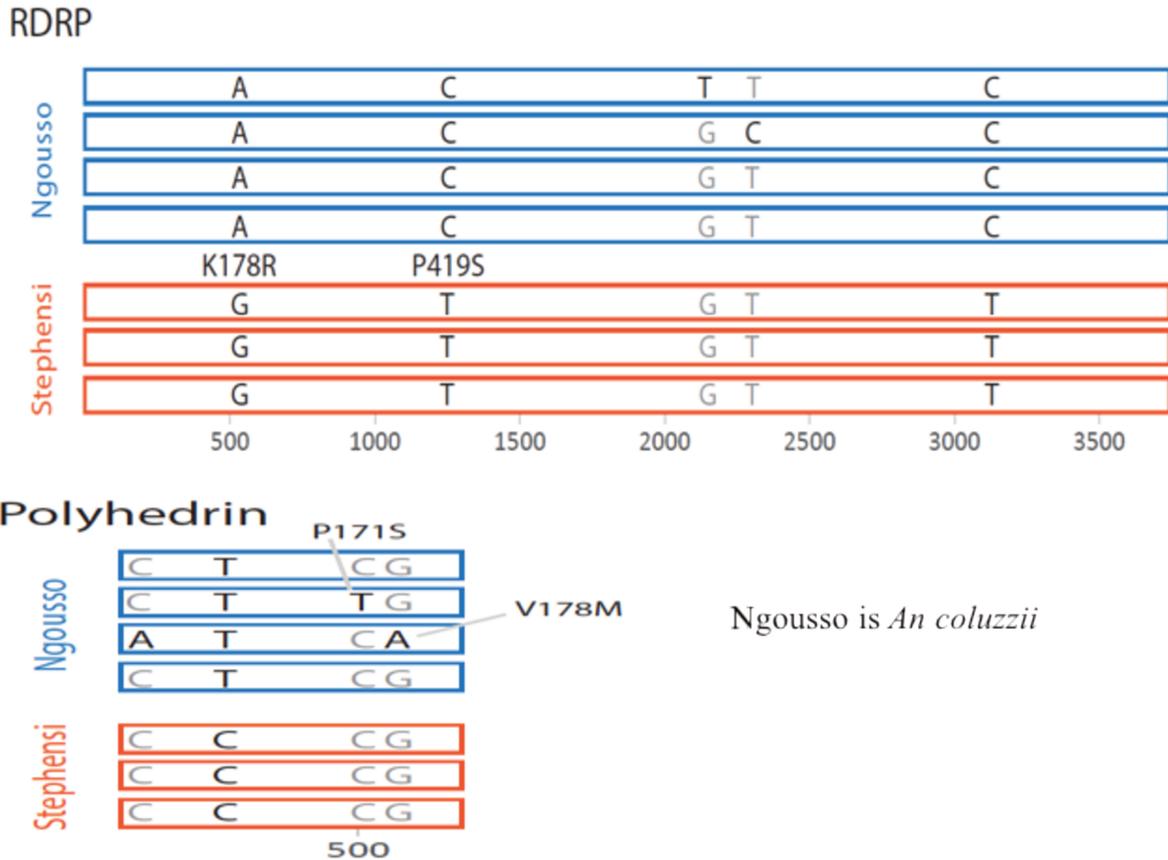


Figure 19: Genomic variation of AnCPV in *An. stephensi* and *An. coluzzii*. Presence of synonymous and non-synonymous substitutions in the RNA-dependent RNA polymerase (RDRP) and polyhedrin segments. Stephensi is *An. stephensi* SDA500 and Ngousso is *An. coluzzii* Ngousso strain.

DISCUSSION

Here we show that AnCPV can be transmitted as an arbovirus from an infected *Anopheles* line to an uninfected one by feeding on an infected vertebrate host, a mode of transmission typical of arboviruses. In addition, we observed that AnCV and AnCPV, which are two distinct ISVs can be used in an artificial infective blood meal model with an *Anopheles* virus-free line in order to study interactions between *Anopheles* and RNA viruses. In this model, while *An. stephensi* ingested both viruses in the infective bloodmeal, only AnCPV appears to infect the midgut epithelium or disseminate, while AnCV appears unable to infect and/or replicate in the epithelium (Fig. 13A). This result is consistent with the major role of the midgut barrier in mosquito competence to viruses, including arboviruses (Franz et al., 2015; Smith et al., 2008). AnCPV infection appears to impose a fitness cost on *An. stephensi*, as infected females displayed higher mortality as compared to females from the non-infected control group.

RESULTS

Using this artificial infection system, we also performed a transcriptomic analysis of AnCPV infection. The primary infection of *An. stephensi* with AnCPV on day 3 post-bloodmeal significantly changed expression of 261 genes (Fig. 15). Genes involved in apoptosis and the Toll immune pathway, among others, are regulated after midgut infection, suggesting an implication of both cellular and humoral factors in the control of AnCPV (Table 3). In persistently co-infected *An. coluzzii*, Toll and JAK/STAT pathways controls AnCPV during the disseminated infection, although the RNAi and Imd pathways play no significant role in control of either virus (Chapter 3.2).

In the current study, we found 171 genes (Fig. 17) that are commonly modulated in the two time points, day 3 and day 7 post-ingestion, and that correspond to the primary infection with AnCPV and the disseminated infection with AnCPV respectively. Most of the regulated genes have unknown molecular and biological functions. Candidate genes from the RNAseq experiment need to be individually validated before follow-up functional studies, for example gene silencing and virus challenge. Strong candidates for functional study include ADP-ribosylation factor-like 6 (Arl), salivary gland protein-1 and LRIM15. ADP-ribosylation factor-like 6 (Arl) is involved in diverse biological processes such as immune response to viruses, DNA repair, mitosis, transcription and signalling (Eckei et al., 2017). Leucine-rich repeat immune proteins (LRIM) are a large protein family in mosquitoes. In *Anopheles*, LRIM proteins are involved in the control of *Plasmodium* infection, for instance LRIM1, is an antagonist of *P. berghei* in *Anopheles* (Povelones et al., 2009). LRIM4 is upregulated during the primary midgut infection of ONNV in *An. coluzzii*, whereas LRIM10 is downregulated, highlighting the complexity and the multifunction of these proteins (Carissimo et al., 2018).

For the first time to our knowledge, we showed that an ISV can be transmitted by a mode like an arbovirus. This highlights the need for a better characterization of other ISVs in evaluating their potential impact in terms of public health. In addition, it is still necessary to assess the influence of these ISVs on *Plasmodium* and ONNV infection of the mosquito. Nothing is known about the potential impact of these viruses on the vector competence of *Anopheles* for other pathogens.

In our study, we also exposed Vero cells to both viruses. Cytopathic effects such as cytolysis, cellular individualization or detached cells are indicators of arbovirus replication (Barreto-Vieira et al., 2017). While AnCV was not transmitted to the *An. stephensi* mosquitoes fed on an infected rabbit or virus-containing larval extract, curiously AnCV can replicate in Vero cells

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(Fig. 12C) with no cytopathic effects, and is detectable in rabbit blood. Therefore, we could not exclude that AnCV could also be transmitted as an arbovirus to a different mosquito host.

The detection of viruses in the rabbits, as well as mosquito feeding on them, was done each time at 1 week after feeding of infected mosquitoes on the rabbit, suggesting that viruses are replicating rather than passively persisting in rabbit blood. Antibodies against viral proteins would be a useful tool to demonstrate replication in the rabbit, which would strengthen the interpretation that they are cryptic arboviruses. However, the low viral loads of both viruses in rabbit blood are also characteristic of arboviral infections in vertebrates. In addition to low viremia, the genome of arboviruses can disappear after a few weeks in vertebrate hosts, while still transmitting as an asymptomatic and molecularly negative host (Duong et al., 2015). For these reasons, serological assays are gold standard tests to detect arbovirus infection in vertebrates because some antibodies are more persistent than arbovirus genomes in blood (LaBeaud et al., 2015; Tigoi et al., 2015).

AnCPV is the most genetically polymorphic of the two viruses. In addition, the suite of mutations found in AnCPV vary between the two mosquito species in the study. The synonymous and nonsynonymous substitutions of AnCPV in *An. gambiae* and *An. stephensi* are consistent with the high rate of error of the RNA polymerase, leading to rapid evolution and generation of quasispecies (Andino and Domingo, 2015). In contrast, though, sequences of AnCV are virtually devoid of variation, even though both viruses display worldwide distribution in *Anopheles* (Carissimo et al., 2016), suggesting radically different mechanisms for their successful host adaptation. Many mutations have also been discovered in ONNV over 35 years, but information on genomic adaptation, if any, between the different *Anopheles* vector species is lacking (Lanciotti et al., 1998). High polymorphism and high evolution rate of AnCPV could also be involved in its adaptation to new species and may explain the infection by AnCPV of *An. stephensi* in the current study, which was not infectable by AnCV. Mutation of CHIKV allowed its adaptation to *Ae. albopictus*, provoking new outbreaks in Indian Ocean countries (Kek et al., 2014; Schuffenecker et al., 2006).

RESULTS

CONCLUSIONS

An experimental model was established for AnCPV infection of *Anopheles* by infective bloodmeal, in order to better study the interactions between AnCPV and *Anopheles*. The primary and disseminated infections of *An. stephensi* by AnCPV modulated expression of genes belonging to immune responses, metabolic pathways, unknown genes and others. *An. stephensi* infected with AnCPV died faster as compared to control mosquitoes fed a normal bloodmeal. AnCPV was transmitted by a mode like an arbovirus through a rabbit to uninfected *Anopheles*. Sequences of AnCPV were highly polymorphic in individual mosquitoes, while AnCV displays only minor variation.

METHODS

Mosquito colonies

Anopheles rearing is described in article II of this thesis.

Mosquito and blood feeding

An. coluzzii (Ngouso colony) and *An. stephensi* mosquitoes from CEPIA were maintained on rabbits. *An. stephensi* from Lille were blood fed on mouse. Mice were anesthetized by an intra-peritoneal injection of 80 µl of a mixing of Rompun 2% (Bayer), ketamine (imalgene 1000) and 1X PBS (0.5:1:2.5). Rabbits (*Oryctolagus cuniculus*) were anesthetized by 1.8ml of intramuscular injection of ketamine and Rompun (1:1.25). The duration of blood meal was between 15 and 30 mins.

