Impact of the blood meal on humoral immunity and microbiota in the gut of female Culicoides sonorensis

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Bacteria,
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Innate immunity,
Microbiota.

Summary
Although *Culicoides sonorensis* is an important vector of orbiviruses causing significant disease in domestic and wild ruminants in the US, little is known about factors contributing to midge vector competence. In other vectors such as mosquitoes, interactions among the humoral immune response, microbiota, and ingested pathogens within the vector gut directly impact pathogen survival and therefore vectoring potential. We recently described components of the humoral immune response in the reference transcriptome for adult female *C. sonorensis* and analysed their temporal expression profiles across several dietary states (unfed, blood, or sugar fed). Blood feeding altered the transcription of several humoral immune components of the Immune deficiency (Imd), dual-oxidase (DUOX), and Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathways. Genes for immune effectors, such as antimicrobial peptides, were in particular highly induced. Since blood feeding also stimulated proliferation and diversification of bacterial populations colonising the gut of female midges, we infer that changes in immune gene expression were a result of fluctuations in gut microbiota. Thus, diet can indirectly (via microbiota) impact gut immune status and therefore should be carefully considered in subsequent studies assessing vector competence in biting midges.

Impatto dell'assunzione di sangue su immunità umorale e microbiota nell'intestino di femmine di Culicoides sonorensis

Parole chiave
Batteri,
Competenza vettoriale,
Culicoides sonorensis,
Immunità innata,
Microbiota.

Riassunto
Culicoides sonorensis è un importante vettore di *Orbivirus*. Nonostante negli Stati Uniti causi importanti patologie in ruminanti domestici e selvatici, le informazioni sui fattori che contribuiscono alla loro competenza vettoriale sono scarse. Per altri vettori, come le zanzare, si è riscontrato come la sopravvivenza degli agenti patogeni ingeriti, presenti all'interno dell'intestino del vettore, venga ridotta dalle interazioni reciproche con il sistema immunitario umorale e il microbiota, riducendo il potenziale vettoriale dell'insetto. Gli autori di questo studio hanno recentemente descritto le componenti che caratterizzano la risposta immunitaria umorale in relazione al trascrittoma di riferimento delle femmine adulte di *C. sonorensis*. Hanno analizzato il loro profilo temporale di espressione considerando diversi regimi dietetici (digiuno, dieta a base di sangue o a base di zucchero). L'alimentazione a base di sangue altera la trascrizione di numerosi componenti immunitari umorali, dual-ossidasi (DUOX) e Janus Chinasi e Trasduttore di Segnale e Attivatore di Trascrizione (JAK/STAT). In modo particolare, i geni per alcuni effettori del sistema immunitario, come i peptidi antimicrobici, sono risultati fortemente indotti. Considerato che l'alimentazione a base di sangue stimola la proliferazione e la diversificazione delle popolazioni batteriche che colonizzano l'intestino dei moscerini femmina, si deduce che i cambiamenti dell'espressione genica relativa alla risposta immunitaria siano il risultato delle fluttuazioni della flora intestinale e che la dieta possa avere un impatto indiretto (tramite il microbiota) sullo stato immunitario intestinale. Questo aspetto deve essere valutato attentamente negli studi sulla competenza vettoriale dei *Culicoides*.

Introduction

Culicoides midges are globally important vectors of protozoan, helminth, and viral pathogens of animals. Two of these pathogens are arboviruses with enzootic impacts in the United States (US): Bluetongue virus (BTV) and Epizootic haemorrhagic disease virus (EHDV). In the US, *Culicoides sonorensis* Wirth and Jones (formerly *Culicoides variipennis* sonorensis) is considered the primary vector of BTV throughout most of the country (Foster *et al.* 1963, Price and Hardy 1954) and is also a proven vector of EHDV (Foster *et al.* 1977).

