Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Insect Science

Culicoides–virus interactions: infection barriers and possible factors underlying vector competence

Mary K Mills[1], Kristin Michel[1], Robert S Pfannenstiel[2,*], Mark G Ruder[3], Eva Veronesi[4,**] and Dana Nayduch[2]

In the United States, *Culicoides* midges vector arboviruses of economic importance such as Bluetongue Virus and Epizootic Hemorrhagic Disease Virus. A limited number of studies have demonstrated the complexities of midge–virus interactions, including dynamic changes in virus titer and prevalence over the infection time course. These dynamics are, in part, dictated by mesenteron infection and escape barriers. This review summarizes the overarching trends in viral titer and prevalence throughout the course of infection. Essential barriers to infection and dissemination in the midge are highlighted, along with heritable and extrinsic factors that likely contribute to these barriers. Next generation molecular tools and techniques, now available for *Culicoides* midges, give researchers the opportunity to test how these factors contribute to vector competence.

Addresses
[1] Division of Biology, Kansas State University, Manhattan, KS 66506, USA
[2] United States Department of Agriculture, Agricultural Research Service, Arthropod Borne Animal Diseases Research Unit, Manhattan, KS 66502, USA
[3] Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA
[4] Vector-borne Viral Diseases Programme, The Pirbright Institute, Ash Road, Pirbright, Surrey GU24 0NF, United Kingdom

Corresponding author: Nayduch, Dana (dana.nayduch@ars.usda.gov)
[*] Present address: USDA-APHIS PPQ, 4700 River Rd, Riverdale, MD 20737, USA.
[**] Present address: National Centre for Vector Entomology, Institute of Parasitology, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland.

Current Opinion in Insect Science 2017, **22**:7–15

This review comes from a themed issue on **Vectors and medical and veterinary entomology**

Edited by **Mariangela Bonizzoni** and **Geoffrey Attardo**

For a complete overview see the Issue and the Editorial

Available online 5th May 2017

http://dx.doi.org/10.1016/j.cois.2017.05.003

2214-5745/Published by Elsevier Inc.

Introduction

Culicoides midges are hematophagous dipterans in the family Ceratopogonidae. For most species, females are anautogenous, requiring a blood meal to produce eggs. Immature *Culicoides* develop through four larval stages that live in moist habitats [1]. Complete larval development can occur in as fast as 14 days (d) after hatching [2], but can be altered by temperature and substrate quality. Adults are sexually mature 28–48 hours post-eclosion, and, after mating and blood feeding, females oviposit 2–4 days later. Adult females are capable of 3–4 gonotrophic cycles within their lifetimes [3], but survival for more than one or two cycles is unlikely.

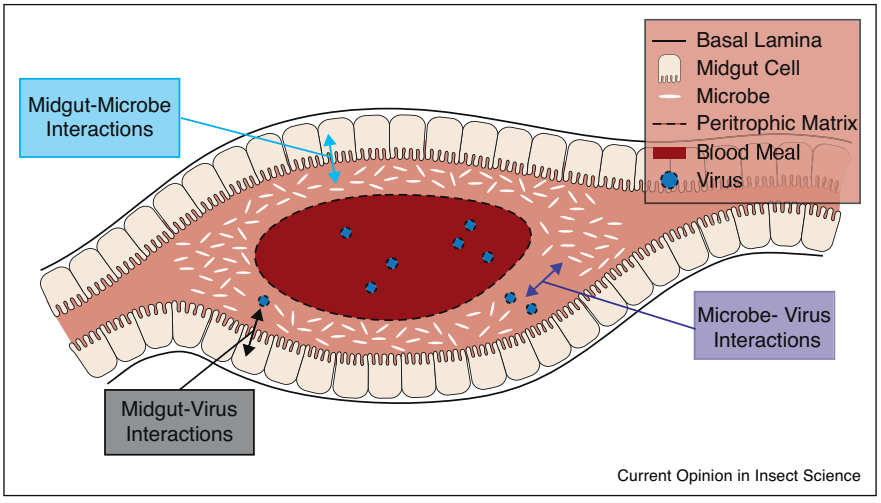
Female *Culicoides* spp. can transmit non-viral or viral pathogens after feeding on infected hosts (see Table 10.1 in Ref. [1]), and serve as vectors for several important arboviruses world-wide [4], specifically orbiviruses, double stranded RNA viruses in the family Reoviridae. Within the United States (US) two closely-related orbiviruses, Bluetongue (BT) Virus (BTV) and Epizootic Hemorrhagic Disease (EHD) Virus (EHDV), are important pathogens of some domestic and wild ruminant species. Subclinical infection is common, although BTV and EHDV are capable of explosive outbreaks of severe febrile disease in multiple ruminant hosts [5]. In the US, severe disease is most common in white-tailed deer (EHD and BT) [6] and some domestic sheep breeds (BT) [7], although other species, including cattle, can also be affected [5,8]. In addition to direct impact of animal mortality on livestock production systems and natural resources, BTV and EHDV have further economic impact through decreased productivity [9,10], cost of preventive and control measures [11,12], and disruption of international trade [13].

Many *Culicoides* species are endemic to the US, but only *Culicoides sonorensis* and *Culicoides insignis* are confirmed vectors of BTV or EHDV [14–17]. While *C. sonorensis* is an important vector of BTV and EHDV in the western US, this species is infrequently recovered in studies of *Culicoides* populations east of the Mississippi river [18,19] and is unlikely to be the primary vector in this region [20]. *C. insignis*, which primarily resides in the far southeast US [20], is also unlikely to be an important vector outside of Florida. Since disease transmission occurs outside the range of these two midge species, other vectors of importance remain to be identified [5,21].