RNA extraction, cDNA synthesis, RT-PCR, SYBR Green and duplex taqman

RNA extraction, cDNA synthesis, RT-PCR, SYBR Green qPCR and duplex Taqman RT-qPCR are described in article II of this thesis. The list of primers is given in table 4.

Tableau 4: List of primers

RT-PCR primers		
Viruses/Genes	Primers	Sequences
AnCV	AnCV-F	CAAGGAGGCTTTTGTAGTGC
	AnCV-R	GCTTTGGGTAAGCTGTCCGAG
AnCPV	AnCPV-F	TGAGCGAATCGTGACCATG
	AnCPV-R	GGTTTTCCGACTAGCCTTCC
Ag-S7	Ag-S7-F	AGGCGATCATCATCTACGTGC
	Ag-S7-R	GTAGCTGCTGCAAACCTCGG
qPCR-sybr green		

RESULTS

18S rRNA	18S-qF	ATCAGATACCGTCGTAGTTC
	18S-qR	TTCCGTCAATTCCTTTAAG
qPCR-Taqman		
AnCV	AnCV-Taq-F	ACCAGGAAAAGAACGACGTAGACA
	AnCV-Taq-R	CGCACCCCTTAACAGCTTTGG
	AnCV-Probe (FAM)	TTTCATGCGCAGGCTCGACAGC
AnCPV	AnCPV-Taq-F	CAGCTCAAGGTAAACAGGTTGGT
	AnCPV-Taq-R	CGTCTGAGTTGTCGCGAATAAC
	AnCPV-Probe (VIC)	ATTCAATGGACCTTCAAGAC
Ag-S7	Ag-S7- Taq-F	CAAGCGTATCCGCGTCAAG
	Ag-S7- Taq-R	GGTGGTCTGCTGGTTCTTATCC
	Ag-S7- Probe (NED)	TCGACGGATCCAGCTGATAAAGGTG
Aste-S7	ASTE-S7-Taq-F	TGGTGCCTGAATTGGAGAAG
	ASTE-S7-Taq-R	GCGACGCTCCGCAATG
	ASTE-S7-Probe (NED)	AGTTCTCCGCAAGCACGTCGTGT

***Anopheles coluzzii* extract preparation and infective blood meal**

Approximately 0.5 g of *An. coluzzii* larvae (L3-L4) were homogenized in 2.5ml of 1X PBS (pH 7), and centrifuged at 12000g for 15 min at 4°C. The supernatant from centrifugation was transferred into a new tube and was centrifuged again for 5 min (12 000xg at 4°C). The final supernatant was successively passed through 5µm (Non-pyrogenic filter, Sartorius Stedium Biotech) and 0.2µm (Acrodisc syringe filter, PALL life sciences) filters in order to remove debris, fungi and bacteria. The final filtered extract was mixed with human blood (1:6) and was given to 7 h starved mosquitoes. A membrane-based human blood-feeding system was pre-heated at 37°C before the blood feeding.

Infection of Vero cells

VeroE6 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin+Streptomycin (PS) mix 1/10000 (Gibco,) at 37°C and 5% CO₂.

24 hours before infection, cells were seeded at a density of 1e⁵ cells per well in 24 well plates. The *An. coluzzii* larvae carrying AnCV and AnCPV are toxic for Vero cells. Therefore, 0.5 g of adults was instead used to prepare extract for infecting Vero cells.

For the day of infection, complete media was removed and replaced either with 200 µL Ngousso adult extract diluted in PBS or with 200µL of serum free media (non-infected controls) for 2 hours.

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After 2 hours of incubation, mosquito extracts and serum free media were removed, and cells were washed twice in PBS before adding DMEM + 2% FBS (Gibco) + PS (1/10000).

Supernatants were collected on days 3, 5 and 7 post-infection. After centrifugation at 300g, 4°C for 5 minutes, 250µL of supernatant were mixed with 500µL of TriReagent (Zymo Research). RNA extractions were performed as described in article II of this thesis.

RNA and RNAseq

Sample Quality Assessment: Total mosquito RNA isolates were quantified by using a fluorimetric RiboGreen assay. Total RNA integrity was assessed by Agilent BioAnalyzer 2100, generating an RNA Integrity Number (RIN). To pass the initial QC step for library production, samples must be quantified at least 1 µg and have a RIN of 8 or greater.

Library Creation: Total RNA samples were converted to Illumina sequencing libraries using Illumina Truseq RNA Sample Preparation Kit (Cat. # RS-122-2001 or RS-122-2002 or stranded mRNA Sample Preparation kit (Cat. # RS-122-2101). Briefly, for the Stranded Total RNA protocol, ribosomal RNA was depleted from total RNA using Ribozero beads. After rRNA depletion, the remaining RNA was fragmented and primed for cDNA synthesis. The cDNA was blunt-ended, ligated to index adapters and amplified for 15 cycles. For the Stranded mRNA workflow, total RNA was purified using oligo-dT coated magnetic beads, fragmented and then reverse transcribed into cDNA. The cDNA was adenylated then ligated to dual-indexed (barcoded) adapters and amplified using 15 cycles of PCR. Final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen). Indexed libraries were then normalized and pooled.

Cluster generation and sequencing: Truseq libraries were hybridized to the NextSeq flowcell (either Single Read or Paired End). Clustering occurs on-board where the bound library molecules are clonally amplified and sequenced using Illumina's SBS chemistry. NextSeq uses 2-color chemistry to image the clusters. Upon completion of read 1, a 7 base pair index read was performed in the case of single indexed libraries. If dual indexing was used during library preparation, 2 separate strands 8 or 10 base pair index reads were performed. Finally, the clustered library fragments were re-synthesized in the reverse direction thus producing the template for paired end read 2.

Primary analysis and demultiplexing: Base call (.bcl) files for each cycle of sequencing are generated by Illumina Real Time Analysis (RTA) software. The base call files and run folders were streamed to servers maintained at the Minnesota Supercomputing Institute. Primary analysis and de-multiplexing were performed using Illumina's bcl2fastq v2.20. The end result

RESULTS

of the bcl2fastq workflow was demultiplexed FASTQ files that were used for subsequent analysis.

RNAseq experiment analysis

The quality of the raw reads was checked with FastQC version 0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and multiqc version 0.7 (Ewels et al., 2016) and cutadapt version 1.9.1 (<https://doi.org/10.14806/ej.17.1.200>) was used to correct the reads and to remove adapters sequences (parameters $-u$ 10, $-m$ 30 and $-q$ 30). STAR version 2.5.0a (Dobin et al., 2013) with default parameters was used for alignment against the reference genome of *Anopheles stephensi* str. SDA500 version AsteS1 (VectorBase). Genes were counted using featureCounts (Liao et al., 2014) version 1.4.6-p3 with the annotation version AsteS1.6 and the parameters $-t$ gene $-g$ ID.

Counts data were analyzed using R version 3.3.1 (R Core Team) and the Bioconductor package DESeq2 version 1.14.1 (Love et al., 2014) using default parameters. Since the two experiments (day 3 and day 7) were done on mosquitoes of different ages, a distinct analysis was done for each experiment. Thus for both analyses a generalized linear model including the treatment (infected vs non-infected) was set up in order to test for the infection effect. Raw p-values were adjusted for multiple testing using the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Genes with adjusted p-values below 0.05 were considered to be differentially expressed.

De novo-assembly

The trimmed reads were mapped by using STAR software to the reference genomes, *An. gambiae* genome (Agam P3.8) and *An. stephensi* (AsteS1). Unmapped reads were de novo assembled into contigs using the assembly software Velvet/ AOSSES with a range of k-mer values from 11 to 67. Assembled contigs were examined for similarity to known viruses by BLAST searches against the NCBI virus genome database.

Statistical analysis

All experiments were carried out at least in 2 independent replicates. Survival curves were generated by R package and the cox proportional hazards regression analysis was used to make statistical analysis.

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3.4 Article IV (In preparation): DNA forms and host range of partiti-like virus in *Anopheles stephensi*

3.4 Article IV (In preparation): DNA forms and host range of partiti-like virus in *Anopheles stephensi*

ABSTRACT

The virome of the Asian malaria vector *Anopheles stephensi* has not previously been described. Here, high throughput sequencing and de novo assembly was used to explore the virome of *An. stephensi*. We discovered a partiti-like virus and a chaq-like virus in the virome. Both viruses were found to replicate in *An. stephensi* while no replication was detected in the African malaria vector *An. coluzzii* or in mammalian cell lines. Vertical transmission was involved in viral persistence in *An. stephensi*, and DNA forms were detected of the partiti-like virus. DNA forms of nonretroviral RNA viruses can be produced and integrated into the host genome by endogenous retrotransposon activities. The integrated viral DNA forms are called non-retroviral integrated RNA virus sequences (NIRVS). The impact of NIRVS on mosquito biology including immunity and vector competence is unknown, and their presence reveals a co-evolutionary potential between viruses and mosquitoes.

Keywords: *Anopheles stephensi*, malaria, virome, sequencing

RESULTS

INTRODUCTION

Anopheles stephensi is the vector of malaria in Asia where it mainly transmits *Plasmodium vivax* and *P. falciparum* (Thomas et al., 2017). Malaria is endemic in 19 Asian countries, and South-East Asia is the most affected subregion, with approximately 27 000 deaths in 2016 (Bhatia et al., 2013; WHO, 2017). *An. stephensi* is widespread and is an anthrozoophilic mosquito (Thomas et al., 2017). Although *An. stephensi* has not been considered as a vector for arboviruses, a *Cypovirus* was identified in the midgut of *An. stephensi*, but it was not sequenced or molecularly characterized, and experimental infection experiments indicated that the mosquito is competent for Chikungunya virus (CHIKV, *Togaviridae*, *Alphavirus*) (Bird et al., 1972; Yadav et al., 2003). These studies imply that *An. stephensi* may be infected with unknown viruses in nature, and their co-infection with malaria parasites could potentially influence its malaria vector competence.