Studies on *Culicoides* vector competence for orbiviruses have revealed several barriers to infection within midges (Fu *et al.* 1999). These include: the mesenteron infection barrier (MIB), which limits initial establishment of arbovirus infections in gut epithelial cells; the mesenteron escape barrier (MEB), which restricts virus infection to the gut cells and prevents breaching of the gut barrier; and the dissemination barrier (DB), which inhibits viruses that had breached the MEB from disseminating and infecting secondary target organs in the body, such as the salivary glands. The ability to artificially select 'susceptible' and 'refractory' lines of *Culicoides* implies that there is a genetic component of vector competence (Tabachnick 1991), which may influence the MIB (*e.g.*, virus-binding gut receptors, antimicrobial defense molecules) and the MEB or DB (*e.g.*, innate immune responses). Consequently, vector competence encompasses these 3 barriers, since they all influence arbovirus infection success, replication, and dissemination.

Like other Nematocera, the adult midge midgut is colonised by a bacterial community whose composition varies across individuals and populations (Campbell *et al.* 2004). Some of these microbes are acquired either trans-stadially from larvae (Parker *et al.* 1977) and/or during blood or nectar feeding. In the gut, which is the initial interface between pathogens in the blood meal and the vector, epithelial innate immune responses impact both the pathogen and microbiota, thereby directly or indirectly affecting pathogen success. Therefore, the MIB involves both molecular components (*e.g.*, receptors, immune factors) and microorganisms, forming a tripartite interaction among host, gut microbiota, and ingested pathogens. Variable vector competence has been observed in both field-caught and colonized *Culicoides* species, yet the mechanism underlying refractoriness (or permissiveness) for virus infection and transmission remains unknown.

Immune deficiency (Imd), Toll, Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) and dual-oxidase (DUOX) pathways play a major role in vector competence of other insects (Azambuja *et al.* 2005, Cirimotich *et al.* 2011, Ramirez *et al.* 2012, Souza-Neto *et al.* 2009, Weiss and Aksoy 2011, Xi *et al.* 2008). We recently described some of the innate immune components of the midge including receptors, signaling molecules, transcription factors, and effectors (Nayduch *et al.* 2014b). Interestingly, blood feeding altered the expression of many genes involved in these immune pathways on a transcriptome-wide level. We subsequently hypothesised that the blood meal does not directly influence differential expression of these genes, and that it rather alters gut microbiota, which subsequently mediates these changes. Our goal is to understand the conditions in the gut of the midge after blood feeding, especially the status of the innate immune response and microbiota, in order to understand what contributes to the primary barriers to pathogen infection (MIB, MEB).

Materials and methods

Differential expression analysis of Imd, JAK/STAT and DUOX pathways in *C. sonorensis*

The adult female midge reference transcriptome has been previously described (Nayduch *et al.* 2014a) and manually annotated to identify genes involved in several insect humoral immune response pathways (*e.g.*, Imd, Toll, JAK/STAT) (Nayduch *et al.* 2014b). In the current study, additional immune genes in the DUOX pathway were also identified in this transcriptome. Methods for digital genome-wide gene expression profiles under different feeding and temporal conditions (teneral, unfed, early or late blood fed female midges) were previously described (Nayduch *et al.* 2014a). Briefly, comparative analyses of differential transcriptome responses among non-feeding (teneral), early blood meal (early blood, 2, 6, 12 hours), late blood meal (late blood, 36 hours), early sucrose meal (early sucrose, 2, 6, 12 hours), and late sucrose meal (late sucrose, 36 hours) were performed using the Tuxedo software package (Trapnell *et al.* 2012). Reads were mapped to the unigene assembly with the Bowtie2 software. Cufflinks was used to generate transcriptome assemblies for each condition, and Cuffmerge merged the transcriptome assemblies for statistical analysis using Cuffdiff (Trapnell *et al.* 2012). Global gene expression profiles were analysed by pairwise comparisons between and within diet source across time, and significant differences in gene expression were reported ($P \leq 0.01$).

