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Review

The role of co-infection and swarm dynamics in arbovirus transmission

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ABSTRACT

Arthropod-borne viruses (arboviruses) are transmitted by hematophagous insects, primarily mosquitoes. The geographic range and prevalence of mosquito-borne viruses and their vectors has dramatically increased over the last 50 years. As a result, the most medically important arboviurses now co-exist in many regions, resulting in an increased frequency of co-infections in hosts and vectors. In addition to concurrent infections with human pathogens, mosquito-only viruses and/or enzootic viruses not associated with human disease are ubiquitous in mosquito populations. Moreover, mosquito-borne viruses are largely RNA viruses that exist within individual hosts as a diverse and dynamic swarm of closely related genotypes. Interactions among co-infecting viruses and genotypes can have profound effects on virulence, fitness and evolution. Here, we review our understanding of how these complex interactions influence transmission of mosquito-borne viruses, focusing on the often-neglected virus interactions in the mosquito vector, and identify gaps in our knowledge that should guide future studies.

1. Introduction

Arthropod-borne viruses (arboviruses) are transmitted by hematophagous insects, primarily mosquitoes. There are over 140 arboviruses known to be associated with human disease and approximately 50 additional arboviruses known to cause disease in wild and/or domestic animals (Gubler, 2001, 2002; Huang et al., 2019; Hubalek et al., 2014). The most prevalent and widespread arboviruses classified as human pathogens are mosquito-borne viruses belonging to the families *Flaviviridae* and *Togaviridae*. *Flaviviruses* (*Flaviviridae*) of medical importance include *dengue virus* (DENV), *West Nile virus* (WNV), *Zika virus* (ZIKV), *yellow fever virus* (YFV), *Japanese encephalitis virus* (JEV), *St. Louis encephalitis virus* (SLEV) and *Murray Valley encephalitis virus* (MVEV). Prevalent *alphaviruses* (*Togaviridae*) of medical importance include *chikungunya virus* (CHIKV), *Ross River virus* (RRV), *O'nyong-nyong virus* (ONNV), *sindbis virus* (SINV), *Barmah Forest virus* (BFV) and *Mayaro virus* (MAYV), as well as *Western*, *Eastern* and *Venezuelan equine encephalitis viruses* (WEEV, VEEV, and EEEV, respectively). Additional human pathogens in the family *Peribunyaviridae* include *LaCrosse virus* (*Peribunyaviridae;orthobunyavirus*; LACV) and *Rift Valley fever virus* (*Peribunyaviridae, phlebovirus;* RVFV). The geographic range and prevalence of these arboviruses and their mosquito vectors has dramatically increased over the last 50 years. As a result, the most medically important arboviruses now co-exist in many regions (Barzon, 2018; Gould et al., 2017; Huang et al., 2019; Mayer et al., 2017) (Fig. 1). In addition to the obvious public health burden, this has created novel diagnostic challenges, particularly for viruses that are serologically and genetically related (Fig. 2). For instance, there are regions in the Americas where the flaviviruses ZIKV, WNV, DENV 1–4, SLEV and YFV, in addition to alphaviruses CHIKV and MAYV are all now endemic (Fig. 1). Diagnostic challenges stem from both overlapping symptomology and cross-reactive clinical assays, a problem that has been a particular challenge when attempting to serologically distinguish DENV and ZIKV in recent years (Priyamvada et al., 2016). The co-circulation of these pathogens has resulted in an increased frequency of co-infections (Caron et al., 2012; Dupont-Rouzeyrol et al., 2015; Furuya-Kanamori et al., 2016; Roth et al., 2014; Rothan et al., 2018; Waggoner et al., 2016a; Zambrano et al., 2016). The consequences of concurrent infections in terms of disease manifestations can be severe. While increases in disease severity are well characterized for sequential infections with heterologous DENV serotypes (Guzman et al., 2013; Hammon, 1973), there is also evidence of enhanced virulence resulting from co- and/or sequential infections with different species, including DENV and ZIKV (Bardina et al., 2017). Although co-infections of mosquitoes are less likely due to shorter lifespans and less frequent exposures, multiple pathogens have been detected from individual mosquitoes and the likelihood of this is increasing with more frequent outbreaks and global expansions. In addition to co-infections with

