Blood meal analysis of *Culicoides* midges collected near horses, donkeys and zebras in the Eastern Cape, South Africa

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Abstract

An upsurge in African Horse Sickness (AHS) in the Eastern Cape, South Africa, since 2006 led to an epidemiological reassessment of the disease there. Light trapping surveys near horses, donkeys and zebras in 2014-2016 collected 39 species of *Culicoides* midges (Diptera: Ceratopogonidae), which are potential vectors of AHS. To establish if these midges fed on equids, DNA sequences were obtained from the gut contents of 52 female midges (35 freshly blood-fed, 13 gravid and four parous), representing 11 species collected across 11 sites. *Culicoides leucostictus* fed on all three equids; *C. bolitinos*, *C. imicola* and *C. magnus* fed on both horses and donkeys; *C. onderstepoortensis* fed on donkeys; *C. similis* and *C. pycnostictus* fed on zebras. Blood meals from cows, pigs, warthogs, impalas and a domestic dog were also identified in various species, but none of the midges tested had fed on birds. These results contribute to establishing the vectorial capacity of several species of *Culicoides* with regard to AHS in the Eastern Cape and point to potential reservoir hosts, of which donkeys, zebras and domestic dogs have previously been established to express AHS. Blood-fed midges were also obtained throughout winter, indicating the potential for endemic AHS in the province.

Key words: African horse sickness (AHS), Equidae, host preference, PCR, vector, vectorial capacity.

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Introduction

Blood-feeding ceratopogonid midges in the genus *Culicoides* are biological vectors of more than 75 arboviruses, belonging mostly to the families Bunyaviridae, Reoviridae and Rabdoviridae, that can cause several veterinarily important diseases in livestock (Meiswinkel *et al*., 2004; Purse *et al*., 2015), the latest addition being a novel Orthobunyavirus, Schmallenberg virus (SBV), that causes congenital defects in ruminants (Purse *et al*., 2015). Among the viruses transmitted by *Culicoides*, those causing African horse sickness (AHS), bluetongue (BT) and epizootic haemorrhagic disease (EHD) are listed by the World Health Organisation for Animal Health as being particularly hazardous to animal health worldwide (World Health Organisation for Animal Health, 2016).

The endemnicity of AHS in southern Africa greatly impedes the movement of horses from South Africa to Europe and the rest of the world (Grewar, 2016). Before 2006, AHS was deemed more abundant in the subtropical northern parts of the country than in the temperate and Mediterranean southern parts (Coetzer & Guthrie, 2004). Since then the number of AHS cases occurring annually in the southern Eastern Cape province increased significantly (AHS Trust, 2017; DAFF, 2017). Subsequent light trapping surveys in the south west of the Eastern Cape (Figure 1) revealed that up to 39 *Culicoides* species could be collected near horses, donkeys and zebras in this area (Riddin, 2017). *Culicoides bolitinos* Meiswinkel was the most abundant species to be collected near horses and donkeys in these surveys (Riddin, 2017). In properties with zebra, *Culicoides pycnostictus* Ingram and Macfie was the dominant species. *Culicoides imicola* Kieffer, the most abundant livestock-associated *Culicoides* species in South Africa (Meiswinkel *et al*., 2004), was the next most abundant, but collected at less than half of the abundance of *C. bolitinos* (Riddin, 2017).

To date, *Culicoides* species have been recorded feeding on several hosts but the detection of antibodies to African horse sickness virus (AHSV) in further mammal species implicates an even greater diversity of species as hosts. Antibodies were detected not only in several carnivore species e.g. hyenas (*Crocuta crocuta*), lions (*Panthera leo*), African wild dogs (*Lycaon pictus*) and domestic dogs (*Canis lupus familiaris*), but also in herbivores such as camels (*Camelus dromedarius*), elephants (*Loxodonta africana*) and equids, including the plains (*Equus quagga*) and mountain zebras (*Equus zebra hartmannae*) (Binepal *et al*., 1992; Alexander *et al*., 1995; Barnard, 1997; Becker *et al*., 2017).