The insect virome includes insect specific viruses (ISVs) which replicate only in insects, and arboviruses which replicate in insects and vertebrates. *Aedes* and *Culex* are the main mosquitoes involved in the transmission of arboviruses such as dengue virus (DENV, *Flaviviridae* *Flavivirus*), West Nile virus (WNV, *Flaviviridae*, *Flavivirus*), Rift Valley fever virus (RVFV, *Phenuiviridae*, *Phlebovirus*) CHIKV, and others (Nanfack Minkeu and Vernick, 2018; Ruckert et al., 2017). *Anopheles* vector competence to most arboviruses and host restriction mechanisms are unknown. *Aedes aegypti* lines were refractory to *Anopheles* C virus and *Anopheles* cypovirus, both discovered in the African malaria vector *An. coluzzii*, but at least AnCPV can replicate in *An. stephensi*, suggesting potential adaptation to *Anopheles* (Carissimo et al., 2016). Because of their non-pathogenicity to vertebrates, ISVs are candidates for a simple benchtop model to study interactions between *Anopheles* and RNA viruses (Carissimo et al., 2016). Interestingly, ISV sequences have been found in insect genomes, suggesting that ISVs and their insect hosts have co-evolved. Moreover, DNA forms of arboviruses may allow them to be tolerated by their hosts (Goic et al., 2016; Lequime and Lambrechts, 2017; Suzuki et al., 2017). Generation of DNA forms by RNA viruses is necessary for their integration into the host genome, a process called endogenization. Integrated viruses into host genomes are named endogenous viral elements (EVEs) or non-retroviral integrated RNA virus sequences (NIRVS) for non-retroviruses (Lequime and Lambrechts, 2017; Palatini et al., 2017; Suzuki et al., 2017). *Aedes* mosquitoes have more described NIRVS compared to *Anopheles* mosquitoes and no

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NIRVS have been described to date in *An. stephensi* (Palatini et al., 2017). In some *Anopheles* species, NIRVS and EVEs are described in the families *Rhabdoviridae*, *Flaviviridae*, *Reoviridae*, *Bunyaviridae* but no EVE and nor NIRVS of the family *Partitiviridae* have been described (Lequime and Lambrechts, 2017; Palatini et al., 2017).

Partitiviruses infect mainly plants, fungi and protozoa and develop persistent infection in their hosts but the mechanism behind their persistence is not known (Liu et al., 2010; Nibert et al., 2014). Partitiviruses carry a double stranded RNA genome comprised of two open reading frames (ORFs), encoding for an RNA-dependent RNA polymerase (RdRp) and a capsid respectively (Nibert et al., 2014). Partitiviruses have been observed in insects but their transmission modes are unknown (Fauver et al., 2016; Webster et al., 2015).

In this study, genomes of a partiti-like virus and chaq-like virus were reconstructed from RNA sequencing of individual *An. stephensi* mosquitoes. They are both specific for *An. stephensi* and are both absent from the *An. coluzzii* Ngousso strain. DNA forms of partiti-like virus were identified but the structure of the DNA molecules is unknown, and we find no evidence of virus DNA integration into the host genome.

RESULTS

Discovery of partiti-like virus and chaq-like virus in *Anopheles stephensi* by deep sequencing

Bird and co-authors previously detected a cypovirus in *An. stephensi* Delhi strain by microscopy of Giemsa-stained midguts, by observation of the characteristic cytoplasmic polyhedrosis inclusions (Bird et al., 1972). However, to our knowledge, to date there has not been a metagenomic virome discovery project in *An. stephensi*. We carried out deep sequencing and de novo assembly for virome discovery in RNA from the *An. stephensi* SDA500 strain maintained in the CEPIA facility of *Institut Pasteur-Paris*. Sequence contigs corresponding to the RdRp and the capsid segments of partiti-like virus (PV) were reconstructed from RNA sequenced from individual whole *An. stephensi*. In addition to PV, a contig corresponding to a chaq-like virus was also reconstructed, with higher read coverage suggesting that this latter is more abundant as compared to PV.

RESULTS

The presence of partiti-like virus and chaq-like virus was confirmed by RT-qPCR in both adults and larvae of *An. stephensi*, suggesting these viruses are vertically transmitted in this Asian vector. We designed RT-qPCR primers targeting the polymerase of each virus. Interestingly, these viruses were not detected in *An. coluzzii* (Ngousso strain) which are kept in another Insectary of the *Institut Pasteur CEPIA* facility (Fig. 20).

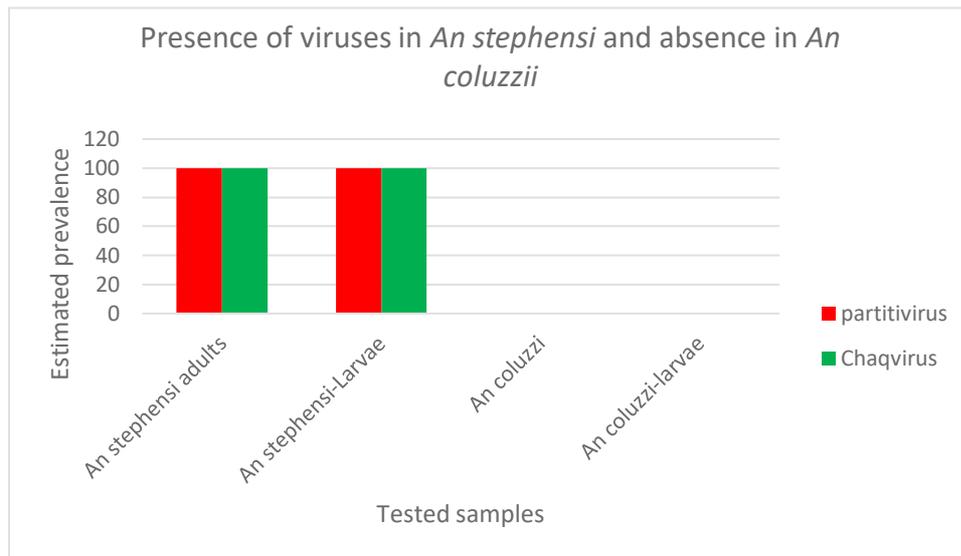


Figure 20: Detection of partiti-like virus (PV) and chaq-like virus in *An. stephensi* and their absence in *An. coluzzii*. Prevalence is estimated because individual and mosquito pools were tested. In different replicates, all tested *An. stephensi* were positive for both viruses and all tested *An. coluzzii* were negative.

Specificity of partiti-like virus and chaq-like virus in *An. stephensi*

The absence of partiti-like virus and chaq-like virus in *An. coluzzii* allowed us to test whether it was possible to transmit them to uninfected mosquitoes by using oral infection of larvae, infective bloodmeal and injection of viruses (Carissimo et al., 2016; Waldock et al., 2012). In the absence of isolated partiti-like virus and chaq-like virus, we used extract of infected *An. stephensi* larvae or adults as source of viruses.

All *An. coluzzii* larvae that were fed with chaq-like virus and partiti-like virus remained negative for both viruses. The same results were obtained when *An. coluzzii* adults were fed with a mix of human blood and both viruses. But, when both viruses were injected in *An. coluzzii* adults, partiti-like virus (Fig. 21a) and chaq-like virus (Fig. 21b) were found at day 3 and 7 post injection, suggesting that bypassing the midgut barrier allowed both viruses to infect in *An.*

RESULTS

coluzzii. Comparison of viral loads 3 and 7 d time points demonstrated a decrease of partiti-like virus (Fig. 21c) and chaq-like virus (Fig. 21d) in *An. coluzzii* after injection. Taken together, these results indicate that *An. coluzzii* is not permissive to either of these viruses.