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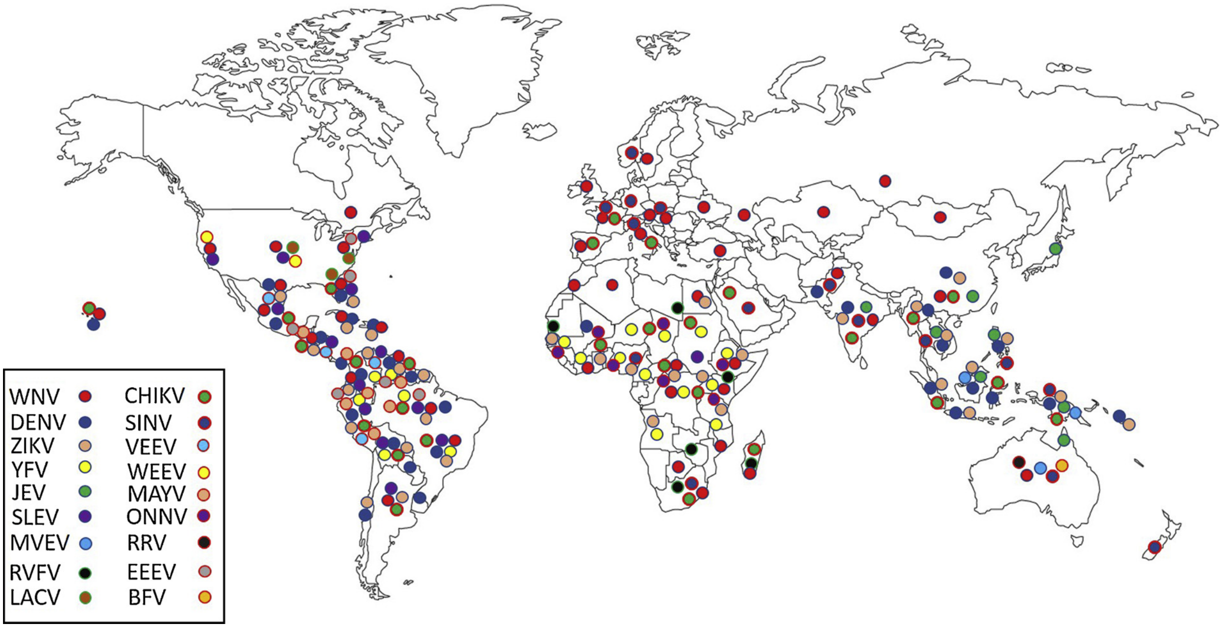

Fig. 1. The geographic range of mosquito-borne viruses of medical importance. Presence of individual viruses are estimated based on reports of human cases, virus isolation and/or serological evidence. Flaviviruses (Flaviviridae), including dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV), are indicated by circles with blue outlines. Alphaviruses (Togaviridae), including chikungunya virus (CHIKV), Ross River virus (RRV), sindbis virus (SINV), O'nyong-nyong virus (ONNV), Mayaro virus (MAYV), Barmah Forest virus (BFV), western equine encephalitis virus (WEEV), eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV), are indicated by circles with red outlines. Viruses in the family Peribunyaviridae including LaCrosse virus (Peribunyaviridae; orthobunyavirus; LACV) and Rift Valley fever virus (Peribunyaviridae, phlebovirus; RVFV) are indicated by circles with green outlines.