Factors underlying vector competence in *Culicoides*

Multiple events occur within female midges after they have ingested viruses during hematophagy: release of viruses from the blood meal, infection of midgut cells,

Figure 1



The midgut and its tripartite interactions of the mesenteron infection barrier (MIB).
To pass the mesenteron barrier, virus particles must escape the blood meal and survive extrinsic factors, such as the midgut microbiota, and heritable traits, such as midgut epithelial defenses and modulation of microbiota. Together microbe–midgut–virus interactions are likely to play a critical role in vector competence at this first barrier of infection.

possible proliferation within midgut cells, escape from the midgut, dissemination to extraintestinal tissues, infection of the salivary glands and, finally, transmission from the salivary glands to the next host. Viruses must pass several infection barriers to be transmitted to the next host. First, viruses must access and infect midgut epithelial cells, which are considered to be the first major barrier to infection (mesenteron infection barrier, MIB, Figure 1) [22••]. Amplified infectious virus particles that are released from midgut epithelial cells have successfully escaped the mesenteron escape barrier (MEB). At this stage, the virus must survive the host defenses within the rest of the body (dissemination barrier) [22••], including hemolymph immune effectors and cellular responses in midge extraintestinal tissues, to arrive at the salivary glands. No salivary gland infection barrier is believed to exist in the midge [22••].

Heritable antiviral defenses

Both field-caught and colonized *Culicoides* show inter-individual variability in vector competence, yet the mechanism underlying refractoriness or permissiveness for virus infection and transmission remains unknown. Selection experiments have generated refractory midge lines [23], which demonstrates heritable vector competence traits in *Culicoides* [22••,24]. Refractoriness may result from selection against midgut receptors for virus entry or selection for robust antiviral defenses. Such antiviral defenses within the midgut epithelia (MIB) include JAK-STAT and siRNA pathways (Figure 1) [25]. The dissemination barrier also presents multiple antiviral defenses, including hemocytes, prophenoloxidases, complement-like pathways, and autophagy [25–32]. Finally, antimicrobial peptides (AMPs) produced by Toll and IMD pathways may serve as humoral antiviral defenses in the hemolymph [33,34].

Putative orthologs of the JAK-STAT, siRNA, Toll, and IMD pathways, and AMPs were previously identified in *Culicoides* (Table 1). For this review, we used the midge transcriptome, ImmunoDB, and reciprocal blast searches (Table 1) [35••,36] to identify members of the melanization, autophagy, and complement pathways, specifically an ortholog to the *Aedes aegypti* antiviral complement-related factor *AaMCR* [31,37]. Further *in vivo* experiments are needed to determine the role of these pathways in *Culicoides* antiviral defense.

The siRNA pathway has been shown to reduce virus titer in *Culicoides* cells that were provided with double stranded RNA (dsRNA) specific to the BTV NS1 gene [38•]. Putative siRNA pathway members were identified in *C. sonorensis* [39••], suggesting this pathway is functional within adult midges. However, the siRNA pathway may never 'see' the dsRNA genome segments necessary for activating this defense pathway, as Reoviruses are thought to produce single stranded positive sense mRNAs and synthesis of the dsRNA genome only occurs within the viral core [40]. Therefore, while the siRNA pathway is a critical antiviral defense in other dipterans [41,42], its function against BTV and EDHV in *Culicoides* requires further study.

Extrinsic antiviral defenses

In addition to heritable antiviral defenses, extrinsic factors can also influence vector competence (*e.g.*, gut

Table 1

Heritable factors of vector competence identified from *C.sonorensis* transcriptome

Pathway	Description	Members	Accession no.	Reference no.	e value
IMD AMPs	Receptors	Peptidoglycan Recognition Protein (Long; PGRP-LC)	GAWM01004359	AAEL013112	4.49E-23
		PGRP-LC	GAWM01003592	AAEL014640	1.9E-40
		PGRP-LC	GAWM01011033	AAEL014640	1.9E-40
		PGRP-LC	GAWM01011035	AAEL014640	1.9E-40
		PGRP-LC	GAWM01011037	AAEL014640	7.24E-43
		PGRP-LC	GAWM01011039	AAEL014640	1.9E-40
		PGRP-LC	GAWM01000194	AAEL014989	1.3E-22
	Signaling	DREDD (Caspase-8)	GAWM01000519	AAEL014148	4.93E-66
		Inhibitor of apoptosis (IAP)	GAWM01008211	AAEL006633	4.4E-146
		tak1-associated binding protein (TAB)	GAWM01006076	CPIJ000820	5.27E-21
		tak1 (MAP3 K)	GAWM01010356	AAEL007035	3.82E-62
		tak1 (MAP3 K)	GAWM01012184	AAEL012659	8.2E-54
		I-Kappa-B Kinase 2 (IKK2, IKK-gamma), key/kenny	GAWM01018250	AAEL012510	2.02E-43
		I-Kappa-B Kinase 1 (IKK1, IKK-beta), ird5	GAWM01013537	AAEL003245	0
	Transcription	NF-kappaB transcription factor, Relish	GAWM01014884	AAEL007624	5.09E-52
		NF-kappaB transcription factor, Relish	GAWM01014885	AAEL007624	2.64E-27
	AMPs	attacin-like AMP	GAWM01008443	n/a	3.53E-07
		attacin	GAWM01017969	AAEL003389	1.39E-22
		defensin	GAWM01019039	n/a	0.000146
		defensin	GAWM01019040	n/a	5.14E-08
		cecropin	GAWM01000005	n/a	3.93E-14
	Regulators	Caudal homeobox protein	GAWM01004228	AAEL014557	1.83E-82
		poor imd response upon knock-in (PIRK); PIMS; RUDRA	GAWM01010231	CPIJ014088	3.27E-09
		FAS-associated factor 1, caspar	GAWM01012793	AAEL003579	0
		Peptidoglycan Recognition Protein (Short form); PGRPSC2/SC3	GAWM01018647	AAEL007039	4.37E-84
Toll	Upstream signaling	Peptidoglycan Recognition Protein (Short); PGRP-SA	GAWM01018051	AAEL009474	6.18E-17
		Gram-Negative Binding Protein (GNBP), or Beta-1,3-Glucan Binding Protein (BGBP); GNBP-1	GAWM01002165	AAEL009176	3.81E-90
		GNBP-1/BGBP-1	GAWM01003712	AAEL009176	3.81E-90
		GNBP-1/BGBP-1	GAWM01004143	AAEL009176	3.81E-90
		GNBP-3/BGBP-3	GAWM01011997	AAEL000652	2.96E-38
		Spaetzle-like cytokine, Spz3	GAWM01001358	AAEL014950	3.56E-129
		Spaetzle-like cytokine, Spz5	GAWM01006049	AAEL001929	1.29E-44
		Spaetzle-like cytokine, Spz6	GAWM01012721	AAEL012164	2.42E-37
		Spaetzle-like cytokine, Spz1?	GAWM01015015	AAEL000499	1.59E-35
	Receptors	Toll receptor	GAWM01015594	AAEL009551	5.73E-106
		Toll receptor	GAWM01019001	AAEL000633	0
		Toll receptor	GAWM01015706	AAEL009551	4.24E-152
		Toll receptor	GAWM01013057	AAEL002583	0
		Toll receptor	GAWM01013058	AAEL002583	0
	Cell signaling	myeloid differentiation primary response protein 88 (MYD88)	GAWM01018790	AAEL007768	1.44E-46
		Ser/Thr Kinase, Pelle (IRAK1)	GAWM01001221	AAEL006571	1.62E-92
		Ser/Thr Kinase, Pelle (IRAK1)	GAWM01011117	AAEL006571	1.62E-92
		Tube (IRAK4)	GAWM01007838	AAEL007642	1.93E-42
		cactus (IkappaB)	GAWM01009580	AAEL001584	1.95E-16
	Transcription	dorsal/dif (REL1)	GAWM01010293	AAEL014821	4.73E-95
		dorsal/dif (REL1)	GAWM01010294	AAEL014821	4.73E-95
		dorsal/dif (REL1)	GAWM01010296	AAEL014821	4.73E-95
		dorsal/dif (REL1)	GAWM01010297	AAEL014821	4.73E-95
JAK-STAT	Receptors	Domeless (Dome)	GAWM01016058	AAEL012471	4.38E-85
			GAWM01016156	AAEL012471	4.38E-85
	Cell signalling	Hopscotch janus kinase (Hop)	GAWM01005626	AAEL012553	0
	Transcription	Signal transducer and activator of transcription (STAT)	GAWM01007780	AAEL013265	1.01E-43
			GAWM01011778	AAEL013265	1.01E-43
			GAWM01013279	AAEL013265	1.01E-43