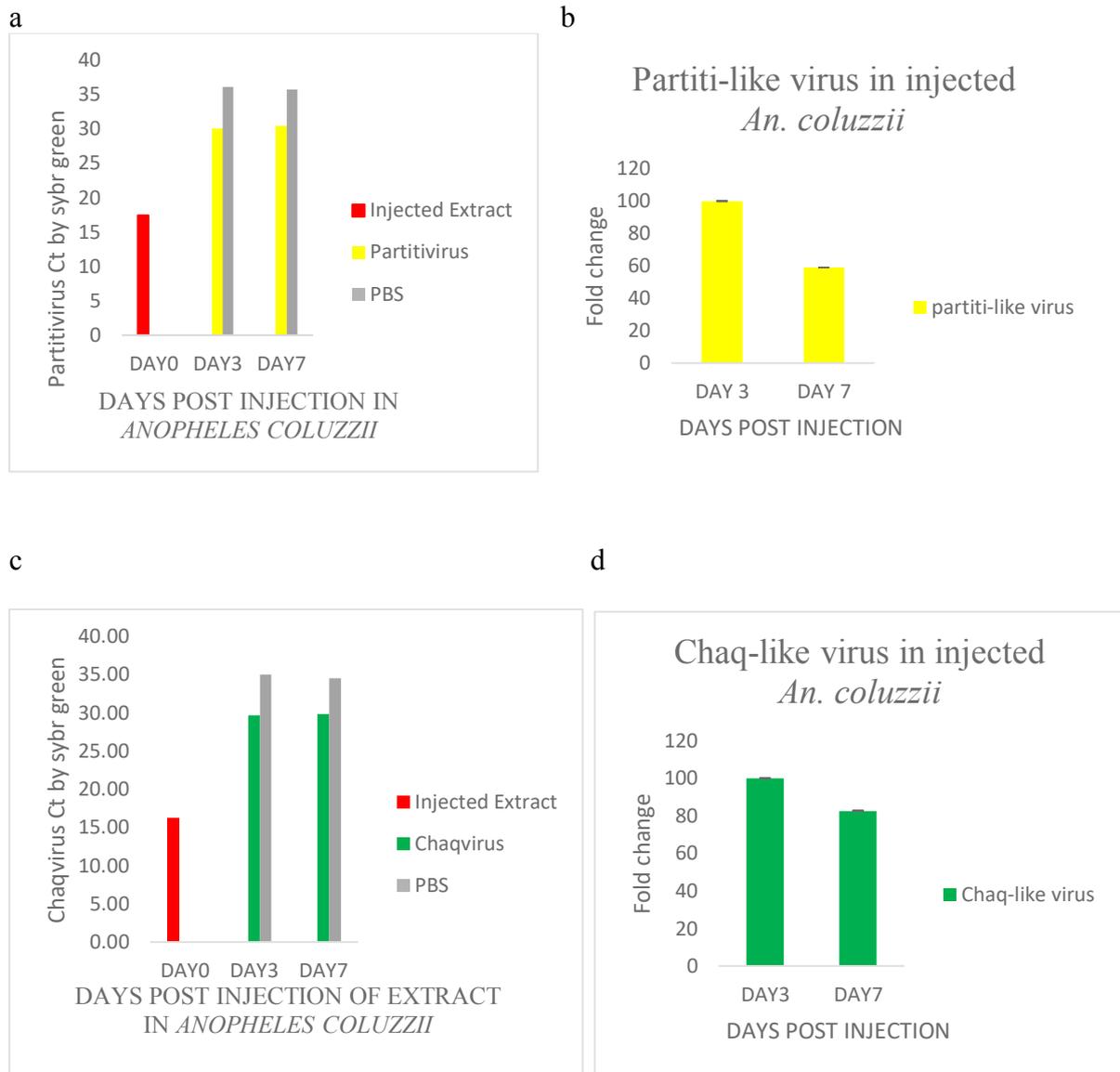


Figure 21: *An. coluzzii* (Ngouso strain) is refractory to partiti-like virus and chaq-like virus. A: Detection of partiti-like virus (PV) in *An. coluzzii* after injection, PBS is used as negative control and Ct greater than 35 are considered as negative samples. B: The PV load decreases in *An. coluzzii* after injection. C: Detection of chaq-like virus in *An. coluzzii* after injection. D: The chaq-like virus load decreases in *An. coluzzii* after injection.

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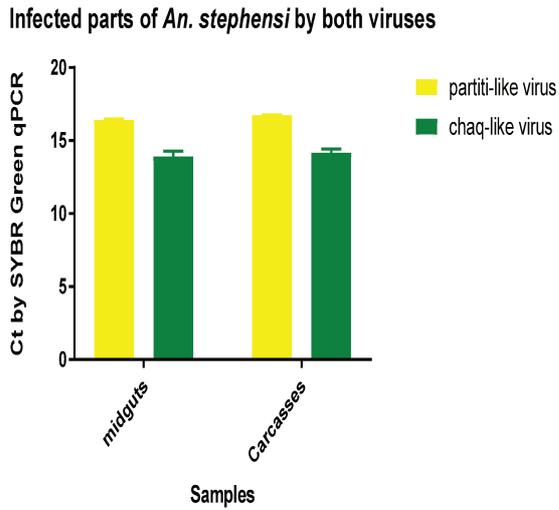
Partiti-like virus and chaq-like virus replicate in *An. stephensi*

We did not sequence small RNA in *An. stephensi*, and thus were not able to interrogate whether viral RNAs generated by the RNAi pathway were generated for the two viruses. (Carissimo et al., 2016; Webster et al., 2015). We measured their presence in other tissues in infected *An. stephensi* in the midgut and the carcass of the same *An. stephensi* pools.

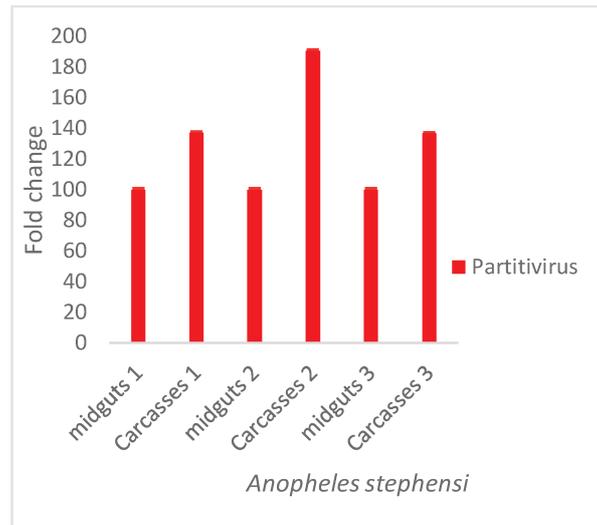
Both viruses were detected by RT-qPCR in the midguts and the carcasses of *An. stephensi*, indicating that they are replicating (Fig. 22A). The partiti-like virus load was higher in the carcasses as compared to the midguts (Fig. 22B). There was almost no difference in the chaq-like virus load between the carcasses and the midguts of *An. stephensi* (Fig. 22C). Infection of the mammalian cell Vero line showed no cytopathic effect and no replication of partiti-like virus and chaq-like virus (Table 5), indicating that they are insect specific viruses with replication only in *An. stephensi* or we did not find a compatible cell line. Absence of replication of both viruses in *An. coluzzii* cell line 4a-3A may indicate an overall lack of permissiveness of *An. coluzzii* to chaq-like virus and partiti-like virus infection.

RESULTS

a



b -Partiti-like dissemination



c

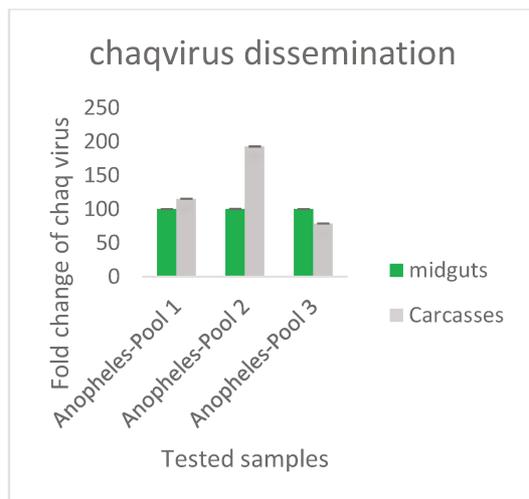


Figure 22: Dissemination of both viruses in *An. stephensi*. A: Chaq-like virus and partiti-like virus (PV) are present in the midgut and the carcasses of *An. stephensi*. B: Comparison of the partiti-like virus load in the carcasses and the midgut. C: Comparison of the chaq-like virus load in the carcasses and the midgut.

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Tableau 5: Absence of replication of chaq-like virus and partiti-like virus in cell lines. Mammalian Vero cell line and *An. gambiae* cell line 4a-3A. Supernatants were tested by qPCR on 0, 2 and 5 days post infection.

Viruses	Cell lines	Vero cell line	4a-3A
Chaq-like virus replication		No	No
Partiti-like virus replication		No	No

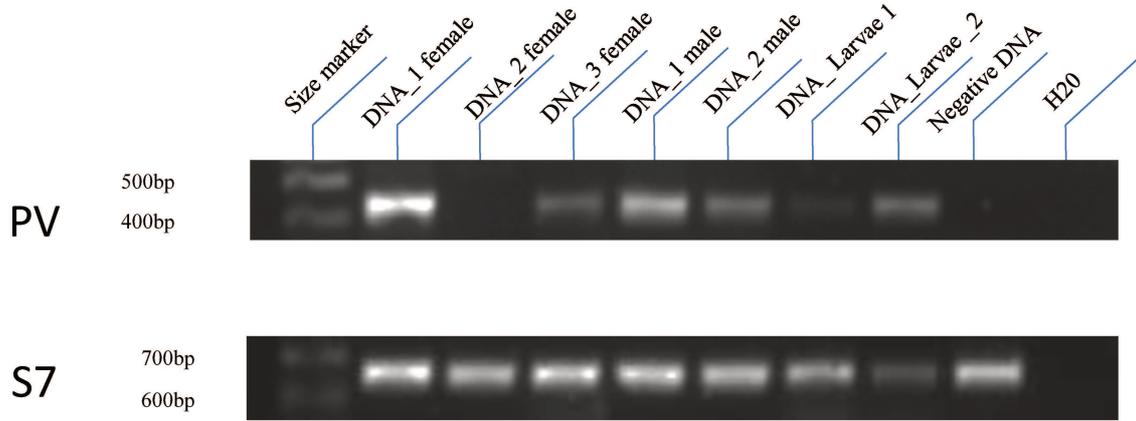
***An. stephensi* produces non-integrated DNA forms of partiti-like virus**

Partiti-like virus has been described from plants and other insects but very limited information was obtained on chaq-like virus from the literature (Fauver et al., 2016; Webster et al., 2015). For instance, chaq-like virus is considered as an unassigned virus or a satellite virus (Webster et al., 2015). For these reasons, partiti-like virus was used to assay for the presence of possible DNA forms by *in silico* and *in vivo* analysis. *In silico* analyses are limited by information availability and genome annotation.

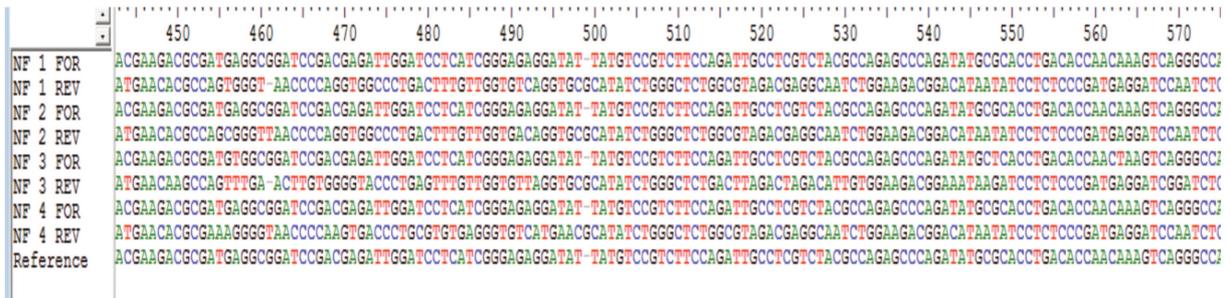
The expected size of partiti-like virus amplicons was obtained after its amplification from the genomic DNA of *An. stephensi* (Fig. 23A), indicating that this mosquito harbors DNA forms of partiti-like virus. DNA forms of partiti-like virus were detected in male, females and larvae. The DNA forms of partiti-like were confirmed in *An. stephensi* using Sanger sequencing. This sequence matched the assembled contigs found by the metagenomic approach (Fig. 23B). Moreover, we also detected DNA forms of partiti-like virus in *An. stephensi* Indian strain from Virginia Tech, USA (Fig. 23C). However, when the partiti-like virus genome, capsid and polymerase segments were blasted against *Anopheles* genomes (*An. stephensi* Indian and SDA500 strains), no match was obtained, suggesting that it is not integrated into the genome of these *Anopheles* strains. Fluorescence *in situ* hybridization (FISH) experiments further supports the non-integration of partiti-like virus in *Anopheles* genomes. Our FISH results did not detect integration of partiti-like virus in polytene chromosomes, suggesting that the DNA forms may be cytoplasmic and possibly episomal in the cells (Fig. 23D).