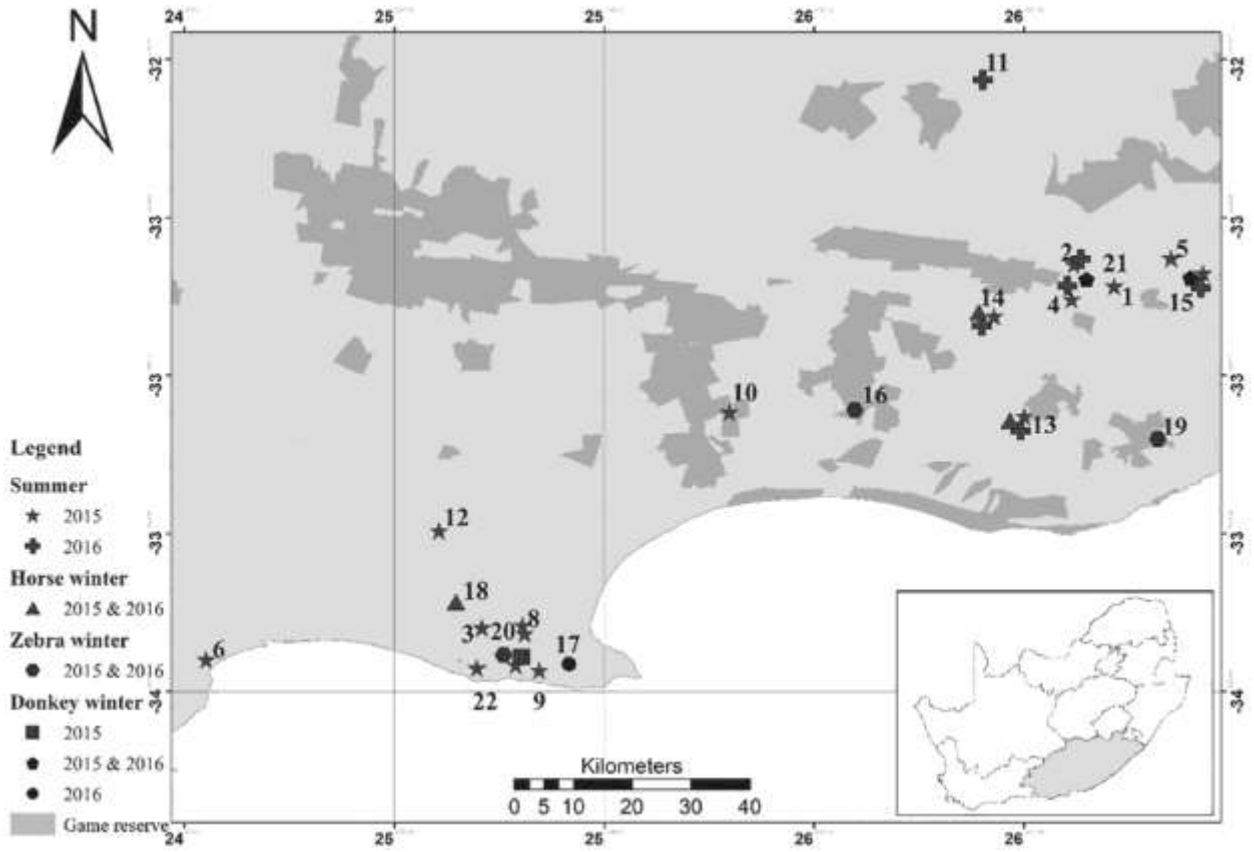

Figure 1. Sites in the southwest region of the Eastern Cape province of South Africa at which light traps were operated for the collection of *Culicoides* midges from 2014 to 2016. 1, Fairview Estate; 2, Grahamstown Riding Club; 3, Greenbushes; 4, Highlands; 5, Hoolton Park; 6, Jeffreys Bay; 7, Kaba Farm; 8, Kragga Kamma; 9, Sardinia Bay; 10, Schotia Reserve; 11, Thornkloof Farm; 12, Uitenhage; 13, Alexandria; 14, Assegaai; 15, Hogwallow Farm; 16, Amakhala Reserve; 17, Eastern Cape Horse Care Unit (Animal Welfare Society) AWS; 18, Hopewell Horse Trails; 19, Kariega Reserve; 20, Kragga Kamma Game Park; 21, Society for the Prevention of Cruelty to Animals (SPCA) Grahamstown; 22, Theesecombe.