Enumeration and identification of bacteria associated with colonized *C. sonorensis*

To assess the microbial community (abundance and diversity) within developmental stages of *C. sonorensis*, larvae (n = 30) and newly-emerged teneral adult females (n = 30) were collected from a single-source rearing pan from the AK colony (Jones and Foster 1978) at the Arthropod Borne Animal Diseases Research Unit in Manhattan, Kansas, US. Additionally, 10 teneral adult females were fed sterile defribrinated sheep blood at 37°C through an artificial membrane feeding system, and were held at 27°C for 24 hours to allow blood meal digestion. Midge specimens were killed with carbon dioxide then surface sanitised by submersion (2-5 min each) in 10% bleach, 70% ethanol, and rinsed in sterile water. Individual larvae or adults were homogenized in 100 µl sterile distilled water or sterile PBS using a motorised pestle. Homogenates were serially diluted in sterile PBS (undiluted followed by several 10-fold dilutions), and cultured on tryptic soy agar (TSA) (Fisher Scientific, Atlanta, GA, USA). Culture plates were incubated at 27°C for 24 hour then examined for distinct morphotypes, which were streaked for isolation on TSA and further identified by polymerase chain reaction (PCR) amplification of the 16S rRNA gene with universal eubacterial primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 806R (5'- CTACCAGG GTATCTAAT-3') following standard protocols (Hugenholtz *et al.* 1998). The polymerase chain reaction products were purified with DNA Clean & Concentrator™ (ZYMO Research, Irvine, CA, USA) and sequenced on Applied Biosystems 3730 DNA analyzer at KSU's Sequencing and Genotyping Facility using the same PCR primers. Sequences were manually edited in CodonCode Aligner (version 3.7.1) (CodonCode Corporation, Centerville, MA, USA) and identified by BLAST (Basic Local Alignment Search Tool) search of the NCBI GenBank database[1].

Results

Expression of Imd, JAK/STAT and DUOX pathways after blood feeding in *C. sonorensis*

Homologs for key components of the insect humoral immune response were identified in our previous study (Nayduch *et al.* 2014b). We further analysed the transcriptome and identified 2 genes involved in the DUOX pathway, *p38* (a MAP kinase) and the dual oxidase *duox*. The latter generates reactive oxygen species that control microbial populations in the gut. Comparisons of these genes across teneral, early (2, 6, 12 hours, pooled) and late (36 hours) blood-fed transcriptomes revealed a fluctuating genetic landscape with expression profiles differing in response to feeding on blood (Figure 1). More genes were differentially expressed between the teneral and early blood-fed profiles, than between the teneral and late blood fed profiles; although there was substantial overlap of typical 'housekeeping' genes (Nayduch *et al.* 2014a). The antimicrobial effectors (*i.e.,* molecules that directly destroy bacteria) *attacin, attacin-like, defensin-1, defensin-2, cecropin*, and *duox* were significantly upregulated in early and/or late blood-fed female midges, compared to the teneral state ($P < 0.01$). Additionally, a key negative regulator of the Imd pathway, *pirk*, which is responsible for all of the effectors except *duox*, was downregulated in both early and late blood fed midges, which would presumably help in enhancing production of these antimicrobial peptides.