Table 1 (*Continued*)

Pathway	Description	Members	Accession no.	Reference no.	e value
	Regulators	suppressor of cytokine signaling 5 (SOCS36E?)	GAWM01008465	AAEL000393	5.7E-102
		suppressor of cytokine signaling (SOCS7?)	GAWM01008657	AAEL006949	1.62E-104
		protein inhibitor of activated stat; PIAS, sumo ligase	GAWM01011450	AAEL015099	1.66E-150
			GAWM01011451	AAEL015099	1.66E-150
Apoptosis	Regulators	IAP1	GAWM01009039	AAEL009074	1.00E-112
		IAP2	GAWM01008211	AAEL006633	6.00E-150
		IAP5	GAWM01018859	AAEL014251	1.00E-54
		IAP6	GAWM01016583	AAEL012446	0
	Caspases	DRONC	GAWM01016707	AAEL011562	1.00E-46
		Drice	GAWM01000206	AAEL012143	1.00E-108
			GAWM01002195	AAEL014348	1.00E-31
Melanization	Prophenoloxidaes	PPO3	GAWM01010754	AAEL011763	0
		PPO6	GAWM01004196	AAEL014544	9.00E-97
			GAWM01015170	AAEL014545	0
			GAWM01004197	AAEL014546	0
Complement		TEP/MCR	GAWM01009528	AAEL012267	0
Autophagy		APG12	GAWM01007498	AAEL009089	4.00E-42
		APG18A	GAWM01006903	AAEL013063	3.00E-173
		APG18B	GAWM01014186	AAEL013995	0
		APG2	GAWM01013097	AAEL003799	0
		APG4A	GAWM01008772	AAEL010516	0
		APG4B	GAWM01004413	AAEL007228	5.00E-150
			GAWM01004412	AAEL007229	5.00E-151
		APG5	GAWM01004821	AAEL002286	2.00E-141
		APG6	GAWM01004552	AAEL010427	0
		APG7A	GAWM01018401	AAEL010641	0
		APG7B	GAWM01011557	AAEL012306	0
		APG8	GAWM01004039	AAEL007162	2.00E-23
			GAWM01012322	AAEL007163	2.00E-81
		APG9	GAWM01011616	AAEL009105	0
	Regulators	BUFFY	GAWM01016181	AAEL001521	3.00E-105
		DEBCL	GAWM01000607	AAEL001515	7.00E-73
		TOR	GAWM01000709	AAEL000693	0
siRNA		AGO2	GAWM01012834	AAEL017251	5.00E-49
			GAWM01012835	AAEL017251	2.00E-133
			GAWM01012837	AAEL017251	4.00E-48
		Dicer2	GAWM01016560	AAEL006794	0
		R2D2	GAWM01013568	AAEL011753	1.00E-53
			GAWM01017705	AAEL011753	1.00E-21
Other	Neural protein	AaHig	GAWM01013591	AAEL004725	0
			GAWM01013592	AAEL004725	0

microbiota). In mosquitoes, certain bacterial microbiota species enhance permissiveness while others bolster refractoriness [43,44]. In the midgut of *A. aegypti*, Dengue virus infection elicited an epithelial immune response which in turn activated antibacterial responses that modulated gut flora [44]. Therefore, in mosquitoes, and presumably in *Culicoides*, the MIB is likely comprised of a tripartite interaction between gut microbiota, epithelial defense responses (immune elements), and pathogens in the blood meal (Figure 1), which ultimately impact midgut infection and subsequent vector competence.