Although a wide range of *Culicoides* species can be collected in relatively large numbers with light traps, these traps do not necessarily reflect biting activity on livestock due to their abiotic attractants (Gerry *et al*., 2009; Viennet *et al*., 2011; Scheffer *et al*., 2012). The presence, and even abundance, of a potential vector at hosts may be irrelevant if it does not actually feed on the host species. Host preference studies are therefore essential for identifying the vectors and reservoirs of arboviruses for appropriate control measures (Hadj-Henni *et al*., 2015). Despite collectively being putative vectors, the host range of many individual species remains largely unknown. Studies in Europe (Bartsch *et al*., 2009; Cerný *et al*., 2011; Garros *et al*., 2011; Lassen *et al*., 2011; Ninio *et al*., 2011; Martínez-de la Puente *et al*., 2017, 2015) indicate that biting

midges acquire blood from a diverse range of mammals and birds, depending upon the relative numbers and availability of the vertebrate hosts.

As an indicator of biting rate, host preference contributes significantly to the vectorial capacity of a species (Mullens *et al*., 2004). Although *Culicoides* species are presently broadly classified as either mammalophilic or ornithophilic feeders, more complex (perhaps opportunistic) behavior was recorded in several species (Braverman & Phelps, 1981; Lassen *et al*., 2012; Santiago-Alarcon *et al*., 2013; Martínez-de la Puente *et al*., 2015). Limited blood meal identifications in South Africa have shown that, although they favor large mammals, *C. imicola, Culicoides zuluensis* de Meillon, *Culicoides brucei* Austen and *Culicoides milnei* Austen will feed opportunistically on birds (Meiswinkel *et al*., 2004b). On the other hand, *C. pycnostictus* is a bird-feeder that feeds opportunistically on large mammals (Meiswinkel *et al*., 2004b). There seems to be little specialization within the mammalophilic species for feeding on specific mammal species. In South Africa, *C. imicola, C. zuluensis, Culicoides magnus* Colaco and *C. brucei* exhibit broad host-specificity, feeding on cattle, horses, sheep and pigs (Nevill *et al.,* 1988; Meiswinkel *et al.*, 2004b). Although generalists and opportunists may have diminished vectorial capacity due to their wide host preference lowering of their impact on particular species, they facilitate transmission of pathogens between a variety of potential reservoir taxa and livestock (Santiago-Alarcon *et al*., 2012).

Knowledge of the blood-feeding behaviour of *Culicoides* midges is essential in assessing their vectorial capacity. The aim of this study was to determine the host preference of *Culicoides* species in the Eastern Cape to clarify their role in the epidemiology of local AHS, and to determine which of the species trapped near equids by Riddin (2017) actually feed on equids.

Materials and methods

Study area and collection method

The study was conducted at 22 sites in the south-western region of the Eastern Cape (Figure 1), an area where populations of horses, donkeys and zebras were relatively dense and outbreaks of AHS occurred regularly (Riddin, 2017). Females midges suitable for blood meal analysis were obtained from six sites dominated by horses, three sites dominated by donkeys and two sites dominated by zebras (Table 2).

A light trap survey was conducted *ad hoc* during suspected viral cases from November 2014 to August 2016, and bimonthly during intervening winters (Riddin, 2017). The traps were modified 220 V Onderstepoort downdraft light traps (Venter *et al*., 2009) refitted with two 8 W G5 fluorescent T5 UV blacklight blue tubes (288 mm) and a 12 V (0.34 A) fan, both connected to a 12 V lead battery. During 2015-2016, the fluorescent tubes were replaced by six UV light-emitting diodes (LEDs), and each trap was fitted with a day-night sensor to improve energy consumption.