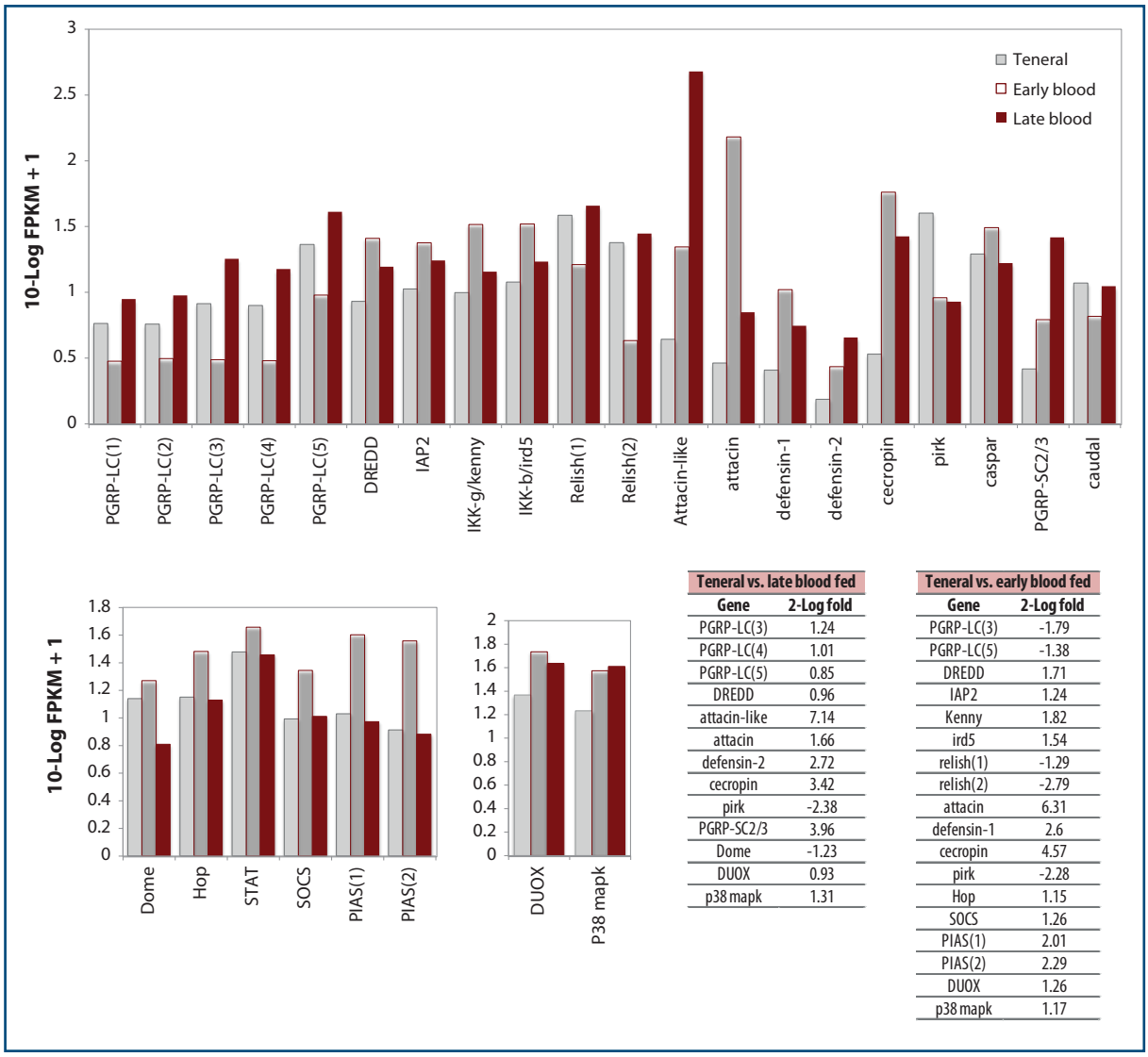
Enumeration and identification of bacteria associated with colonized *C. sonorensis*

All larval cultures (30/30) displayed bacterial growth. Bacteria enumerated from individual larvae ranged from 1.6×10^2 to 7.0×10^6 CFU. The great variation observed in larval samples could be attributed to age and size differences across samples, as the larval instars were not recorded at the time of collection. The number of colony forming units (CFUs) recovered from teneral adults also varied considerably. Of the 30 teneral adults cultured, only 5 adults (16.7%) had recoverable amounts of bacteria ranging from 6.0 to $> 2.5\times10^3$ CFU. Bacteria were cultured from 6/10 of blood fed females from the AK colony (60%), with a range of 2.0 to $> 4.0\times10^3$ CFU per midge. The genera *Aeromonas* and *Pseudomonas* were highly represented in the cultured isolates (Table I). Although *Aeromonas* also was detected in larvae, which could point to the source of this species, *Pseudomonas*, *Morganella*, and *Comamonas* were not.