Some microbial species identified in either rearing media or natural breeding substrate of colonized *Culicoides variipennis* were also culturable from surface-sanitized sp. pupae and newly-emerged adults, which implies transstadial carriage of bacteria contributes to microbial community assembly in the adult midgut [45]. The bacteria species cultured included members of the genera *Acinetobacter*, *Enterobacter* and *Pseudomonas*. These same genera and sister taxa were identified using a culture-independent approach (sequencing 16S rDNA) from field-collected [46•] and culture-dependent approach (culture of viable bacteria, followed by 16S sequencing) from colonized [47] adult *C. sonorensis*, suggesting certain bacterial species are important members of adult gut microbial communities. Interestingly, blood meal alone alters the composition of the gut microbial population, including species diversity and abundance [47]. These results suggest that previous blood feeding can impact the immune

Table 2

Summary of orbivirus transmission dynamics within *C. sonorensis*

Paper	Experimental setup				Viral titer	Prevalence	
	Virus	Infecting dose[a]	Temp [°C]	Detection limit[b]	Proliferation phase [dpi]	Primary eclipse	
						dpi	% decrease
Foster and Jones [60]	BTV[d]	6	23	NA	4	–	–
Chandler *et al.* [61]	BTV-11	6.5	27	NA	5	–	–
Fu *et al.* [22••]	BTV-1	6.7	24	0.75	1	2.5	14.3
Wittman *et al.* [57]	EHDV-1	5.7–6.5	20	1.4	–	2	20
			25		–	1	10
			30		–	3[c]	20[c]
	BTV-10	5.7–6.5	20	1.4	–	4	20
			25		–	1	10
			30		–	4[c]	10[c]
	BTV-16	5.7–6.5	20	1.4	–	4	20
			25		–	5	10
			30		–	1	50
Ruder *et al.* [59•]	EHDV-7	7.03–7.6	22	2.3	6	–	–
		3.1–3.9	22	2.3	–	–	–
Veronesi *et al.* [49••]	BTV-1	≥6	25	0.5	4	3	52
Ruder *et al.* [5]	EHDV-1	7	20	2.3	(13–20)	3	70
			25		6	3	30
			30		(2–6)	3	20
	EHDV-2	6.8	20	2.3	(16–20)	1	40
			25		6	4	10
			30		(4–12)	3	20
	EHDV-7	7	20	2.3	12	3	50
			25		6	4	20
			30		3	(2–3)[c]	20[c]
	EHDV-7	6.2	20	2.3	12	(2–3)[c]	60[c]
			25		4	(2–3)[c]	60[c]
			30		(2–6)	2	62

[a] $log_{10}TCID_{50}$/ml.
[b] $log_{10}TCID_{50}$/midge.
[c] initial 10% decrease at 1 dpi.
[d] serotype not listed.

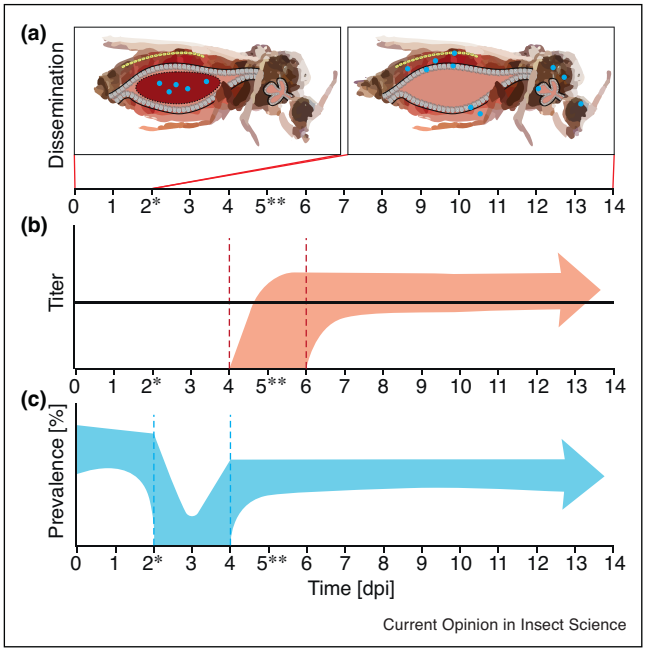
status of the midgut and, therefore, affect vector competence for pathogens in a subsequent blood meal. In addition, innate immune gene expression is upregulated in either sugar or blood fed midges (compared to the teneral state), which further suggests that the meal impacts microflora and subsequently, or concurrently, influences midgut immunity (Figure 1) [47,48•].

Temporal dynamics of virus dissemination

A time course (Box 1) monitoring the dissemination of BTV infection upon first blood meal in the midge was recorded by using an array of detection techniques in a limited number of studies and serotypes (BTV-1, -10, -11, and 17) [22••,49••,50,51]. The peritrophic matrix was formed in the midgut by 1 day post infection (dpi), but did not prevent the entry of virus into the midgut epithelia [51]. In competent midges, the MEB was bypassed by BTV-1 as early as 2 dpi as evidenced by fat body infection (Figure 2a) [22••]. Of note, Sieburth *et al.* [51] found BTV-11 virions associated with the midgut epithelia as early as 1 hpi and viral particles outside the basal lamina by 3 dpi. Data from transcriptomic studies that focused on midge–virus interactions, also align with dissemination observations. Since BTV enters insect cells via clathrin-mediated endocytosis (Lisa M Stevens, PhD thesis, University of Surrey, 2015) the presence of upregulated clathrin-heavy chain transcripts at 2 dpi demonstrates virion entry into midge cells early in infection [52,53].

Several studies observed virus in extra-intestinal tissues, including the salivary glands, by 7 dpi (Figure 2a) [22••,49••,50]. Fu *et al.* [22••] and Veronesi *et al.* [49••] detected infectious BTV in saliva at 5 dpi and 7 dpi, respectively, indicating vector competence. Of note, virions were also observed in neural tissue early after infection (BTV-1:3 dpi) [22••], suggesting that the virus may use the neural network to disseminate. A similar dissemination route for the Reovirus, Rice Dwarf Virus, has been implicated in the leafhopper, *Nephotettix cincticeps* [54]. In addition, AaHig, a membrane protein associated with neural cells was linked to antiviral immunity against Dengue infection in *A. aegypti* [55]. We have identified a putative ortholog to this gene in *Culicoides*, which

Figure 2



Proposed temporal proliferation dynamics of orbivirus infection within *C. sonorensis* at 25°C.
These figures summarize typical findings from previous experimental BTV and EHDV infections listed in Table 2 and compare dissemination **(a)**, viral load **(b)**, and prevalence **(c)** at 25°C. (a) After ingestion of the infected blood meal, the mesenteron escape barrier (MEB) is passed as early as 2 dpi (*) in competent midges, but salivary gland infection does not occur until 5 dpi (**). (b) Upon initial infection, viral load remains at or below the limit of detection until the proliferation phase (4–6 dpi). Viral load continues to fluctuate along the competence threshold (black line), but overall increases/plateaus over time. (c) Prevalence of virus-positive midges is stable early in infection, but undergoes a primary eclipse between 2–4 dpi. Afterwards, prevalence increases and plateaus over the course of infection. Additional environmental and host factors can also significantly impact this scenario.

suggest the midge may also have neural antiviral defenses (Table 1).