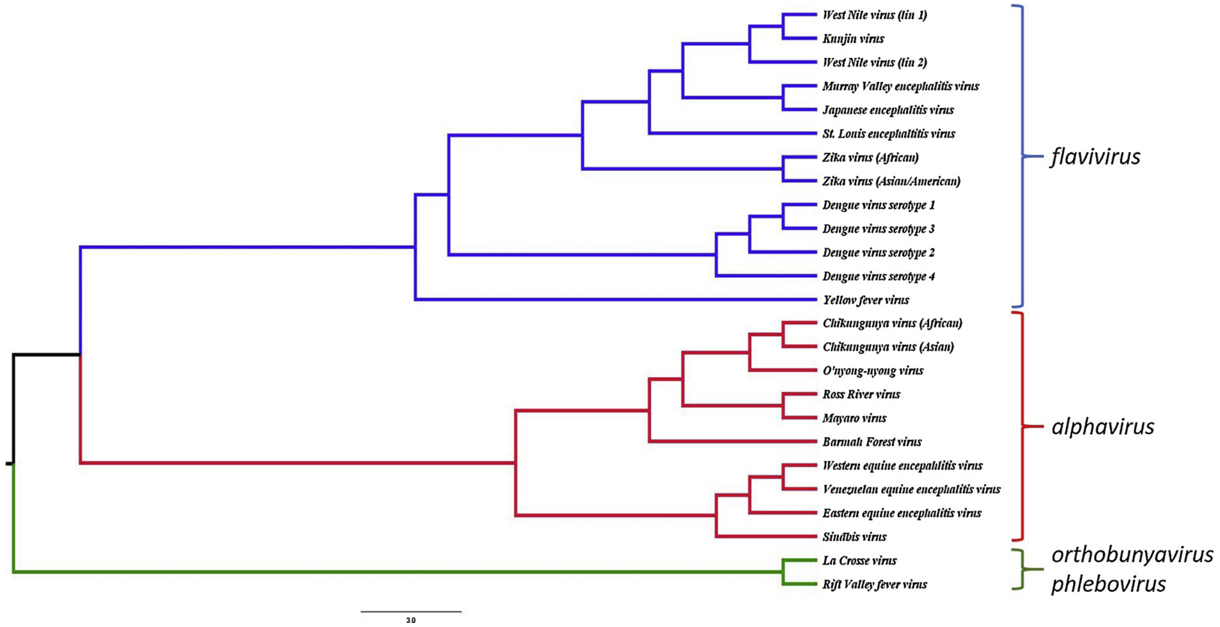

Fig. 2. Genetic relatedness of mosquito-borne viruses of medical importance. An alignment of representative genotypes was created using the software Geneious 11.1.5 and a maximum likelihood tree was generated with 500 bootstraps. Posterior probabilities of all branches are greater than 0.75. Tree editing was completed using FigTree v1.4.

heterologous viruses or strains, co-infections with the same viruses occur. Indeed, as all these pathogens are highly mutable RNA viruses, each individual infection is itself a diverse swarm of closely related, co-infecting haplotypes. Beyond interactions between heterologous and homologous RNA pathogens, there are also numerous mosquito-only viruses that have been identified in recent years (Ohlund et al., 2019). In addition to this diverse virome, there are diverse and abundant microbial co-infections that are known to influence transmissibility (Hegde et al., 2015). Here, we review our understanding of how these complex and diverse interactions influence transmission of mosquito-borne viruses, focusing on the often-neglected virus interactions in the vector, and identify gaps in our knowledge that should guide future studies.