Discussion

Although *Culicoides* larvae tend to be generalist feeders, subsisting on protists, nematodes, and bacteria, it has been shown that larvae of *C. sonorensis* can survive on a diet (*e.g.,* rearing media) comprised solely of bacterial cultures (Jones *et al.* 1969). Parker and colleagues (Parker *et al.* 1977) demonstrated that some of the same species identified from either rearing media or natural breeding substrate also were recoverable from surface-sanitised *C. variipennis* pupae and newly-emerged adults, which implies both trans-stadial carryover and microbial community assembly in the gut. Most of

[1] http://blast.ncbi.nlm.nih.gov/Blast.cgi.



Teneral vs. late blood fed	
Gene	2-Log fold
PGRP-LC(3)	1.24
PGRP-LC(4)	1.01
PGRP-LC(5)	0.85
DREDD	0.96
attacin-like	7.14
attacin	1.66
defensin-2	2.72
cecropin	3.42
pirk	-2.38
PGRP-SC2/3	3.96
Dome	-1.23
DUOX	0.93
p38 mapk	1.31

Teneral vs. early blood fed	
Gene	2-Log fold
PGRP-LC(3)	-1.79
PGRP-LC(5)	-1.38
DREDD	1.71
IAP2	1.24
Kenny	1.82
ird5	1.54
relish(1)	-1.29
relish(2)	-2.79
attacin	6.31
defensin-1	2.6
cecropin	4.57
pirk	-2.28
Hop	1.15
SOCS	1.26
PIAS(1)	2.01
PIAS(2)	2.29
DUOX	1.26
p38 mapk	1.17

Figure 1. *Transcriptome-wide differential expression of selected* Culicoides sonorensis *humoral immune genes.* Teneral midges were unfed adult females, early blood transcriptomes were 2, 6 and 12 h post blood meal (pooled) and late blood was 36 h post blood meal. Two biological replicates were performed, and data were analyzed as previously described (Nayduch *et al.* 2014a). Imd is immune deficiency pathway; JAK/STAT is Janus Kinase/Signal Transduction and Activators of Transcription; DUOX is dual-oxidase pathway. 10-log FPKM (Fragments Per Kilobase of transcript per Million mapped reads) represents relative expression across conditions. Significant pairwise comparative differences are indicated in the inset tables as 2-Log (fold change), with the baseline condition being teneral, unfed adult female midges ($P < 0.01$).

the recovered bacterial cultures included members of the genera *Acinetobacter*, *Enterobacter*, and *Pseudomonas*. Since these same genera were identified using a culture-independent approach (cloning and sequencing of 16S rDNA) from field-collected adult *C. sonorensis* in a later study (Campbell *et al.* 2004), this suggests that some bacterial species are common members of adult gut microbial communities in midges.

In addition to midges, trans-stadial carryover of bacteria from the larval gut has been demonstrated in other hematophagous insect vectors, such as sand flies and mosquitoes, and results in the microbial colonization of the adult midgut (Boissière *et al.* 2012, Peterkova-Koci *et al.* 2012, Straif *et al.* 1998, Volf *et al.* 2002). In our study, only 1 taxon (*Acinetobacter*) was isolated from both teneral adults and larvae, which implies trans-stadial carriage. This microbe was the dominant genus in teneral adults (data not shown), and was previously reported in surveys of *C. sonorensis* (Parker *et al.* 1977, Campbell *et al.* 2004). Our data indicate that a selection or attrition process may occur during metamorphosis (*i.e.,* during the pupal stage, bridging larvae to teneral adults) where only a few bacterial taxa survive for trans-stadial carryover. Since we used a culture-based approach, some of the microbes present in larvae but apparently absent in adults, and vice versa, may be due to low abundance and inability to recover by our culture techniques.