Conclusions

Taken together, these studies demonstrate the complexity of virus infection time course and events within *Culicoides* midges (Figure 2). Nevertheless, we can determine critical time points during infection. The blood meal is present within the midgut from 0–2 dpi, and disseminates from the mesenteron barrier between 2–3 dpi [22••,51]. At these time points, virus titer is normally below the limit of detection (Figure 2b), and sharp decrease in the prevalence of virus-positive midges is observed (primary eclipse, Figure 2c), suggesting the primary eclipse is due to blood meal digestion and/or responses to viral dissemination. Genes and proteins putatively involved vector–virus interactions at the mesenteron barrier would be expressed or activated between 1–3 dpi.

Infection of the salivary glands was not observed until 5 dpi [22••,50], which coincided with increasing prevalence (Figure 2c) and viral proliferation (Figure 2b). The proliferation phase normally results in viral titer $\geq 10^{2.7}$ $TCID_{50}$/ml, which suggests that this threshold titer can be used to assess vector competence and associated salivary gland infection. However, because studies have demonstrated that viral titer [56•] and prevalence [56•,57,58] are temperature-dependent, the detection

Box 1 Time course of viral infection in the midge

Studies focusing on the time course of viral infection within the midge are invaluable in interpreting the dynamics underlying dissemination. These studies describe temporal changes in viral titer and viral prevalence during the course of infection (Figure 2, Table 2).

Viral titer

To quantify viral titer over the course of infection, most studies summarized here utilized $TCID_{50}$ endpoint dilution assays, which measure the amount of virus that causes cytopathic effect in 50% of inoculated cells. These assays have a limit of detection ranging from $10^{0.75–2.3}$ $TCID_{50}$/midge [22••,56•,59•,61,60]. Veronesi *et al.* used an ELISA-based approach with a $10^{0.5}$ $TCID_{50}$/midge detection limit. Early after infection (0–4 dpi), titers are near or below the limit of detection due to the low viremia in the volume of blood ingested (likely in the hundreds of nanoliter range) (Figure 2b) [65]. Viral titer increases to levels above the detection limit between 4–6 dpi when at 25°C; this event is known as the proliferation phase. The proliferation phase is temperature-dependent: high incubation (30°C) hastens the onset of this event to occur as soon as 2–3 dpi, whereas low temperatures (20°C) can delay the event until 12 dpi [56•,58]. In most studies, the proliferation phase results in viral titer above the threshold for presumed vector competence ($\geq 10^{2.7}$ $TCID_{50}$/midge), suggesting competent midges are observed when this event occurs [22••,66]. Viral titer varies over the course of infection [66], and may fluctuate around this apparent vector competence threshold (Figure 2b) [22••,49••,56•,61,59•].

Viral prevalence

Although viral titer may be below the limit of detection using a $TCID_{50}$ approach, cell culture assays were also used to determine the prevalence of virus-positive midges in a population [5,22••,57,59•]. Overall, prevalence of virus-positive midges is high to moderate after feeding (0 dpi) on a high virus titer blood meal, due to detection of ingested virus (Figure 2c). Indeed, the initial prevalence after feeding is dependent on the titer of the infectious blood meal, as Ruder *et al.* [59•] showed all midges were virus-positive at 0 dpi after feeding on high viremic deer ($10^{7.03–7.6}$ $TCID_{50}$/ml); however, prevalence at 0 dpi dropped to 33% after midges fed on deer with low viremia ($10^{3.1–3.9}$ $TCID_{50}$/ml). The blood meal is digested by 2 dpi and any viable virus particles should encounter the mesenteron barriers, which apparently results in a decrease in prevalence, as much as 70% between 2–4 dpi when at 25 °C (primary eclipse, Table 2) [56•]. Prevalence subsequently increases and plateaus later in infection. Prevalence is temperature-dependent, where increasing temperatures leads to a higher prevalence (>80%) during early infection (1–4 dpi) and low temperatures result in consistently lower prevalence [56•]. Jennings and Mellor [66] also found prevalence to be between 0–51.6% at 8 dpi after BTV-4 infection, demonstrating high variation in prevalence.

of competent midges (via this threshold titer), and dissemination dynamics could both be impacted by temperatures used during experimental studies.

Gaps and future directions

Several studies have documented the time course of orbivirus infection in *Culicoides* [5,22••,49••,59•,60,61], but more work is required to understand the critical events that occur at the MIB, MEB, and dissemination barrier. The interface between virus, gut microbiota, and the midgut epithelium needs to be better described in both laboratory and field-caught midges. Such studies would help elucidate the primary mechanisms in the MIB. Heritable components of vector competence could be identified from transcriptome and genome sequencing of refractory and permissive lines. In addition, we suspect that viruses that bypass the MEB may disseminate throughout the hemocoel using neural tissues, but this notion has yet to be tested. There is also a need to better understand the relationships between viral dissemination and temperature, as these dynamics are directly related to the extrinsic incubation period [58]. Differential competence for strains and serotypes of BTV and EHDV in midges is unknown, and therefore an area of study that requires further investigation.