During summer (September to May), light trapping was conducted at sites with suspected or confirmed outbreaks of AHS for single nights. In the absence of outbreaks, winter (June to August) collections were made at ten sites (at least three per equid species, horse, donkey and zebra) in suitable weather for two consecutive nights per winter month. The contemporary climatic conditions include rainfall in the area which varies between 350 and 650 mm/annum (Palmer, 2004; Fadi & Dreyer, 2016). The Algoa Bay region in the south receives about 610 mm annually, varying from 400 to 1200 mm across the area (Klages *et al*., 2011). Being in a transitional zone between the temperate summer and Mediterranean winter rainfall areas of South Africa, rainfall peaks from October to November and March to April. Average maximum temperatures range between 29°C and 32°C at the coast and 19°C and 25°C inland, with a mean monthly minimum of 9°C-18°C and 4°C-6°C, respectively (Palmer, 2004; Klages *et al*., 2011). The area is mostly frost-free with temperature minima seldom below freezing. The Nelson Mandela Metropolitan and Kouga Municipalities frequently experience winds of > 8 m/s (Klages *et al*., 2011; Fadi & Dreyer, 2016). Vegetation biomes are variable and include fynbos, subtropical thicket (Albany thicket), Nama Karoo, grassland and forest (Palmer, 2004; Klages *et al*., 2011). Large areas, previously cleared for agriculture, are gradually being restored to a natural state, with an increase in the number of game farms (Langholz & Kerley, 2006), which often stock plains zebra . The climatic and collection methods are described in detail by Riddin (2017).

Insects were collected overnight in 200 ml of deionized water and 1 ml of Savlon® (chlorhexidine gluconate 0.3 g/100 ml and cetrimide 3.0 g/100 ml; Johnson & Johnson, East London, South Africa). The following morning the trapped insects were transferred to 70% ethanol and stored at 4°C in the dark.

Blood-meal analysis

During morphological identification of the species under a dissection microscope, freshly blood-engorged *Culicoides* females were sorted into 90% ethanol and stored individually in 1.5 ml microcentrifuge tubes at 4°C. For blood-meal identification, suitable blood-engorged, gravid or parous females' abdomens were removed with a razorblade and fine forceps, air-dried and transferred to individual 1.5 ml microcentifuge tubes with 70% ethanol.

Deoxyribonucleic acid (DNA) extraction was performed using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol for animal tissue isolation. The final dilution step was performed with 50 µl buffer AE and eluting, followed by a further 50 µl buffer AE and a second elution to allow for a higher concentration of DNA material in a 100 µl volume. Concentrations of extracted DNA, ascertained with a Nanodrop 2000 Spectrophotometer, ranged from 1 ng/µl to 12 ng/µl. Since these were low concentrations, a polymerase chain reaction (PCR) volume of 5 µl of DNA in a total volume of 25 µl was chosen. Nested PCR of purified products was used in cases where initial amplifications failed.

Three sets of primers targeted for vertebrate, mammalian and avian genetic markers (Table 1) were selected for amplification (Lassen *et al*., 2011; Pettersson *et al*., 2013). Screening was initially performed using the universal vertebrate primers, L14841 and H15149 (Kocher et al., 1989), coding for a region of the cytochrome B (cytb) gene and avian-specific primers, Bird F and Bird R (Cicero & Johnson, 2001). Samples that were unsuccessful, and those that were successful for vertebrate but failed using the avian primers, were further tested with mammal-specific primers (Ngo & Kramer, 2003) in an attempt identify the host or to obtain larger segments for more precise identification.

All molecular procedures used with each primer pair followed the same PCR conditions, adjusted to suite the primer and DNA template. The 25 µl PCR volume was made of 12.5 µl Taq polymerase, 2 µl of each primer (10 ng/µl), 3.5 µl PCR H_2O and 5 µl DNA template. The PCR consisted of an initial heating phase of 94°C for 3 min followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 50-55°C for 45-55 s and elongation at 72°C for 30 s. A final elongation step was performed at 72°C for 10 min. Annealing temperature and time depended on the primer, and were altered if initial annealing at 50°C for 50 s produced multiple bands or no band(s). Negative controls with 8.5 µl PCR H_2O and no DNA were included to confirm the absence of contamination.

Table 1. Primer names, sequences, product size and references for *Culicoides* blood-meal analysis.

Primer pair	5'-3 sequence forward	Product size (bp)	Reference
Cyt b L 14841 (F) Cyt b H15149 (R)	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA AAACTGCAGCCCCTCAGAATGATATTTGTCCTC	305	Kocher *et al*., 1989
Mammal (F) Mammal (R)	CGAAGCTTGATATGAAAAACCATCGTTG TGTAGTTRTCWGGGTCHTCTA	772	Ngo & Kramer, 2003
Bird (F) Bird (R)	GACTGTGACAAAATCCCNTTCCA GGTCTTCATCTYHGGYTTACAAGAC	558	Cicero & Johnson, 2001

PCR products were run on 1% agarose gels, stained with ethidium bromide, at 100 V for 30 min to confirm amplification, followed by capillary electrophoresis by Macrogen using only the forward primer. The resulting nucleotide sequences were assessed using BioEdit sequence alignment software (Hall, 2004) and edited to resolve any ambiguous bases. Sequence matches were confirmed using BLAST (Altschul *et al*. 1997) with a match acceptance threshold of > 95%.