Table I. *Identification of bacterial isolates from larvae, teneral and blood fed adult female* Culicoides sonorensis *by 16S rRNA PCR and sequencing.*

Sample	No. positive/ total	Strain ID	% ID	Length (bp)
Larvae	30/30	*Aeromonas hydrophila*	99	769
		Bacillus/Lysinibacillus sp.	99	775
		Bacillus/Lysinibacillus sp.	98	775
		Aeromonas sp.	99	775
		Bacillus thuringiensis/cereus	99	776
		Chryseobacterium sp.	99	757
		Acinetobacter sp.	97	759
Teneral females[1]	5/30	*Acinetobacter* sp.	98	748
		Providencia sp.	99	761
		Brevudimonas sp.[2]	99	709
		Acinetobacter sp.[2]	99	762
		Flavobacterium sp.[2]	99	756
Blood-fed females	6/10	*Morganella morganii*[3]	98	769
		Aeromonas sp.[3]	99	768
		Aeromonas sp.	99	768
		Aeromonas sp.	99	772
		Pseudomonas sp.	99	761
		Pseudomonas sp.	99	761
		Comamonas sp.	99	762

[1] One of the teneral females had an unidentifiable, yet culturable, bacterial isolate.
[2,3] Same number indicates that each of these isolates were cultured from the same female midge.

Since these data are very preliminary and we only sampled a small population, additional approaches involving selective media and culture-independent analyses (Gupta *et al.* 2012) may be appropriate in future studies.

In addition to the flux in recoverable bacterial taxa across developmental stages, we also observed a shift in the bacterial diversity in blood-fed female colony midges. With the exception of *Aeromonas* sp., the species cultured from blood-fed colony midges were not detected in other stadia (Table I). This implies that diet source (blood meal for example) alters composition of the gut microbial population. Interestingly, we also have isolated *Aeromonas* from wild *C. sonorensis* midges trapped at the KSU Dairy Farm in 2013 (data not shown). Species within the genus *Aeromonas* have previously been cultured from larval media and pupae in colony-reared *C. sonorensis* (Parker *et al.* 1977) but not from adults. However, *Aeromonas* species have been detected within many other dipteran vectors, suggesting that, at least transiently, they persist in the insect gut and remain viable (Gupta *et al.* 2012, Mourya *et al.* 2001, Nayduch *et al.* 2001, Nayduch *et al.* 2002, Pidiyar *et al.* 2002). Interestingly, some *Aeromonas* species impact vector competence for Japanese encephalitis virus in mosquitoes, resulting in increased susceptibility in *Culex quinquefasciatus* (Mourya *et al.* 2001).

We also identified *Pseudomonas* and *Comamonas* species in blood-fed midges (Table I). Campbell and colleagues (Campbell *et al.* 2004) used a culture-independent approach to identify bacteria associated with wild-caught *C. sonorensis*, and detected *Pseudomonas* sp. and *Comamonas* sp. in midgut samples. *Pseudomonas* sp. has been previously cultured from larval media as well, pupae and adults of colonized *C. sonorensis* (Parker *et al.* 1977). *Morganella morganii* also was associated with both blood-fed (Table I) and wild female *C. sonorensis* (data not shown). To our knowledge, there are no previous reports of *Morganella* sp. associating with *C. sonorensis* midges. Both the relationship that these bacteria have with the midges and their acquisition source remain unknown. The ecology of any of these commonly occurring genera within the midge gut including acquisition, persistence, and impact on vector competence would make for an intriguing study due to their apparent ability to colonize and persist in midges.