RESULTS

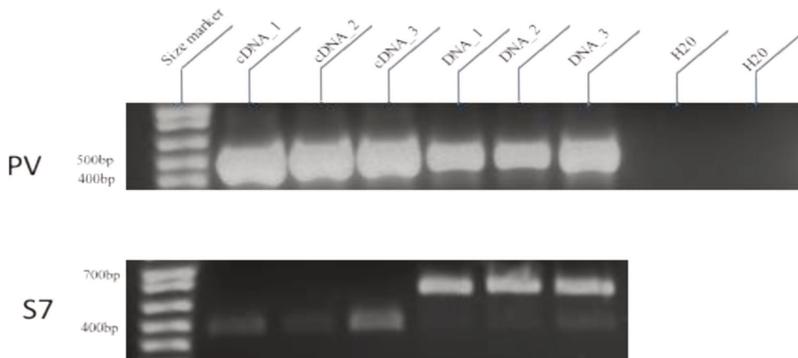
a



b



c



RESULTS

d

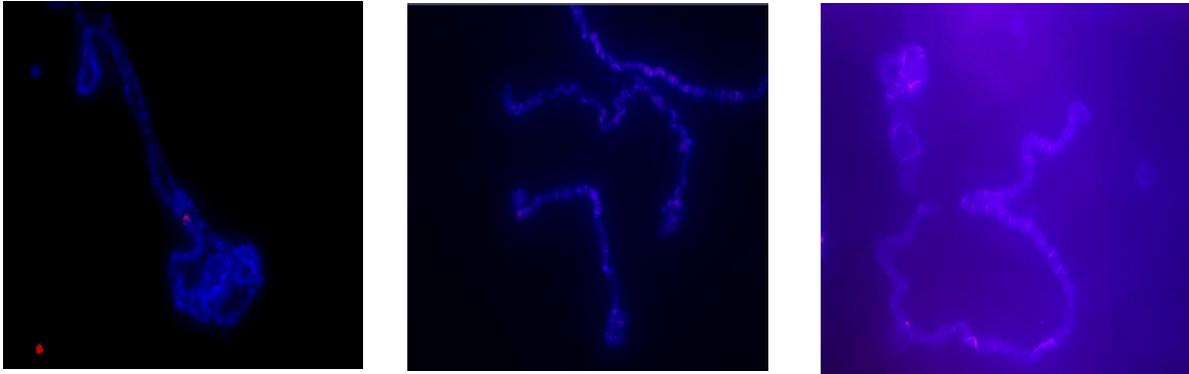


Figure 23: *An. stephensi* harbors the DNA forms of the non-retrovirus partiti-like virus (PV). A) DNA detection of PV by PCR in females, males and larvae of *An. stephensi*. B) DNA amplicon from different mosquito pools match with partiti-like virus. C) DNA forms of partiti-like virus in *An. stephensi*, india strain from Virginia Tech in the United States. D) Absence of consistent signals by using FISH on polytene chromosomes from 3 replicates. DAPI was used during revelation and PV was labeled with Cy3.

DISCUSSION

Anopheles virus discovery is important to study interactions between *Anopheles* and viruses and to prevent arbovirus outbreaks from unknown viruses. To contribute to the knowledge of the *Anopheles* virome, individual *An. stephensi* mosquitoes from a laboratory strain from *Institut Pasteur*, Paris were subjected to RNA sequencing. De novo assembly was used to reveal the presence of contigs related to partiti-like virus and chaq-like virus in this Asian malaria vector and both viruses are maintained in *An. stephensi* by vertical transmission (Fig. 20). *Culex* flavivirus in *Cx pipiens*, *Anopheles* cypovirus and *Anopheles* C virus in *An. coluzzii* similarly persist by vertical transmission and they are detectable in larvae, male and female adults (Bolling et al., 2012; Carissimo et al., 2016; Nanfack Minkeu and Vernick, 2018).

Partial sequences of chaq-like virus and partiti-like virus were obtained by metagenomic studies in *Drosophila* and *Anopheles* species collected in United Kingdom and Africa but those studies were unable to assign a genus/family to chaqvirus (Fauver et al., 2016; Webster et al., 2016; Webster et al., 2015). These authors did not perform *in vivo* studies, and replication was shown

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by *in-silico* analysis (Webster et al., 2015). Here, the replication of chaq- like virus and partiti-like virus was confirmed in *An. stephensi* as demonstrated by their detection both midgut and the carcasses. In parallel, *An. coluzzii* (Ngousso strain) appeared refractory to both viruses, and the only route to transmit them to this African malaria vector was bypassing the midgut barrier (Fig. 21), suggesting a specificity or tropism of chaq-like virus and partiti-like virus to *An. stephensi*. Virus tropism is more studied with mosquito tissues, for example Sindbis virus tropism to haemocytes in *Ae. aegypti* (Parikh et al., 2009). In this latter mosquito, DENV-2 tropism to the midgut, the neural tissue and the salivary glands was demonstrated (Salazar et al., 2007).

Partiti-like virus and chaq-like virus did not display replication in the mammalian Vero cell line (Table 5), but their replication in *An. stephensi* was observed in this study (Fig. 22). Both viruses have been also reported to replicate in *Drosophila* (Webster et al., 2015). These data indicate that they are probably insect specific viruses, and support idea that some ISVs are shared by a broad range of arthropods. A commensal relation is established between many ISVs and their hosts.

Our BLAST and FISH analysis did not provide evidence of partiti-like virus integration. Integration of RNA viruses into host genomes appears to be a rare phenomenon. The proposed mechanism includes two main steps, the production of cDNA by reverse transcription followed by integration into the host genome. Integration of non-retroviruses in host genomes open new perspectives to paleovirology which studies the evolution and influence of ancient viruses on host biology (Patel et al., 2011). Most paleoviruses are retroviruses which infect the germline of their hosts.

CONCLUSIONS

We found two viruses in *An. stephensi*: one is related to chaq-like virus, an unclassified virus and the other is related to partiti- like virus belonging to the family *Partitiviridae* which infect mainly plants, fungi and protozoa. Both viruses were found to replicate in *An. stephensi* but they did not replicate in the mammalian Vero cell line, suggesting that they could be ISVs. *Anopheles coluzzii* was refractory to both viruses, and partiti-like virus was also found as unintegrated DNA in *An. stephensi*.

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METHODS

Mosquito colonies

Anopheles rearing is described in article II of this thesis.

DNA extraction

The reagent, DNAzol (Life Technologies) was used to extract the genomic DNA. Single or mosquito pools were macerated with 100µl DNAzol (500µl for mosquito pools) and a pestle motor mixer (ARGOS), and the homogenate was centrifuged at 11'000 RPM for 10 min at 15°C. The supernatant containing DNA was transferred to a new tube. DNA precipitation was carried out with 0.5 DNAzol volume of absolute ethanol, followed by gentle mixing at room temperature for 1min. The pellet was visible after a centrifugation at 11 000 RPM for 10 min at 15°C. The supernatant was decanted and the DNA washed with 1ml of 75% ethanol and a centrifugation at 11'000 RPM for 10 min at 15°C. This wash step was repeated. An additional centrifugation (11 000RPM/10 min./15°C) was performed to eliminate any residual ethanol, and the pellet was then dried at room temperature for 30 min. DNA was re-suspended with 100µl nuclease-free water followed by a centrifugation at 11'000 RPM for 1 min at 15°C.

RNA extraction, cDNA synthesis, RT-PCR and qPCR

Extraction of RNA, cDNA synthesis and RT-PCR, and PCR are described in article II of this thesis. A SYBR Green qPCR was performed using the SsoAdvanced universal SYBR®Green Kit (BIO-RAD) following the manufacturer's instructions. The Sybr green primers for *Anopheles* transcripts were designed using Primer-Blast to produce product sizes between 140-200bp. Gene expression normalization was done using the ribosomal protein-coding gene, S7. The list of primers is given in table 6.

Table 6: List of primers for the detection of partiti-like virus and chaq-like virus.

RT-PCR primers		
Viruses/Genes	Primers	Sequences
Partiti-like virus (PV)	PV_F	GCTACAGTCCGTCCGAAGAG
	PV_R	TCATGACAGCCTTAGCACCG
S7	S7-F	AGGCGATCATCATCTACGTGC
	S7-R	GTAGCTGCTGCAAACCTTCGG
qPCR-sybr green		
Chaq-like virus	Chaqvirus_qF	AGCAAGTGTCTCTTCCCTG
	Chaqvirus_qR	GCTGAGAGAACC GGAACACA

RESULTS

Partiti- like virus	PV_qF	ACTGGCGTGTTCATTGAGGA
	PV_qR	TCATGACAGCCTTAGCACCG
Ag-S7	Ag_S7_qF	ATCAGATACCGTCGTAGTTC
	Ag_S7_qR	TTCCGTCAATTCCTTTAAG
Aste-S7	Aste_S7_qF	AGGCGATCATCATCTACGTGC
	Aste_S7_qR	CAATGAACACGACGTGCTTG

***Anopheles stephensi* extract preparation and infective blood meal**

Approximately 0.5gr of *An. stephensi* larvae (L3-L4) were crushed in 2.5ml 1XPBS (pH 7), and centrifuged at 12000g for 15 mins at 4°C. The supernatant from centrifugation was transferred into a new tube and was centrifuged again for 5min (12 000xg/4°C). The final supernatant was successively pushed through 5µM (Non-pyrogenic filter, Sartorius stedium biotech) and 0.2µm (Acrodisc syringe filter, PALL life sciences) filters in order to remove debris, fungi and bacteria. The final filtered extract was mixed with human blood (1:6) and was given to 7 hour starved mosquitoes. A membrane-based human blood-feeding system was pre-heated at 37°C before the blood feeding.