Compared to mosquito biologists, researchers who study *Culicoides* midges have a deficit of next generation resources and tools available [62]. However, several modern tools have recently been developed which will help in studying midge–virus interactions including *in vivo* RNAi [39••], a *de novo* transcriptome [35••], and comparative transcriptome projects [48•]. Several microbiome projects are underway to better understand microbial impacts on vector competence, larval development and species distribution. The need for additional molecular tools to study *Culicoides*–virus interactions remains [62,63,64], including an available genome. However, existing tools and techniques allow researchers now to test the respective contributions of annotated immune pathways to vector competence, which will significantly increase our understanding of midge–virus interactions.

Acknowledgements

We thank Dr. Simon Carpenter at The Pirbright Institute (UK) for unpublished data provided for this review. Data on BTV replication rates was produced using studies funded by Defra grant SE: 2616 awarded to Dr. Carpenter. *Culicoides* studies at USDA-ARS are funded by National Program 104, project number 3020-32000-007. M.K.M. was supported by Specific Cooperative Agreement 58-5430-4-022 to K.M. from the USDA Agricultural Research Service. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author and do not necessarily reflect the official policy or position of the U.S. Department of Agriculture, or the U.S. Government. D.N. and R.S.P. are U.S. Government employees. This work was prepared as part of official duties. Title 17 U.S.C. §105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by an employee of the U.S. Government as part of that person's official duties.

References

1. Borkent A: **The biting midges, the Ceratopogonidae (Diptera)**. In *Biology of Disease Vectors.* Edited by Marquardt WH. Academic Press; 2005:113-126.

2. Mullens BA, Rutz DA: **Development of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) at constant laboratory temperatures**. *Ann. Entomol. Soc. Am.* 1983, **76**:747-751.

3. Mullens BA, Schmidtmann ET: **The gonotrophic cycle of *Culicoides variipennis* (Diptera: Ceratopogonidae) and its implications in age-grading field populations in New York State, USA**. *J. Med. Entomol.* 1982, **19**:340-349.

4. Mellor PS, Boorman J, Baylis M: ***Culicoides biting* midges: their role as arbovirus vectors**. *Annu. Rev. Entomol.* 2000, **45**: 307-340.

5. Ruder MG, Lysyk TJ, Stallknecht DE, Foil LD, Johnson DJ, Chase CC, Dargatz DA, Gibbs EPJ: **Transmission and epidemiology of bluetongue and epizootic hemorrhagic disease in North America: current perspectives, research gaps, and future directions**. *Vector-Borne Zoonotic Dis.* 2015, **15**:348-363.

6. Howerth EW, Stallknecht DE, Kirkland P: **Bluetongue, epizootic hemorrhagic disease, and other orbivirus-related diseases**. In *The Infectious Diseases of Wild Mammals.* Edited by Williams ES, Barker IK. Iowa State Press; 2001:77-97.

7. Miller MM, Brown J, Cornish T, Johnson G, Mecham JO, Reeves WK, Wilson W: **Investigation of a bluetongue disease epizootic caused by bluetongue virus serotype 17 in sheep in Wyoming**. *J. Am. Vet. Med. Assoc.* 2010, **237**:955-959.

8. Stevens G, McCluskey B, King A, O'Hearn E, Mayr G: **Review of the 2012 epizootic hemorrhagic disease outbreak in domestic ruminants in the United States**. *PLoS One* 2015, **10**:e0133359.

9. Kedmi M, Van Straten M, Ezra E, Galon N, Klement E: **Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds**. *J. Dairy Sci.* 2010, **93**: 2486-2495.

10. Santman-Berends IMGA, Hage JJ, Lam TJGM, Sampimon OC, van Schaik G: **The effect of bluetongue virus serotype 8 on milk production and somatic cell count in Dutch dairy cows in 2008**. *J. Dairy Sci.* 2011, **94**:1347-1354.

11. Velthuis AGJ, Saatkamp HW, Mourits MCM, de Koeijer AA, Elbers ARW: **Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007**. *Prev. Vet. Med.* 2010, **93**:294-304.

12. Maclachlan NJ, Mayo CE: **Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife**. *Antiviral Res.* 2013, **99**:79-90.

13. MacLachlan NJ, Osburn BI: **Impact of bluetongue virus infection on the international movement and trade of ruminants**. *J. Am. Vet. Med. Assoc.* 2006, **228**:1346-1349.

14. Tabachnick WJ: ***Culicoides variipennis* and bluetongue–virus epidemiology in the United States**. *Annu. Rev. Entomol.* 1996, **41**:23-43.

15. Foster NM, Jones RH, McCrory BR: **Preliminary investigations on insect transmission of bluetongue virus in sheep**. *Am. J. Vet. Res.* 1963, **24**:1195-1200.

16. Tanya VN, Greiner EC, Gibbs EP: **Evaluation of *Culicoides insignis* (Diptera: Ceratopogonidae) as a vector of bluetongue virus**. *Vet. Microbiol.* 1992, **32**:1-14.

17. Foster NM, Breckon RD, Luedke AJ, Jones RH: **Transmission of two strains of epizootic hemorrhagic disease virus in deer by *Culicoides variipennis***. *J. Wildl. Dis.* 1977, **13**:9-16.

18. Holbrook FR, Tabachnick WJ, Schmidtmann ET, McKinnon CN, Bobian RJ, Grogan WL: **Sympatry in the *Culicoides variipennis* complex (Diptera: Ceratopogonidae): a taxonomic reassessment**. *J. Med. Entomol.* 2000, **37**:65-76.

19. Borkent A, Grogan WL: **Catalog of the New World biting midges north of Mexico (Diptera: Ceratopogonidae)**. *Zootaxa* 2009, **2273**:1-48.

20. Vigil SL, Wlodkowski JC, Parris J, De Vargas SE, Shaw D, Cleveland C, Grogan WL, Corn JL: **New records of biting midges of the genus *Culicoides* Latreille from the southeastern United States (Diptera: Ceratopogonidae)**. *Insecta mundi* 2014, **394**: 1-14.

21. Pfannenstiel RS, Mullens BA, Ruder MG, Zurek L, Cohnstaedt LW, Nayduch D: **Management of North American *Culicoides* biting midges: current knowledge and research needs**. *Vector-Borne Zoonotic Dis.* 2015, **15**:374-384.