Results

From the 18,465 female *Culicoides* specimens collected (13,208 in summer outbreak season; 5,257 in winter off-season) at 262 trapping events at 22 sites, from November 2014 to August 2016, fewer than 1% were blood-engorged, which is typical for light trapping. A further total of 7, 524 (40.7%) pigmented/parous, 9, 169 (49.7%) nulliparous/non-pigmented and 3, 019 (16.3%) gravid females were recorded. We tested a total of 117 females' blood meals (46 freshly blood-fed, 21 parous and 50 gravid) representing 17 *Culicoides* species from 12 sites.

The success of the primers varied greatly, with the universal vertebrate primers showing the greatest success rate of amplification. Although 94.9% of the 117 samples returned visible electrophoresis bands, successful BLAST matches were obtained in only 46.4% of these samples. Every effort was made to concentrate DNA samples with low concentration (> 1%), including nested-PCRS with purified product, but failed initial amplifications remained unsuccessful. Bird- and mammal-specific primers had 19.7% and 5.4% amplification successes, respectively, with 95.7% and 50% successful BLAST matches, respectively. Amplification was most successful when using the universal vertebrate primers (i.e. a 305 bp fragment) and least successful with mammal-specific primers (i.e. a 772 bp fragment). Sequencing success was highest with the avian-specific primers (i.e. a 558 bp fragment), although all successful BLAST matches were to mammal species.

Of the 117 specimens tested, 52 (44.4%: 35 freshly blood-fed, 13 gravid and four parous), representing 11 species collected across 11 sites, successfully returned confident BLAST matches to vertebrate species (Table 2). The success rate from freshly blood-fed females (76.1%) was unsurprisingly significantly ($p < 0.001$) better than from gravid (26.0%) or parous flies (19.1%). Unsuccessful amplifications were attributed to a lack of undigested host DNA in parous

Table 2. Site, date of collection, and identity of 52 blood-engorged *Culicoides* females and the most likely associated blood meal host identified by a BLAST search, with its Genbank accession code.