In the vector gut, the microbial community interacts with pathogens that the insect ingests with the blood meal. Further, the microbial communities and pathogens both interact with the insect at the interface of the midgut epithelium. The dynamics and outcome of this interaction have been shown to directly impact the fate and transmission of arthropod-borne pathogens. Numerous studies have shown that the microbial communities in the adult gut of insect vectors influence, either directly or indirectly, pathogen transmission and therefore vector competence. *Anopheles funestus* mosquitoes harbouring Gram-positive bacteria were more likely to be infected with the malarial parasite *Plasmodium falciparum* than mosquitoes that were free of bacteria or harbouring only Gram-negative species (Straif *et al.* 1998). Colonisation of the gut with abundance of *Enterobacteriaceae* was positively correlated with *Plasmodium falciparum* infection in *Anopheles gambiae* (Boissière *et al.* 2012). Notably, bacterial isolates colonising the adult mosquitoes were the same as those from the natural larval breeding habitat. However, other studies in *Anopheles* mosquitoes have shown that bacterial presence in the gut negatively impacted *Plasmodium* infection (Pumpuni *et al.* 1993), and when bacteria were removed by antibiotics, susceptibility increased (Gonzalez-Ceron *et al.* 2003). Although mechanisms by which microbes impact vector competence in *Anopheles* have yet to be determined, the midgut microbiota play an essential role in modulating the local epithelial immune response in the gut, which indirectly impacts mosquito permissiveness to *P. falciparum* infection (Dong *et al.* 2009).

Mosquitoes produce antimicrobial peptides in order to control the midgut microbial community and also unintentionally affect *Plasmodium* parasites (Dimopoulos *et al.* 1997, Dong *et al.* 2006, Dong *et al.* 2009). Furthermore, at least some microbes have direct anti-parasitic activity, *Enterobacter* spp. directly inhibited *Plasmodium* parasites in the gut of *A. gambiae* by generation of reactive oxygen species (Cirimotich *et al.* 2011).

The impact of gut microbes on mosquito vector competence for arboviruses has also varying outcomes, resulting in either enhanced permissiveness or refractoriness. The bacterium *Serratia odorifera* was trans-stadially transmitted to adult mosquitoes and enhanced susceptibility of *Aedes aegypti* to Dengue-2 virus infection by producing molecules that protect the virus from destruction (Apte-Deshpande *et al.* 2012). In contrast, field-derived bacterial isolates negatively influence Dengue virus infections in *A. aegypti* (Ramirez *et al.* 2012, Ramirez *et al.* 2014). It is worth noticing that infection with Dengue virus elicited an epithelial immune response in the gut, which in turn activated antibacterial responses that modulated gut flora. These findings illuminate that the tripartite interaction of vector gut epithelium, pathogen, and microbiota is more dynamic and complex than previously thought.

Microbiota-arbovirus and microbiota-immune interactions, such as those highlighted in this article, have not been explored in *Culicoides* spp. and represent a significant gap in our understanding of the components of midgut infection barriers that directly affect vector competence. Our study provides the first glimpse on the role of some possible players in these barriers, including gut microbes and the induction of antimicrobial effectors. We demonstrated that the blood meal on its own, which in nature may also contain the virus (if the host was infected), influences both abundance and composition of gut microbiota. We also demonstrated that blood feeding also induced expression of microbicidal effector molecules such as antimicrobial peptides. These effectors are likely to be aimed at controlling populations of gut flora. Yet, based on what it has been shown in mosquitoes, these altered gut conditions may directly influence pathogen success, infection barriers, and ultimately vector competence. We intend to expand this study to elucidate the tripartite molecular interactions that contemporaneously occur among the midge gut epithelium and both gut bacteria and viruses. The complex and dynamic interaction among these three factors may shed light on the mechanism and impact that gut microbes, possibly acquired from larval habitats, have on the transmission of orbiviruses in the natural environment. Finally, a better understanding of the interplay between microbiota and host immunity in defending midge guts against *Orbivirus* infection could lead to the development of new strategies to control disease transmission.

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