The extract of *An. stephensi* larvae has partiti-like virus and chaq-like virus and is used to feed *An. coluzzii* larvae, however it is toxic for the VeroE6 cell line. Therefore, 0.5gr adult was used to prepare extract (In the same way as above) for infecting the vero cell line and to inject *An. coluzzii* (Ngousso colony).

Injection of *Anopheles coluzzii*

Viruses in the *An. stephensi* extract were injected (50 nl) into the thorax of ice-anesthetized 1-2 day old *An. coluzzii* females using the Nanoject II (Drummond Scientific) and glass capillary needle as previously described by (Garver and Dimopoulos, 2007). Three and seven days post-injection, viral loads were measured from total RNA from a pool of five to ten mosquitoes.

Infection of Vero cell line

Infection of Vero cell line and RNA extractions were performed as described in article III of this thesis.

RESULTS

Fluorescence in situ hybridization (FISH) Experiment

Partitivirus DNA was amplified from infected *An. stephensi*. Purification of DNA was carried out using the Wizard®SV Gel and PCR Clean-Up System (Promega, USA). For labelling PV and probe: 100ng purified DNA product was mixed with 15µl random primer (Random Primers DNA Labeling System, Invitrogen) and 1.5µl of water. This mix was heated at 95°C for 5min, and then cooled in ice. Three deoxy triphosphates (dATP, dCTP, dGTP) of 2 µl each, 1 µl of cyanine 3 (Cy3) and 1 µl of Klenow were added to the cooled mix and subsequently incubated at 37°C for 90 min. The reaction was stopped with 1µl 0.5M EDTA, 1/10 volume of 3M sodium acetate and 2.5 volume of absolute ethanol. The labelled probe partiti-like virus (PV) was stored at -20°C.

Ovaries of PV-infected half-gravid *An. stephensi* females were dissected, and were prefixed in Carnoy's solution (a 3:1 mixture of ethanol and glacial acetic acid). Coverslips were put on the follicles and gently pressed to squash the cells. Slides were dehydrated for 5 min each in 50, 70, 95, and then 100% ethanol. The dehydrated slides were dried for 20 min. at room temperature (RT) and dissolved dried probes were added to them. Pre-warmed (42°C) hybridization buffer [60% formamide, 10% dextran sulfate and 1.2X sodium chloride (SSC)] was used to dissolve labelled probes. Coverslips were used to cover chromosome preparations with probes and were denatured at 73°C for 5 min. Denatured slides were incubated at 42°C in humid chambers for 5 hours. At the end of incubation, Coverslips were removed and the slides were washed with 1X SSC at 42°C for 20 min. The fluorescent stain, DAPI, was used to view the probed chromosomes. Signals were detected by using a Zeiss laser scanning microscope.

4. GENERAL DISCUSSION

GENERAL DISCUSSION

Despite all efforts made, malaria is one of the deadliest vector-borne diseases in sub-Saharan Africa and South Asia (WHO, 2017). Vector control is essential and remains an efficient tool for reducing malaria transmission by avoiding contact between human malaria vectors (*Anopheles* mosquitoes) and humans. However, with the increase of insecticide resistance in wild populations of *Anopheles* vectors, new vector control strategies are required. Paratransgenesis, aimed at eliminating a pathogen from vector populations through transgenesis of a symbiont of the vector, could be a potential strategy (Ren et al., 2008). Many functional studies of the virus-insect interaction have been done in *Drosophila* because of the availability of non-pathogenic viruses to humans, and genetic tools available in this model. Unfortunately, results from this model are not directly applicable to mosquitoes because *Drosophila* is not a hematophagous insect.

The objectives of this PhD work were to explore the *Anopheles* virome and to biologically characterize two novel viruses identified in the *Anopheles* virome.

Based on our retrospective analysis, we observed that the *Anopheles* virome was diverse and complex with at least 51 viruses identified or associated with this mosquito genus. In addition to the O'nyong nyong arbovirus transmitted by *Anopheles* species, other arboviruses were detected in *Anopheles* mosquitoes but their epidemiological status was often not studied. This situation points out the likely underestimation of arbovirus prevalence in malaria-endemic countries due to limited surveillance. Moreover, there were more insect specific viruses (ISVs) in *Anopheles* species as compared to arboviruses associated with *Anopheles* mosquitoes. Those ISVs that were found to replicate only in *Anopheles* were named *Anopheles* specific viruses (ASVs). Most ASVs are uncharacterized and their interactions with *Anopheles* are under-analyzed.

Focusing mainly on two ASVs: *Anopheles* C virus (AnCV) and *Anopheles* cypovirus (AnCPV), we deciphered the immune responses of *An. coluzzii* during the disseminated infection phase and we found the implication of two signaling pathways in the control of AnCPV. In addition to interacting with their mosquito host, the infections of the two ASVs are not independent, and interact with each other. For the first time, we showed that *Anopheles* mosquitoes use classical signaling pathways of immune response to control ASVs in the natural virome. Indeed, we hypothesize that the host need to limit replication of the ubiquitous members of the natural

GENERAL DISCUSSION

virome to prevent pathogenicity may be the evolutionary source of the selection pressure that shaped the mechanisms of antiviral immunity. These antiviral immune and restriction mechanisms can then be addressed by the host against arboviruses when they are sporadically encountered.

We showed that *Anopheles* cytovirus (AnCPV), which was discovered as a vertically transmitted ISV, can also be transmitted like an arbovirus, because it was transmitted from mosquitoes to a vertebrate and then to uninfected mosquitoes. This finding suggests that the evolutionary pathway from vertical “insect-specific” virus to infectious blood transmission, and potential host-shift and emergence, may be remarkably simple (Fig. 24). A potential implication is that during blood feeding on humans, *Anopheles* females could inoculate unknown viruses in addition or not to *Plasmodium* parasites.

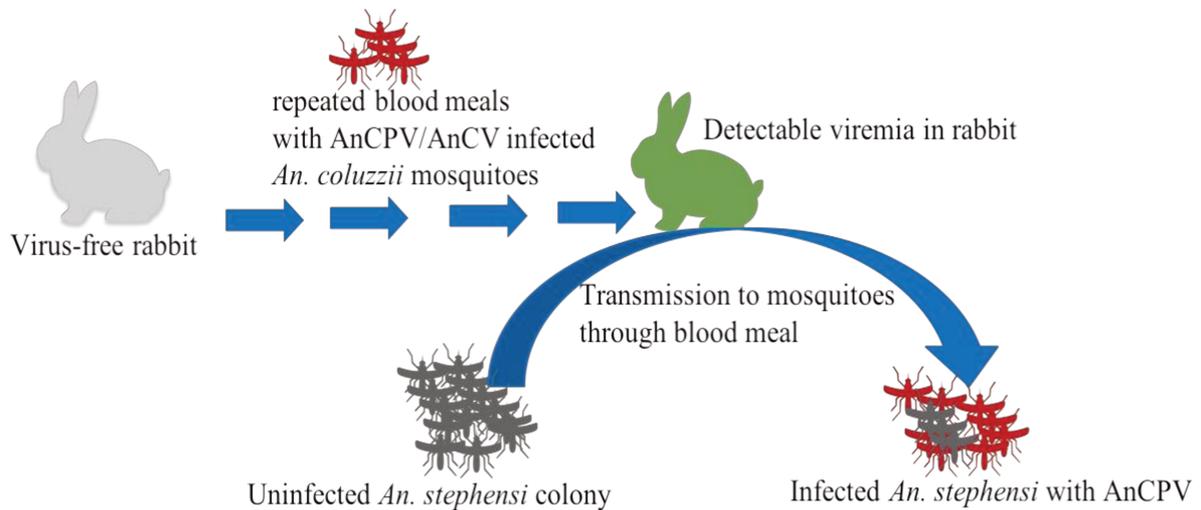


Figure 24: AnCPV is transmitted like an arbovirus between mosquito and vertebrate hosts.

Finally, we discovered a partiti-like virus and a chaq-like virus in *An. stephensi*, thus increasing the discovered repertoire of ISVs. Both viruses were absent from the *An. coluzzii* colonies tested, and attempts to infect *An. coluzzii* with them were unsuccessful, highlighting an apparent specific host restriction mechanism between different species of the same genus.

GENERAL DISCUSSION

Composition and implications of the *Anopheles* virome

Bibliographic work showed that *Anopheles* in addition to transmitting ONNV might also be involved in transmission of other arboviruses such as Venezuelan equine encephalitis virus, Sindbis virus, Semliki Forest virus, Rift Valley fever virus, West Nile virus, Japanese encephalitis virus, Tensaw virus and others (Nanfack Minkeu and Vernick, 2018). All of those arboviruses have been detected in *Anopheles* mosquitoes in nature. Semliki Forest virus (SFV) is phylogenetically close to the arboviruses CHIKV and ONNV within the family *Togaviridae*. Artificial infections showed infectivity to laboratory *Anopheles* mosquitoes of SFV and CHIKV, indicating that under some conditions, *Anopheles* malaria vectors might also be competent to transmit arboviruses of the family *Togaviridae* (Nanfack Minkeu and Vernick, 2018; Vanlandingham et al., 2005; Yadav et al., 2003). An open question is why *Anopheles* mosquitoes are apparently unable to transmit other families of arboviruses such as flaviviruses including DENV, YFV, ZIKV. For instance, *An. albimanus* is refractory to DENV and mechanisms involved in this resistance have not yet been determined (Ramos-Castaneda et al., 2008). Arboviruses are transmitted to vertebrate hosts by bloodfeeding arthropod vectors, and these viruses have the capacity to replicate in both hosts (Kuno and Chang, 2005). This dual replication capacity allows distinguishing biological transmission from mechanical transmission that occurs by insect mouthparts contaminated by a previous blood meal on an infected vertebrate. Additional studies on *Anopheles* susceptibility to arboviruses are required to verify those that can be biologically or mechanically transmitted to uninfected hosts. Arbovirus infections could be exacerbated by co-infection with *Plasmodium* parasites in the human host, and may increase mortality in malaria endemic countries. Studies of the potential of *Anopheles* to transmit other arboviruses would help to focus control of arbovirus outbreaks, because current vector control of arboviruses is mainly focused on culicine mosquitoes (Beaty, 2005).