Site	Date	*Culicoides* species (and number of specimens)	Blood meal identification	Genbank BLAST match(es) [Ident %]
Horse-dominated				
S 33.3239, E 26.4821 – Highlands	2016/02/17	*C. zuluensis* (1)	*E. caballus*	KT757764.1 [98%]
S 33.9836, E 25.4639 – Kragga Kamma	2015/04/01	*C. bolitinos* (4)	*E. caballus*	LC088145.1 [97-99%]
		C. zuluensis (3)	*E. caballus*	LC088145.1 [96-100%]
		C. magnus (1)	*E. caballus*	KT998647.1; KT757762.1 [99%]
S 33.7645, E 25.3489 – Uitenhage	2015/04/01	*C. imicola* (13)	*E. caballus*	MH586816.1 [97-99%]; MG761996.1 [97-99%]; LCO88145.1 [98%]; KT757758.1 [99%]; AY819736.1 [100%]
S 33.5676, E 26.3598 – Alexandria	2016/05/29	*C. gulbenkiani* (1)	*E. caballus*	KT757762.1 [98%]
		C. gulbenkiani (1)	*C. lupus*	JX849653.1 [100%]
S 33.3746, E 26.3051 – Assegaai	2015/08/24	*C. bolitinos* (1)	*E. caballus*	KT998647.1; KT792938.1 [98%]
		C. bolitinos (1)	*B. taurus*	EU365345.1 [99%]
	2016/06/16	*C. imicola* (1)	*E. caballus*	LCO88145.1 [99%]
		C. bolitinos (1)	*E. caballus*	LCO88145.1 [99%]
	2016/07/23	*C. imicola* (1)	*E. caballus*	KT757762.1 [99%]
S 33.8923, E 25.3699 – Hopewell	2016/08/12	*C. imicola* (2)	*E. caballus*	KT757762.1 [98%]; KT757763.1 [99%]
Donkey-dominated				
S 33.3136, E 26.6866 – Hogwallow	2015/07/09	*C. bolitinos* (1)	*S. scofra*	FM205714.1 [99%]
	2015/08/27	*C. bolitinos* (1)	*E. asinus*	KX669267.1 [98%]
	2015/08/28	*C. zuluensis* (1)	*E. caballus*	KT757758.1 [99%]
		C. isioloensis (1)	*B. taurus*	KT151960.1 [99%]
	2016/06/26	*C. imicola* (1)	*E. asinus*	KT829585.1 [99%]
		C. bolitinos (1)	*E. asinus*	FJ428516.1 [96%]
		C. bolitinos (1)	*S. scofra*	KT943507.1 [99%]
	2016/08/06	*C. leucostictus* (1)	*E. caballus*	LCO88145.1 [98%]
	2016/08/07	C. sp. #94 (2)	*E. caballus*	KT757764.1 [99%]
S 34.0035, E 25.5719 – ECHU AWS	2016/08/12	*C. leucostictus* (1)	*E. asinus africanus*	KX669267.1 [98%]
		C. bolitinos (1)	*E. asinus*	KT829585.1 [97%]
	2016/08/13	*C. zuluensis* (1)	*E. caballus*	KT757762.1 [99%]
S 33.3191, E 26.4979 – SPCA GHT	2015/08/24	*C. magnus* (1)	*E. asinus*	KX669267.1 [98%]
	2016/05/24	*C. onderstepoortensis* (1)	*B. taurus*	JX472264.1 [99%]
	2016/05/24	*C. onderstepoortensis* (1)	*E. asinus*	KT829585.1 [98%]
Zebra-dominated				
S 33.6005, E 26.6247 – Kariega Game Reserve	2016/07/29	*C. similis* (1)	*E. burchelli*	MG847597.1 [96%]
		C. leucostictus (1)	*E. burchelli*	MG847597.1 [97%]
S 33.9836, E 25.4639 – Kragga Kamma Game Reserve	2015/08/04	*C. magnus* (1)	*A. melampus*	AF036289.1 [99%]
	2016/07/03	*C. onderstepoortensis* (1)	*P. africanus*	KX697540.1 [97%]
	2016/08/01	*C. pycnostictus* (1)	*E. quagga*	MG847597.1 [97%]

and gravid females, which was apparent through spectrophotometric assessment of individual DNA extracts.

Horses, donkeys and zebras were the most abundant mammals at six, three and two of the sites, respectively (Table 2). Other species present at horse- and donkey-dominated sites were predominantly domestic livestock, including cows, pigs, sheep and dogs. Sites with zebras were inhabited by an unestimated number of common, free-roaming species of wildlife. There was a strong link between the most common mammal at a site and the origin of the blood meals of associated midges (Table 2). At sites where horses were the most numerous mammal, 93.5% of the flies had fed on a horse; one *C. bolitinos* on a cow and one *C. gulbenkiani* on a domestic dog (Table 2). At donkey-dominated sites, 43.8% of flies fed on donkey, 31.3% on horse, 12.5% on cow, and 15.5% on pig (Table 2). Zebra-dominated sites yielded meals from zebra (60%), impala (20%) and warthog (20%). Estimated blood meal diversity at donkey-dominated sites was generally wider than at horse-dominated sites. Despite the omnipresence of wild birds in various numbers at all of the sites, none of the blood meals were matched to birds.

Culicoides leucostictus fed on all three equids; *C. bolitinos, C. imicola, C. magnus* and *C. leucostictus* fed on both horses and donkeys (Table 3); *C. onderstepoortensis* fed on donkeys; and *C. leucostictus, C. similis* and *C. pycnostictus* collected in game reserves containing zebras had fed on zebra (Tables 1, 3). Other game-associated meals included impala by one *C. isioloensis* and warthog by one *C. onderstepoortensis* (Tables 2, 3).

Discussion

The frequency of blood-feeding of an insect vector on susceptible and non-susceptible vertebrate hosts will be closely related to pathogen amplification and transmission risk for different vertebrates (Martínez-de La Puente *et al*., 2015; 2017). Although light traps attract few freshly blood-engorged females, blood meals from at least 11 species collected near equids were analyzed. Although DNA extraction and identification was significantly more successful using freshly blood-engorged females, parous and gravid females could also be used to estimate host preference. It remains to be seen how long after feeding the origin of the blood meal can still be identified, but this study showed that amplification success decreased significantly from blood-engorged females to parous and gravid females, in line with a negative relationships between digestion time and amplification success (Hadj-Henni *et al*., 2015).