22. •• Fu H, Leake CJ, Mertens PP, Mellor PS: **The barriers to bluetongue virus infection, dissemination and transmission in the vector, *Culicoides variipennis* (Diptera: Ceratopogonidae)**. *Arch. Virol.* 1999, **144**:747-761.

Authors identified the barriers to infection within *Culicoides* and confirmed titers $\geq 10^{2.7}$ $TCID_{50}$/midge resulted in infectious virions in the saliva, indicating vector competence. This was the first study to focus on all dynamics of BTV infection (titer, prevalence, and dissemination).

23. Tabachnick WJ: **Genetic variation in laboratory and field populations of the vector of bluetongue virus, *Culicoides variipennis* (Diptera: Ceratopogonidae)**. *J. Med. Entomol.* 1990, **27**:24-30.

24. Tabachnick WJ: **Genetic control of oral susceptibility to infection of *Culicoides variipennis* with bluetongue virus**. *Am. J. Trop. Med. Hyg.* 1991, **45**:666-671.

25. Cheng G, Liu Y, Wang P, Xiao X: **Mosquito defense strategies against viral infection**. *Trends Parasitol.* 2016, **32**:177-186.

26. Yordy B, Iwasaki A: **Autophagy in the control and pathogenesis of viral infection**. *Curr. Opin. Virol.* 2011, **1**:196-203.

27. Calvo E, Pham V, Marinotti O, Andersen J, Ribeiro J: **The salivary gland transcriptome of the neotropical malaria vector *Anopheles darlingi* reveals accelerated evolution of genes relevant to hematophagy**. *BMC Genomics* 2009, **10**:57.

28. Sim S, Ramirez JL, Dimopoulos G: **Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior**. *PLoS Pathog* 2012, **8**:e1002631.

29. Zhao P, Lu Z, Strand MR, Jiang H: **Antiviral, antiparasitic, and cytotoxic effects of 5, 6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response**. *Insect Biochem. Mol. Biol.* 2011, **41**:645-652.

30. Rodriguez-Andres J, Rani S, Varjak M, Chase-Topping ME, Beck MH, Ferguson MC, Schnettler E, Fragkoudis R, Barry G, Merits A *et al.*: **Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest Virus**. *PLoS Pathog.* 2012, **8**:e1002977.

31. Xiao X, Liu Y, Zhang X, Wang J, Li Z, Pang X, Wang P, Cheng G: **Complement-related proteins control the flavivirus infection of *Aedes aegypti* by inducing antimicrobial peptides**. *PLoS Pathog.* 2014, **10**:e1004027.

32. Ip YT: ***Drosophila* innate immunity goes viral**. *Nat. Immunol.* 2005, **6**:863-864.

33. Kingsolver MB, Huang Z, Hardy RW: **Insect antiviral innate immunity: pathways, effectors, and connections**. *J. Mol. Biol.* 2013, **425**:4921-4936.

34. Merkling SH, van Rij RP: **Beyond RNAi: antiviral defense strategies in *Drosophila* and mosquito**. *J. Insect Physiol.* 2013, **59**:159-170.

35. •• Nayduch D, Lee MB, Saski C: **The reference transcriptome of the adult female biting midge (*Culicoides sonorensis*) and differential gene expression profiling during teneral, blood, and sucrose feeding conditions**. *PLoS One* 2014, **9**:e98123.

This study provided the first de novo reference transcriptome for this genus, and identified genes associated with blood feeding and reproduction.

36. Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM *et al.*: **Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes**. *Science* 2007, **316**:1738-1743.

37. Cheng G, Liu L, Wang P, Zhang Y, Zhao YO, Colpitts TM, Feitosa F, Anderson JF, Fikrig E: **An *in vivo* transfection approach elucidates a role for *Aedes aegypti* thioester-containing proteins in flaviviral infection**. *PLoS One* 2011, **6**:e22786.

38. • Schnettler E, Ratinier M, Watson M, Shaw AE, McFarlane M, Varela M, Elliott RM, Palmarini M, Kohl A: **RNA interference targets arbovirus replication in *Culicoides* cells**. *J. Virol.* 2013, **87**:2441-2454.

Authors demonstrate RNAi can be used to reduce infection by providing dsRNA to BTV Ns1 in *Culilcoides* cells. This is the first study to utilize RNAi in *Culicoides* cell lines to target viral transcipts.

39. •• Mills MK, Nayduch D, Michel K: **Inducing RNA. interference in the arbovirus vector, *Culicoides sonorensis***. *Insect Mol. Biol.* 2015, **24**:105-114.

Injecting dsRNA specific to the *Culicoides* ortholog to inhibitor of apoptosis gene 1 into adult midges resulted in targeted transcipt knockdown and increased mortality. This study was the first to use RNAi to target -*Culicoides* transcripts *in vivo*.

40. Patel A, Roy P: **The molecular biology of Bluetongue virus replication**. *Virus Res.* 2014, **182**:5-20.

41. Ding SW: **RNA-based antiviral immunity**. *Nat. Rev. Immunol.* 2010, **10**:632-644.

42. Marques JT, Imler J-L: **The diversity of insect antiviral immunity: insights from viruses**. *Curr. Opin. Microbiol.* 2016, **32**:71-76.

43. Apte-Deshpande A, Paingankar M, Gokhale MD, Deobagkar DN: ***Serratia odorifera* a midgut inhabitant of *Aedes aegypti* mosquito enhances its susceptibility to dengue-2 virus**. *PLoS One* 2012, **7**:e40401.

44. Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Pascale JM, Dimopoulos G: **Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota: innate immune system and dengue virus influences vector competence**. *PLoS Negl. Trop. Dis.* 2012, **6**:e1561.

45. Parker MD, Akey DH, Lauerman LH: **Microbial flora associated with colonized and wild populations of the biting gnat *Culicoides variipennis***. *Entomol. Exp. Appl.* 1977, **21**:130-136.

46. • Campbell CL, Mummey DL, Schmidtmann ET, Wilson WC: **Culture-independent analysis of midgut microbiota in the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae)**. *J. Med. Entomol.* 2004, **41**:340-348.

This study used a sequencing approach to identify bacterial microflora associated with *C. sonorensis*.