2. Viral co-infection in mosquitoes

Co-infections can occur either simultaneously or sequentially and can result in myriad interactions. These include: (a) competitive interactions ranging from exclusion to resource sequestration resulting in suppression of one or both viruses; (b) cooperative interactions, resource sharing or immune evasion resulting in enhancement of one or both viruses; or (c) a benign coexistence in which interactions do not significantly alter fitness and transmission of either virus. Although not well-studied on a cellular level *in vivo*, competitive exclusion, both by the same virus (homologous interference) and different viruses (heterologous interference) has been frequently reported in mosquito cell culture (Abrao and da Fonseca, 2016; Johnston et al., 1974; Karpf et al., 1997a). While this has most often been reported with superinfection, interference has also been noted with simultaneous infections. Suppression has often been attributed to competition for intracellular host factors as RNA which is transfected into persistently infected cells generally fails to replicate (Stollar and Shenk, 1973). As homologous exclusion and heterologous exclusion among closely related viruses is more common than exclusion with more distantly related viruses, there is some specificity to the mechanisms, perhaps due to trans-acting proteases that process non-structural proteins (Karpf et al., 1997a). For instance, interference has been noted between DENV and YFV (Abrao and da Fonseca, 2016), as well as SINV and RRV, yet not between SINV and YFV (Karpf et al., 1997a). While there is less data on simultaneous infections in cell culture, LACV-SINV co-infection enhanced replication of SINV without affecting LACV replication in mosquito cells (Bara and Muturi, 2014). Co-infections of mosquito cells with different strains or serotypes of DENV, on the other hand, were generally suppressive (Pepin and Hanley, 2008; Pepin et al., 2008).

Although the magnitude of cellular co-infection may not be as extensive within a vector, the potential for direct and indirect interactions is more complex. The majority of *in vitro* studies have been completed in C6/36 cells, which lack a functional RNA interference (RNAi) response. RNAi is the primary invertebrate immune response to RNA virus infections (Blair, 2011). Although RNAi targeting is highly specific, upregulation of the pathway could either work to enhance reactivity to co-infecting RNA pathogens or sequester this response for an individual pathogen due to limited resources, thus permitting increased replication of the co-infecting pathogen, *i.e.* multiple fronts costs (McKean et al., 2008). Additional suppression of the RNAi pathway could be achieved through specific viral inhibitors, for instance the flavivirus NS4B protein (Kakumani et al., 2013). Classical innate immune pathways normally associated with bacterial infections including IMD, Toll and JAK-STAT, as well as conserved signals of cellular damage resulting in autophagy, have also been associated with viral infections of invertebrates (Eng et al., 2016; Paradkar et al., 2012; Souza-Neto et al., 2009; Xi et al., 2008). Primary infections could similarly divert or enhance the capacity of these pathways to react to co-infecting pathogens. Secreted flavivirus NS1 protein, present in blood of infected hosts, has also been shown to be important in flavivirus infectiousness of mosquitoes, likely through regulation of the ROS pathway (Liu et al., 2016). While there is some strain and virus specificity with this mechanism (Liu et al., 2017), the general effect on innate immunity in *Ae. aegypti* suggests that an indirect influence on co-infecting viruses is likely (Liu et al., 2016). It is important to note that, while these viruses persistently infect mosquitoes and are not known to be highly pathogenic to their invertebrate hosts, most evidence supports the idea that arboviruses have the potential to impart measurable virulence in mosquitoes. In fact, the maintenance of specific, canonical pathways to combat these viruses would make little evolutionary sense if uncontrolled infections were not virulent. Indeed, when the RNAi response is inhibited, longevity of infected invertebrates has been shown to decrease (Poirier et al., 2018). In addition, pathology, as well as decreased longevity and fecundity has been noted in mosquitoes infected with WNV, DENV, CHIKV, WEEV and EEEV (Ciota et al., 2013, 2011; Girard et al., 2005; Girard et al., 2007; Maciel-de-Freitas et al., 2011; Martin et al., 2010; Moncayo et al., 2000; Scott and Lorenz, 1998; Weaver et al., 1992). Characterizing this potential for virulence is critical if we are to accurately define the potential effect of co-infection on transmissibility.