Table 3. Blood-meal sequence matches from 52 freshly blood-fed, pigmented *Culicoides* females collected with light traps near equids in the Eastern Cape from 2014 to 2016.

***Culicoides* species**	**Host species**								
	Perissodactyla			**Artiodactyla**				**Carnivora**	**Total**
	Equidae			**Suidae**		**Bovidae**		**Canidae**	
	***Equus caballus* horse**	***Equus asinus* donkey**	***Equus quagga* zebra**	***Sus scofra* pig**	***Phacochoerus africanus* warthog**	***Bos taurus* cow**	***Aepyceros melampus* impala**	***Canis lupus familiaris* dog**	
C. imicola	17	1	-	-	-	-	-	-	18
C. bolitinos	6	3	-	2	-	1	-	-	12
C. zuluensis	6	-	-	-	-	-	-	-	6
C. magnus	1	1	-	-	-	-	1	-	3
C. leucostictus	1	1	1	-	-	-	-	-	3
C. onderstepoortensis	-	1	-	-	1	1	-	-	3
C. gulbenkiani	1	-	-	-	-	-	-	1	2
C. sp. #94	2	-	-	-	-	-	-	-	2
C. isioloensis	-	-	-	-	-	1	-	-	1
C. similis	-	-	1	-	-	-	-	-	1
C. pycnostictus	-	-	1	-	-	-	-	-	1
Total	34	7	3	2	1	3	1	1	52

Meiswinkel *et al*. (2004b) listed 13 species of *Culicoides* that feed on horses in South Africa; the present study adds *C. leucosticus* and C. #94 (a yet undescribed species that morphologically resembles *C. brucei*, and which is numbered following the system of Meiswinkel (1995) that is used by South African specialists). Of the five species that fed on donkeys, four also fed on horses. Of the three species that fed on zebras, *C. leucosticus* fed on all three equids represented in this study and *C. pycnostictus* has previously been shown to feed on horses (Meiswinkel *et al*., 2004b). This work presents the first evidence that the host range of *C. onderstepoortensis* and *C. similis* can include equids.

At least nine species (*C. imicola*, *C. bolitinos*, *C. gulbenkiani*, *C. leucostictus*, *C. pycnostictus*, *C. magnus*, *C. zuluensis*, *C. brucei* and *C. engubandei*) of the 13 that were previously shown to be orally susceptible to AHSV under laboratory conditions (Venter, 2016) are now shown to feed on equids under natural conditions. To date, AHSV has been isolated in the field from only *C. imicola* and *C. bolitinos* (Meiswinkel and Paweska 2003; Meiswinkel *et al*., 2004b).

This study confirms generalist feeding for *C. bolitinos*, *C. magnus*, *C. leucostictus* and *C. onderstepoortensis*. The catholic feeding of *Culicoides* species with recognized high vectorial capacity on both wild and domestic animals may provide a bridge mechanism for the transmission of AHSV between wild and domesticated hosts (cf. Santiago-Alarcon *et al*., 2012; Talavera *et al*., 2015). Bloodmeal analysis confirmed the large contribution of *C. bolitinos* and *C. imicola* to AHSV transmission because their host selection included horses at sites with confirmed AHS cases. This also supports their previously-determined apparent preference (Nevill & Anderson 1972; Fall *et al*., 2015). *Culicoides imicola* was found to feed exclusively on horses at horse-dominated sites, affirming its vectorial capacity, but it feeds on a variety of livestock species (Nevill *et al*., 1988; Meiswinkel *et al.*, 2004b), including a single donkey host sample at a donkey-dominated site (Table 2), which may broaden its transmission potential.

Culicoides bolitinos fed on diverse livestock, including donkey, pig and cow, which can be related to their known larval development in bovid dung (Meiswinkel, 1989). This may also contribute to potential transmission of bluetongue virus (BTV) among ruminants within the selected sites, as proven generalist feeding and the identification of a cow host here would facilitate BTV transmission by *C. bolitinos*. *Culicoides gulbenkiani* is also found near large mammals and apparently breeds in horse dung (Nevill *et al*., 1988; Venter & Meiswinkel, 1994),

increasing its vector potential. The broad feeding habits of *C. bolitinos*, *C. magnus*, *C. leucostictus* and *C. onderstepoortensis* might lessen their vectorial capacity for AHSV but will promote AHS transmission and potential reservoir status if other livestock are shown to be reservoirs.