47. Nayduch D, Erram D, Lee MB, Zurek L, Saski CA: **Impact of the blood meal on humoral immunity and microbiota in the gut of female *Culicoides***. *Vet. Ital.* 2015, **51**:385-392.

48. • Nayduch D, Lee MB, Saski CA: **Gene discovery and differential expression analysis of humoral immune response elements in female *Culicoides sonorensis* (Diptera: Ceratopogonidae)**. *Parasit. Vectors.* 2014, **7**:388.

This study identified components of the innate immune response in *C. sonorensis*, including Toll, Imd and JAK-STAT pathways, and their expression under several feeding conditions over time.

49. •• Veronesi E, Antony F, Gubbins S, Golding N, Blackwell A, Mertens PP, Brownlie J, Darpel KE, Mellor PS, Carpenter S: **Measurement of the infection and dissemination of bluetongue virus in *Culicoides* biting midges using a semi-quantitative rt-PCR assay and isolation of infectious virus**. *PLoS One.* 2013, **8**:e70800.

Using a variety of techniques, the authors recorded the dynamics of BTV-1 infection within *C. sonorensis* and the poor vector, *Culicoides nubeculosus*. This is the most recent study to track titer, prevalence, and dissemination of BTV-1 over time, and the only study utilize both qPCR and titering techniques when observing prevalence.

50. Ballinger ME, Jones RH, Beaty BJ: **The comparative virogenesis of three serotypes of bluetongue virus in *Culicoides variipennis* (Diptera: Ceratopogonidae)**. *J. Med. Entomol.* 1987, **24**:61-65.

51. Sieburth PJ, Nunamaker CE, Ellis J, Nunamaker RA: **Infection of the midgut of *Culicoides variipennis* (Diptera: Ceratopogonidae) with bluetongue virus**. *J. Med. Entomol.* 1991, **28**:74-85.

52. Campbell CL, Wilson WC: **Differentially expressed midgut transcripts in *Culicoides sonorensis* (Diptera: ceratopogonidae) following Orbivirus (Reoviridae) oral feeding**. *Insect Mol. Biol.* 2002, **11**:595-604.

53. Campbell CL, Vandyke KA, Letchworth GJ, Drolet BS, Hanekamp T, Wilson WC: **Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae)**. *Insect Mol. Biol.* 2005, **14**:121-136.

54. Chen H, Chen Q, Omura T, Uehara-Ichiki T, Wei T: **Sequential infection of Rice dwarf virus in the internal organs of its insect vector after ingestion of virus**. *Virus Res.* 2011, **160**:389-394.

55. Xiao X, Zhang R, Pang X, Liang G, Wang P, Cheng G: **A neuron-specific antiviral mechanism prevents lethal flaviviral infection of mosquitoes**. *PLoS Pathog.* 2015, **11**:e1004848.

56. • Ruder MG, Stallknecht DE, Howerth EW, Carter DL, Pfannenstiel RS, Allison AB, Mead DG: **Effect of temperature on replication of epizootic hemorrhagic disease viruses in *Culicoides sonorensis* (Diptera: Ceratopogonidae)**. *J. Med. Entomol.* 2015, **52**:1050-1059.

Using temperatures varying from 20–30°C, the authors EHDV serotypes to increase in titer and prevalence within the midge as temperature increases. The experimental design and data make an essential contribution to the field.

57. Wittman EJ, Mellor PS, Baylis M: **Effect of temperature on the transmission of arboviruses by the biting midge, *Culicoides sonorensis***. *Med. Vet. Entomol.* 2002, **16**:147-156.

58. Carpenter S, Wilson A, Barber J, Veronesi E, Mellor P, Venter G, Gubbins S: **Temperature dependence of the extrinsic incubation period of orbiviruses in *Culicoides* biting midges**. *PLoS One* 2011, **6**:e27987.

59. • Ruder MG, Howerth EW, Stallknecht DE, Allison AB, Carter DL, Drolet BS, Klement E, Mead DG: **Vector competence of *Culicoides sonorensis* (Diptera: Ceratopogonidae) to epizootic hemorrhagic disease virus serotype 7**. *Parasit. vectors* 2012, **5**:236.

Authors followed titer and prevalence throughout *Culicoides* infection. This study also compared the infection of *Culicodies* after feeding on deer of different viremia, indicating titer fed is potentiall an essential component to vector competence.

60. Foster NM, Jones RH: **Multiplication rate of bluetongue virus in the vector *Culicoides variipennis* (Diptera: Ceratopogonidae) infected orally**. *J. Med. Entomol.* 1979, **15**:302-303.

61. Chandler LJ, Ballinger ME, Jones RH, Beaty BJ: **The virogenesis of bluetongue virus in *Culicoides variipenis***. *Prog. Clin. Biol. Res.* 1985, **178**:245-253.

62. Nayduch D, Cohnstaedt LW, Saski C, Lawson D, Kersey P, Fife M, Carpenter S: **Studying *Culicoides* vectors of BTV in the post-genomic era: resources, bottlenecks to progress and future directions**. *Virus Res.* 2014, **182**:43-49.

63. Carpenter S, Veronesi E, Mullens B, Venter G: **Vector competence of *Culicoides* for arboviruses: Three major periods of research, their influence on current studies and future directions**. *Rev. Sci. Tech. Off. Int. Epiz.* 2015, **34**:97-112.

64. Drolet BS, van Rijn P, Howerth EW, Beer M, Mertens PP: **A review of knowledge gaps and tools for orbivirus research**. *Vector-Borne Zoonotic Dis.* 2015, **15**:339-347.

65. Leprince D, Higgins J, Church G, Issel C, McManus J, Foil L: **Body size of *Culicoides variipennis* (Diptera: Ceratopogonidae) in realtion to bloodmeal size estimates the ingestion of Onchocerca cervicalis**. *J. Am. Mosq. Control Assoc.* 1989, **5**:100-102.

66. Jennings DM, Mellor PS: **Variation in the responses of *Culicoides variipennis* (Diptera, Ceratopogonidae) to oral infection with bluetongue virus**. *Arch. Virol.* 1987, **95**:177-182.