Recent experimental studies have focused largely on the highly invasive and cosmopolitan *Ae. aegypti,* which is the primary vector of DENV, ZIKV, YFV and CHIKV. With concurrent outbreaks now common (Roth et al., 2014), incidents of co-infection with these pathogens in both humans and mosquitoes have been reported more frequently in recent years (Caron et al., 2012; Furuya-Kanamori et al., 2016; Norman et al., 2016; Sardi et al., 2016; Waggoner et al., 2016a; Zambrano et al., 2016). Magalhaes et al (2018) demonstrated that sequential infection of *Ae. aegypti* with CHIKV and ZIKV resulted in co-transmission and enhanced ZIKV transmission at 7 days post-infection. Co-transmission of CHIKV and ZIKV has also been demonstrated with simultaneous infection (Goertz et al., 2017; Ruckert et al., 2017), and a similar enhancement of DENV replication in salivary glands was measured following simultaneous infection with CHIKV (Le Coupanec et al., 2017). Co-infections with CHIKV and MAYV had no measurable effect on transmission of either pathogen by *Ae. aegypti*, but superinfection exclusion was noted for CHIKV in MAYV-infected mosquitoes (Kantor et al., 2019). Ruckert et al measured vector competence following co- and triple infection with DENV, CHIKV and ZIKV and found overall effects to be generally modest and at times in contrast with previous findings. Specifically, ZIKV infection rates and DENV dissemination rates were slightly reduced with CHIKV co-infection and CHIKV transmission rates were reduced following DENV infection. Importantly, this study also demonstrated that simultaneous transmission of all three pathogens by a single mosquito can occur. Together, these studies demonstrate the potential for co-infection and co-transmission and the range of possible consequences of these interactions. The variability in measurable effects could stem from virus strain or mosquito population differences, and/or from the nuanced differences in experimental design including timing of sequential infections or age of mosquitoes.

In addition to understanding the effect that co-infections with multiple human pathogens have on transmission, interactions between RNA pathogens and insect-specific viruses (ISVs), which are likely ubiquitous, are important to define (Chandler et al., 2015; Coffey et al., 2014; Cook et al., 2013). These interactions many be quite specific given that many ISVs belong to the same genera as human pathogens. This is particularly the case with flaviviruses, for which at least 12 ISVs have been identified, often in mosquito species which are primary vectors of human pathogens (Blitvich and Firth, 2015). The ISV *Nhumirin virus* (NHUV) has been shown to suppress replication of WNV, JEV and SLEV (Kenney et al., 2014), while *Palm Creek virus* has the capacity to suppress replication of WNV and MVEV (Hobson-Peters et al., 2013). Similarly, the sole ISV identified in the *alphavirus* genus, *Eliat virus*, can suppress replication of SINV, EEEV, VEEV, WEEV and CHIKV (Nasar et al., 2015). Conversely, cells persistently infected with *Culex flavivirus* (CxFV) do not suppress infection or replication of JEV and DENV, yet enhanced cell pathology was noted in dual infections with JEV and CxFV (Kuwata et al., 2015).

While fewer studies have evaluated how interactions with ISVs could alter transmissibility *in vivo*, evidence for the potential importance of these interactions exists. Surveillance data suggests an association between CxFV and WNV positivity in mosquitoes (Newman et al., 2011), yet experimental data is mixed. Specifically, Kent et al (2010) demonstrated an enhancement of WNV infection with CxFV in *Cx. quinquefasciatus*, yet Bolling et al (2012) demonstrated delayed WNV transmission in *Cx. pipiens* (Bolling et al., 2012; Kent et al., 2010). Differences between these species were also noted with NHUV, for which co-inoculation with WNV decreased transmission of WNV in *Cx. quinquefasciatus* but not *Cx. pipiens* (Goenaga et al., 2015). Establishment of NHUV infection in *Ae. aegypti* was also found to significantly decrease both infection and transmission of ZIKV (Romo et al., 2018).

Similarly, EILV infection was found to delay CHIKV infection in *Ae. aegypti* (Nasar et al., 2015). What remains unclear is the tropism of these ISVs, if co-infections with human pathogens occur on a cellular level, and how these viruses are maintained. These questions need to be more thoroughly addressed before we can begin to understand the mechanistic basis of these interactions and their effect on viral transmission.