That *C. zuluensis* and *C. magnus* fed on horses in this and previous studies (Braverman & Phelps, 1981), are orally susceptible to AHSV (Paweska *et al*., 2003), and were abundant at sites with horses (Riddin, 2017), this increases their probable vectorial capacity for AHSV in the Eastern Cape. *Culicoides similis* was abundant around horses in Senegal (Bakhoum *et al*., 2016) and feeds on zebras (Table 2), which could indicate its potential as another vector species.

Little is known of the host preference of *C. onderstepoortensis* and *C. isioloensis*. The first species appeared to be opportunistic, feeding on cow, warthog and donkey (Table 3). If it can be confirmed that *C. isioloensis* feeds on cattle (Table 3), it may act as a vector of BTV.

Domestic dogs can develop peracute fatal infections after ingesting AHSV-infected horsemeat, but they were not considered preferred hosts for *Culicoides* species and therefore unlikely to play a role in the epidemiology of AHS (Braverman & Chizov-Ginzburg, 1996). AHSV infections in dogs ascribed to vector transmission have been documented in South Africa, which supports the susceptibility of canids to the virus and suggests a role in the transmission of AHSV (O'Dell *et al*., 2018). Slama *et al*. (2015) and Martínez-de La Puente *et al*. (2017) recently reported that *C. imicola* fed on dogs in Tunisia and Spain, respectively. The present study found a blood-engorged female of *C. gulbenkiani* that had fed on a domestic dog at a site dominated by horses living in the proximity of a number of domestic dogs bred for hunting. The recent detection of AHSV in South Africa (van Sittert *et al*., 2013) and SBV in Europe (Sailleau *et al.*, 2013) in dogs with no history of eating horse meat, is evidence for vector-mediated transmission of these viruses to dogs. The roles of dogs and other carnivores in AHS need further investigation as they may provide potential reservoirs for AHSV (O'Dell, 2018).

Despite the inclusion of putatively ornithophilic species (*C. pycnostictus, C. leucosticus*), none of the midges tested had fed on birds but, given the sample sizes involved, this does not exclude birds as hosts. More than half of the analyses were unsuccessful due to too little DNA remaining in the partially-digested blood meal (Martínez-de la Puente *et al*., 2013), inappropriate primer selection for the bird DNA (cf. Ngo & Kramer, 2003), and/or mixed signals due to multiple templates within the samples. Mixed signals have not been reported in previous studies

(Hadj-Henni *et al*., 2015; Slama *et al*., 2015). The failure of nested PCR of purified samples strongly suggests that the host's DNA was largely digested. This topic could be confirmed by using species-specific primers to identify each host in a mixed diet independently.

Conclusion

Livestock-associated *Culicoides* species have relatively wide mammalian host spectra (Pettersson *et al*., 2013; Martínez-de la Puente *et al*., 2015). Our data represent the first records of *Culicoides* species that feed on donkeys and zebras, potential reservoir hosts of AHSV, and revealed an overlap in the host preferences and associated vector potential of these midge species. Several *Culicoides* species besides *C. imicola* and *C. bolitinos* will feed on equids and may therefore also be involved in the transmission of AHSV in the Eastern Cape. Although the oral susceptibility of these species has been proven in the laboratory for both AHSV and BTV (Paweska *et al*., 2003; Mullens *et al*., 2004), transmission studies are still lacking and research should be focused here. The involvement of a number of *Culicoides* species, each with a potentially unique and mostly unstudied biology and vector competence, increases the complexity of the epidemiology of livestock orbiviruses like AHSV, BTV and epizootic haemorrhagic disease virus (EHDV). Multi-vector potential in these viruses would increase their capacity to spread rapidly between ecosystems.

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the Nature Conservation Act. 1987 (Act No. 10 of 1987, former Ciskei)). The National Research Foundation and the African Horse Sickness Trust provided funding. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Research Foundation. The authors have no conflicts of interest to declare.

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