There are probable *Anopheles* arboviruses such as Nyando virus, Ilesha virus, Ngari virus and others, for which more information is needed. There is not yet enough data to classify them either as arboviruses or ISVs, and assuming that they are ISVs could constitute a risk (Nanfack Minkeu and Vernick, 2018).

The *Anopheles* virome also includes ISVs, which unlike arboviruses infect only arthropods. It is not known whether these ISVs interact with known arboviruses (such as ONNV) in *Anopheles*. Theoretically this group of ISVs does not constitute any danger for humans and could be used to better study interaction between viruses and mosquitoes. The correlation

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between ISVs and arboviruses in *Anopheles* is understudied. In culicine mosquitoes, ISVs can influence the competence of mosquitoes to arboviruses (Roundy et al., 2017).

Viral interference between *Anopheles C* virus and *Anopheles cypovirus* in *An. coluzzii*

Most studies on virus interactions have been done using cell culture, and there is little reported work on *Anopheles* viruses (Zhang et al., 2017). *In vitro* studies can be more efficient to do than studies in the whole organisms, although the *in vitro* system may not be an accurate model for host immune responses, particularly in specialized cell types like the midgut epithelium (Kimoto, 1986). In terms of nomenclature, ‘homologous interference’ corresponds to negative interaction between two viruses from the same family while ‘heterologous interference’ corresponds to negative interaction between viruses from different families.

Anopheles C virus and *Anopheles cypovirus* infections are disseminated in co-infected *An. coluzzii* adults, which allowed simultaneous *in vivo* studies on the two viruses. We showed that high viral load of AnCV decreased or inhibited AnCPV and reciprocally in *An. coluzzii*. Therefore, both viruses displayed a heterologous viral interference. The absence of purified AnCV and/or purified AnCPV impeded *in vitro* studies. In studies of Eilat virus, an insect specific alphavirus, dissemination of CHIKV was delayed for three days by homologous interference in *Ae. aegypti* and Eilat virus also reduced the replication of Sindbis virus and Venezuelan equine encephalitis virus by heterologous interference in the *Ae. albopictus* C7/10 cell line (Nasar et al., 2015). Another ISV, Nhumirim virus, can inhibit replication of dengue and West Nile viruses in culicine cell lines (Goenaga et al., 2015; Zhang et al., 2017). Mechanisms underlying viral interference are probably diverse. The negative correlation or heterologous interference between AnCV and AnCPV may be explained by resource competition between both viruses, production of factors by one virus that inhibit the replication of the other virus, or an immune response of *An. coluzzii* triggered by one virus that negatively impacts the other (Masson and Lemaitre, 2017). Further work will be required to measure the influence of AnCV or AnCPV infection upon the infection and dissemination of ONNV and *Plasmodium* parasites in *Anopheles* mosquitoes. These results could raise the possibility of using ISVs to control transmission of other viruses or *Plasmodium*.

Immune responses of *An. coluzzii* against insect specific viruses

AnCV and AnCPV persistently infect many colonies of *An. coluzzii*. We showed that AnCV and AnCPV are not present as DNA forms in *Anopheles*, and thus transmission occurs by RNA genomes. At least AnCV is maintained by in mosquitoes by transovarial transmission. Because

GENERAL DISCUSSION

of the vertical transmission and the widespread presence of the two viruses in laboratory and wild populations of *Anopheles*, the two viruses may be commensals of *Anopheles* mosquitoes. The functional mechanisms used by mosquitoes to control and limit commensal viruses of the virome has been very little examined.

We approached this problem by using functional genomic gene silencing tools to query the immune responses of *Anopheles* mosquitoes against these two ISVs. *An. coluzzii* deploys humoral responses mediated by the Toll and JAK/STAT pathways to limit AnCPV abundance. Constitutive activation of the Toll pathway by silencing the negative regulator, Cactus, reduced AnCPV prevalence while silencing of the JAK/STAT positive regulator, STAT-A, decreased AnCPV prevalence. These results may suggest cross talk or cooperation between the Toll and JAK/STAT pathways to control AnCPV. Interestingly, both pathways influenced infection prevalence but not virus abundance within infected mosquitoes, suggesting that their primary action was to blockade initial establishment of infection in individual mosquitoes. For detailed studies of immunity to these two viruses, it would be valuable to have *Anopheles* lines free of AnCV and AnCPV, but susceptible to infection, for experimental infections with a negative control state.

In *An. coluzzii*, the Toll, RNAi, JAK/STAT, and Imd pathways had no impact on the dicistrovirus AnCV. The RNAi suppressors DCV-1A and CrPV-1A have been reported in the genome of the dicistroviruses DCV and CrPV respectively (Nayak et al., 2010; van Rij et al., 2006), and so the presence of immune suppressors may explain this lack of AnCV control by the classical immune pathways. Another hypothesis could be that AnCV is not ‘detectable’ by *An. coluzzii* immune surveillance, or produces defective genomes to divert the antiviral response (Poirier et al., 2018), resulting in absence of impact of classical pathways on AnCV.

***An. stephensi* is not a competent host for *Anopheles* C virus**

The *An. stephensi* colony studied was free of AnCV and AnCPV (Carissimo et al., 2016). Therefore, we developed an experimental infection system by membrane feeding to infect *An. stephensi* by ingestion of both viruses. This allowed us to assess the adaptation of ISVs to transmission by blood feeding, and to establish a model for functional interaction studies between RNA viruses and *Anopheles* mosquitoes. We found that AnCPV was able to infect, replicate and disseminate in *An. stephensi*, while AnCV could not infect *An. stephensi* under the same conditions. After blood feeding, AnCV was detected in *An. stephensi* only until three days post-feeding. Different hypotheses could explain the apparent inability of AnCV to infect

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the *An. stephensi* midgut by blood, including local midgut antiviral responses, biochemical properties of the midgut bloodmeal environment could inhibit AnCV, or resource competition or interference from other microbes in *An. stephensi* midgut. Interestingly, this result sheds light on the fact that *Anopheles* competence to viruses may depend in part on the combined influence of the host mosquito species, and the background of virome composition. Different hypotheses have been proposed to explain why *Anopheles* mosquitoes display poor competence for some arboviruses: i) their intrinsic physiology ii) their antiviral responses iii) the structure and morphology of the viruses (Romoser et al., 2005). Unlike *Anopheles*, *Aedes* species are competent to CHIKV, DENV and other flaviviruses, indicating that the underlying causes of differential arbovirus competence between *Anopheles* and *Aedes* mosquitoes are complex.

***Anopheles* cypovirus: a potential candidate for viral paratransgenesis?**

AnCPV colonizes larvae and adults of *An. coluzzii* and *An. stephensi* with a high systemic dissemination in both mosquitoes. Our experimental infection model showed that *An. stephensi* infected with AnCPV displayed greater mortality as compared to controls. In addition, AnCPV modulated gene expression during its early and disseminated infection phases in *An. stephensi*. Moreover, there may be a super-infection exclusion between AnCPV and AnCV, indicating that both may reduce or prevent the replication of other viruses in co-infected systems.

This evidence suggests the possibility that AnCPV could potentially be used as a paratransgenic agent against malaria vectors and arboviruses. To achieve this, several scenarios are possible: i) AnCPV has to be purified and sequenced in order to be genetically manipulated by potential insertion of an anti-parasite or anti-viral factor, ii) The genetically modified AnCPV must exclude arboviruses such as ONNV or SFV during a co-infection in *Anopheles*, iii) The genetically modified AnCPV must reduce the lifespan of newly infected *Anopheles* mosquitoes to prevent them from transmitting *Plasmodium* parasites or arboviruses. AnCPV is currently being sequenced in the laboratory to generate a reference genome assembly, to aid further work. The scenario iii) is plausible because AnCPV imposes a fitness cost in newly infected *An. stephensi*. Indeed, reducing *Anopheles* lifespan would have a strong impact on malaria parasite transmission, because the mosquito needs to live long enough after the infective blood meal to transmit sporozoites (Ricci et al., 2012). The scenarios i) and ii) will be explored in further studies.

Another promising viral paratransgenic agent in malaria vectors is *Anopheles gambiae* densovirus, which is vertically transmitted and has a small genome that is easily engineered. It is considered an ISV, and it has no detectable effect on the transcriptome and lifespan of *An.*

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gambiae (Ren et al., 2008; Ren et al., 2014). Interestingly, another strain, Thai densovirus, is vertically transmitted in the *An. minimus* population with no effect on fecundity (Rwegoshora and Kittayapong, 2004). Therefore, densoviruses could be used to deliver or express anti-pathogenic molecules within malaria vectors (Ren et al., 2008; Ren et al., 2014).

Further basic biological research is necessary to explore the feasibility of viral paratransgenesis for the control of malaria and arbovirus vectors.