3. Mutant swarm dynamics and transmission

Although there are bottlenecks associated with transmission, infection and traversing morphological barriers, diverse mutant swarms are maintained throughout the life cycle of arboviruses and constantly regenerated through high levels of error-prone replication. So, while studies show that individual variants can be lost during the extrinsic incubation period of arboviruses (time from mosquito exposure to subsequent transmission; Ciota et al., 2012a; Forrester et al., 2012), diverse swarms are still often transmitted by mosquitoes (Brackney et al., 2011; Forrester et al., 2014). In addition, each subsequent transmission event from a single mosquito could comprise unique subpopulations (Grubaugh et al., 2017). Since the result of this intra-host diversity is persistent co- and super-infection of closely related strains, understanding mutant swarm dynamics is in essence simply an extension of the characterization of interactions of co-infections with more taxonomically diverse pathogens. There have been a number of studies that have characterized the phenotypic impact of these diverse mutant swarms and the unique selective pressures they are subject to. As with more taxonomically diverse viruses, interactions between genotypes that comprise a viral swarm can be both direct and indirect and may be largely dependent on the level of co-infection within individual cells. It is well established that both the breadth and specific composition of the RNA virus mutant swarm influences viral fitness, tropism, pathogenesis, adaptability and evolutionary trajectories (Ciota et al., 2007a, b; Coffey and Vignuzzi, 2011; Duarte et al., 1994; Ebel et al., 2011; Fitzpatrick et al., 2010; Novella, 2003; Novella and Ebendick-Corp, 2004; Vignuzzi et al., 2006). There are multiple explanations for why diverse swarms are maintained within individual hosts, the simplest being mutation-selection balance. Although mutation rates vary among RNA viruses, on average arboviruses accumulate one new mutation in every nascent genome (Drake and Holland, 1999). Given that these viruses may replicate to levels as high as 1 billion RNA genomes in individual vertebrate and invertebrate hosts, it is not surprising that intra-host diversity is high. While new mutations are largely deleterious and therefore subject to purifying selection, the strength and pace of this selection is not likely sufficient to overcome the pace of mutation. In addition, viruses under high mutation rates are predicted to evolve towards robust sequence space, which in effect decreases the probability that new mutations will alter phenotype (Montville et al., 2005; Wilke et al., 2001). Although there is indeed evidence for the evolution of robustness (Montville et al., 2005), it has also been shown that genetic diversity does correspond to phenotypic diversity, *i.e.* diverse fitness landscapes (Ciota et al., 2012b). There are a few possible explanations for this. First, variants could at times be subject to density-dependent selection. Given that RNAi is known to be highly specific in terms of sequence targeting, rare variants with minor mutations could be at an advantage in terms of RNAi escape. In support of this, a study with WNV demonstrated that the regions of the genome that were most frequently targeted by RNAi corresponded to regions for which intra-host diversity was highest (Brackney et al., 2009). A second contributor to the maintenance of diversity could be that unique hosts and intra-host environments impose unique and dynamic selective pressures. In other words, maintaining diverse swarms allows for the maintenance of individual variants that are specialized for vertebrate or invertebrate hosts and/or unique tissues within these hosts (Coffey et al., 2011; Vignuzzi et al., 2006). Given the requirement for high levels of fitness in taxonomically diverse hosts, this could be particularly advantageous for arboviruses. Unique variants could be specialized for mosquito midgut infection, midgut replication, midgut escape, replication in external tissues, salivary gland infection, salivary gland replication, transmission, establishment of vertebrate infection, replication in diverse vertebrate tissues and maximizing viremia. While these specialized variants may be transiently selected against at some points during these cycles, they can be maintained at low levels awaiting selection in subsequent environments, a concept that has previously been referred to as 'molecular memory' (Ruiz-Jarabo et al., 2000). Lastly, diversity could be maintained as a result of cooperative interactions among co-infecting variants. Studies with WNV in mosquito cells demonstrate that co-infection can promote the maintenance of both genetic and phenotypic diversity through strain complementation, and that cooperative interactions among co-infecting variants can result in population fitness levels that exceed that of any individual variant (Ciota et al., 2012b). Although the extent to which complementation could be a relevant mechanism *in vivo* is not entirely clear, additional studies with WNV demonstrated that attenuated non-consensus variants are generated and maintained during mosquito infection despite suppressing fitness *in vivo* (Ebel et al., 2011). Further, Aaskov et al (2006) previously reported the maintenance and transmission of a defective DENV genome which was repeatedly recovered from mixed wildtype populations. Studies with *measles virus* have shown advantageous interaction through heteroologomer formation of a viral protein (Shirogane et al., 2012, 2016). While co-packaging would be one clear explanation for cooperative interactions, such molecular mechanisms have not been well characterized for arboviruses. All of these explanations for swarm maintenance predict that diversity is itself advantageous, rather than simply a by-product of error-prone pathogens maximizing replicative fitness. Indeed, high-fidelity variants of arboviruses have been shown to be attenuated in mosquitoes and/or vertebrate hosts in the absence of replication deficits (Coffey et al., 2011; Van Slyke et al., 2015), further supporting the idea that the capacity to produce variability is independently bound to arbovirus fitness and transmission.