From insect specific virus to arbovirus status

Insect specific viruses are restricted to insects and most of them, which were discovered by metagenomic discovery projects, are still uncharacterized and with unknown host ranges and no biological data (Colmant et al., 2017a; Fauver et al., 2016; Nanfack Minkeu and Vernick, 2018). We showed that the ISV, AnCPV was detectable in rabbit blood 1 week after exposure to infected insects. Biting by naïve *Anopheles* mosquitoes on the infected rabbit resulted in the infection of those mosquitoes. Unlike ISVs, arboviruses have dual host tropism with replication in vertebrates and arthropods, and their evolutionary origins are unclear. In our case, we do not have direct evidence of replication of AnCPV in rabbit blood, hence we use the term ‘like an arbovirus’. This result and our infection model with human blood may suggest an ability of AnCPV to adapt rapidly to survival and potentially replication in a mammal bloods, with persistence of at least seven days. The fact that AnCPV could potentially be transmitted like an arbovirus could lead to two implications:

- i) If AnCPV replication is confirmed in vertebrates then it is a new arbovirus commonly present in *Anopheles* laboratory colonies. If true, this may suggest that uncharacterized arboviruses could be transmitted by *Anopheles* species, and that *Anopheles* insectaries should revise their safety confinement procedures.
- ii) If AnCPV does not replicate in vertebrates, then AnCPV at least survives or persists in vertebrate bloods. In that case, additional questions remain: why does an ISV has a mechanism to persist in mammalian blood, and what is the mechanism of protection from inactivation or clearance? Is it possible for AnCPV to adapt to replicate in vertebrates, if it does not do so already? Our observations suggest that ISVs could be just a short step from evolving into arboviruses, that is, they could be arbovirus precursors (Bolling et al., 2015; Junglen et al., 2017). The adaptation of ISVs to vertebrates may require frequent exposure to vertebrate blood by mosquito bite. Indeed, ISVs are mainly RNA viruses with high mutation rates, and consistent exposure to any new condition, including the vertebrate blood environment, may be the essential missing ingredient driving adaptation to the new condition. The high mutation rates

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are exemplified in this study by the high polymorphism of AnCPV and its genomic variation between *An. stephensi* and *An. coluzzii*.

Thus, uncharacterized ISVs in the viromes of hematophagic arthropods may comprise a pool of candidate arboviruses in waiting, adding to the interest in describing and studying their biology. In addition to consistent exposure to vertebrates, other factors involved in the evolution and potential emergence of novel arboviruses could be related to anthropological behavioral changes such as irrigation, deforestation, and travel, as well as climate change (Liang et al., 2015).

Immune responses and tissue barriers of vertebrates are considered as the main obstacles to the evolution of ISVs into arboviruses. However, the existence of arbo-reoviruses, such as Bluetongue virus, and insect specific reoviruses such as Fako virus may indicate that vertebrate immunity is not an impervious barrier against vertebrate host tropism (Auguste et al., 2015). Arboviruses and ISVs from the same family also exist among flaviviruses and bunyaviruses, and like AnCPV, these could also be used as models to understand the origin and evolution of arboviruses from ISVs (Marklewitz et al., 2015).

Discovery of new viruses by RNAseq and de novo assembly

Metagenomic approaches using deep sequencing, or cell culture screening of extracts, has allowed the discovery of many ISVs, arboviruses and analysis of complete virus genomes (Colmant et al., 2017a; Fauver et al., 2016; Nanfack Minkeu and Vernick, 2018).

In this thesis, we discovered by RNAseq a partiti-like virus and a chaq-like virus in *An. stephensi*. Chaqvirus is considered as a satellite virus of galbut virus in *Drosophila* because of their co-infection in many samples and both viruses are unclassified (Shi et al., 2018). Galbut virus was absent in our samples, as well as absent from *Anopheles* species from other studies where partiti-like virus was present. Therefore, the claim that chaqvirus is a satellite virus of galbut virus needs to be revisited. Unlike chaq-like virus, partiti-like virus is often a plant virus, and belongs to the family *Partitiviridae*. The presence of plant viruses such as partitiviruses and totiviruses in *Anopheles* may suggest an adaptation of viruses to different hosts via horizontal transmission, but phylogenetic studies and *in vivo* experiments are needed before drawing conclusions. In plants, partitiviral genes can be transferred into the host nuclear genome (Liu et al., 2010). In our study, partiti-like virus is present as non-integrated DNA of unknown structure in *An. stephensi*. In addition to infecting plants and insects, partiti-like virus has also been reported in fungi, protozoa and other taxa (Fauver et al., 2016; Shi et al., 2016;

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Webster et al., 2015). The chaq-like virus and partiti-like virus are present in both males and females.

Although deep sequencing is a powerful tool for virus discovery, most of the focus has been on RNA viruses, which has led to a scarcity of discovery studies on mosquito DNA viruses. The DNA arbovirus African swine fever virus, transmitted by soft ticks, has been responsible for epizootics in Africa (Galindo and Alonso, 2017; Parker et al., 1969). A DNA virus responsible for myxomatosis in rabbits, Myxoma virus, can be spread by mechanical transmission by mosquitoes (Kerr and Best, 1998). Therefore, ISV and arbovirus discovery studies should ideally target DNA as well as RNA viruses.

5. GENERAL CONCLUSIONS

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The objectives of this PhD thesis were to explore the virome of *Anopheles* malaria vectors, study interactions between viruses and *Anopheles*, and understand the genomic variation and evolution of RNA viruses.

The *Anopheles* virome is composed of insect specific viruses, arboviruses, and potential arboviruses. We identified a phenomenon of viral interference between *Anopheles* C virus (AnCV) and *Anopheles* cypovirus (AnCPV) in naturally co-infected *An. coluzzii* mosquitoes. *An. coluzzii* displayed a disseminated systemic infection with both viruses, and this mosquito limits AnCPV infection by antiviral activity of the Toll pathway, while activity of the JAK/STAT pathway promotes AnCPV infection. Our experimental blood infection model of AnCPV in previously uninfected *An. stephensi* revealed pathogenicity of AnCPV in this new host. This experimental model was used for transcriptome-wide profiling of *An. stephensi* genes regulated upon viral infection. AnCPV can be transmitted by a mechanism like an arbovirus from infected mosquitoes to rabbits and finally to uninfected mosquitoes. AnCPV is highly polymorphic, and the virus originating in *An. coluzzii* displayed fixed nucleotide differences once a persistent infection was established in previously uninfected *An. stephensi*. The fixed positions may indicate adaptation of AnCPV in the new *An. stephensi* host species. The sequencing and assembly of a reference AnCPV genome, currently in progress, will permit mapping of all variable sites to the virus genome to facilitate interpretation of their positions and meaning. Analysis of the variation data will provide important information to understand the genomic alterations required for AnCPV infection and adaption in *An. stephensi*. We also used a metagenomic approach to reconstruct the genomes of chaq-like virus and partiti-like virus, and these can be used as tools to study further *Anopheles* antiviral immunity. Partiti-like virus generates non-integrated DNA forms whose impact on vector competence and co-evolution between host and virus need to be assessed.

This study provides to the community new tools to study interactions between viruses and *Anopheles* mosquitoes, thus filling a gap. Our data enrich the RNA virosphere of mosquitoes with new perspective on viral diversity. We shed light on the fact that some viruses discovered as insect-specific viruses can potentially evolve to be arboviruses with an impact on public health and research. Nevertheless, ISVs seem to be encouraging tools for controlling malaria

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vectors, parasites and arboviruses. In addition, they bring other arguments to study paleovirology in non-retroviruses in mosquitoes. Indeed, the existence in the double stranded and single stranded RNA viruses of DNA forms, endogenous viral elements and non-retroviral integrated RNA virus sequences show that paleovirology which is mainly focused on retroviruses should be extended to all virus families.

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Résumé :

Les moustiques sont colonisés par un virome très peu étudié. Comme les bactéries, le virome influence probablement la biologie et l'immunité des populations de moustiques vecteurs, mais les modèles expérimentaux sont rares. Nous avons récemment découvert deux nouveaux virus chez le virome des vecteurs sauvages du paludisme, anophèles et des colonies d'*Anopheles coluzzii* : *Anopheles C virus* (AnCV) et *Anopheles cypovirus* (AnCPV). L'un ou les deux virus sont présents dans toutes les colonies de laboratoire d'*An. gambiae* et *An. coluzzii*. La prévalence des virus varie en fonction des stades du moustique. L'abondance des deux virus est négativement corrélée chez les moustiques individuels. L'analyse fonctionnelle révèle l'implication des voies de signalisation immunitaire des moustiques sur la réplication du virus, avec une influence différentielle sur les deux virus. Un modèle expérimental a été développé pour l'infection d'AnCPV chez les anophèles non porteurs de ces virus, en utilisant du sang infecté afin d'étudier les réponses antivirales chez ces moustiques. Les séquences de l'AnCPV sont hautement polymorphiques chez les moustiques individuels, alors que l'AnCV est pratiquement dépourvue de mutations. AnCPV entraîne une plus grande mortalité chez *An. stephensi*, mais certaines mutations semblent impliquées dans son adaptation à cette espèce. AnCPV peut être potentiellement transmis comme un arbovirus à travers un hôte mammifère à des moustiques non infectés, ce qui suggère une voie évolutive relativement simple. Le virome d'*An. stephensi* contient un chaq-like virus et un partiti-like virus. Ce dernier appartenant à la famille des *Partitiviridae* a des formes d'ADN.

Mots clés : anophèle, virus, interactions, métagénomique, mutation, voies immunitaires.

Abstract:

Mosquitoes are colonized by a little-studied natural virome. Like the bacterial microbiome, the virome also probably influences the biology and immunity of mosquito vector populations, but tractable experimental models are lacking. We recently discovered two novel viruses in the virome of wild *Anopheles* and in colonies of the malaria vector *Anopheles coluzzii*: *Anopheles C virus* and *Anopheles cypovirus*. One or both viruses are present in all tested laboratory colonies of *An. coluzzii* and *An. gambiae*. Viral abundance varies reproducibly during mosquito development. Relative abundance of the two viruses is inversely correlated in individual mosquitoes. Functional genomic analysis reveals the implication of mosquito immune signaling pathways on virus replication, with differential influence on the two viruses. An experimental model was developed for AnCPV infection of *Anopheles* by bloodmeal, in order to study mosquito antiviral responses. Sequences of AnCPV are highly polymorphic in individual mosquitoes, while AnCV is virtually devoid of variation. AnCPV is pathogenic to *An. stephensi* but some viral mutations seem to be involved in its adaptation to this species. AnCPV can be transmitted like an arbovirus through a vertebrate host to uninfected mosquitoes, suggesting that the evolutionary pathway from vertical “insect specific” to infective blood transmission may be remarkably simple. The *Anopheles stephensi* virome harbors a chaq-like virus and partiti-like virus. This latter belonging to *Partitiviridae* is present in *An. stephensi* as DNA forms of the virus genome.

Key words: *Anopheles*, virus, interactions, metagenomic, mutation, immune pathways.