4. Concluding remarks and future directions

What is clear from all these studies is that understanding arbovirus fitness and transmission requires consideration of the consortium of interactions within hosts and vectors. The importance of these interactions is also clearly applicable to other pathogen systems, and as the global reach of pathogens expand, characterizing how these relationships impact patterns of transmission becomes more critical. Beyond the interactions between co-infecting viruses discussed here, it is necessary, while daunting, to additionally consider interactions with the diverse and dynamic microbial communities of hosts and vectors. Both extracellular bacteria and obligate intracellular bacteria such as Wolbachia have been shown to have significant effects on competence and transmission of arboviruses in mosquitoes (Hegde et al., 2015; Rainey et al., 2014). As our capacity to sequence the holobiome increases, as well as our ability to readily and efficiently generate transcriptomic and metabolomic data, so too will our understanding of the complexity of these relationships. Such studies should be used to inform detailed experimental work to better define the mechanistic basis of these interactions. Why do different studies with similar questions and design report conflicting findings regarding suppression and enhancement with co-infection? We need to understand the mechanistic nuance in, for instance, how strain variability and timing of co-infections effect outcomes, so that we are able to create better predictive models and perhaps inform novel control strategies. In addition, the focus of mosquito studies thus far has been on the effect of co-infection on vector competence only, yet competence is just one aspect of vectorial capacity (*i.e.* transmission potential). Even modest alterations to life-history traits can have profound effects on the vectorial capacity of a population. Since infections with individual arboviruses have been shown to have the capacity to significantly alter mosquito longevity and feeding behavior (Ciota et al., 2013, 2011; Maciel-de-Freitas et al., 2011; Styer et al., 2007) it seems likely that mixed infections could have a greater influence in this regard. The last, and perhaps greatest challenge is to not just characterize the consequences of interactions in the present, but also to predict how the evolution of these interactions will influence pathogen transmission in the future. As the frequency of co-infections increase, and rising temperatures have the potential to accelerate the evolutionary rates of viruses that replicate in ectothermic organisms, unique evolutionary pressures exerted on co-infecting pathogens will contribute more to shaping the evolutionary paths of these rapidly mutating viruses. Antagonistic relationships now could ultimately evolve to be synergistic if there is a viable evolutionary path to cooperation that maximizes transmission. As shown with experiments with bacteriophages (Sachs and Bull, 2005) distinct viral species with appropriate selective pressures can evolve cooperative interactions that increase fitness and result in novel molecular subtypes. The consequences of this for transmission of human pathogens could be substantial.

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