Culicoides species as potential vectors of African horse sickness virus in the southern regions of South Africa

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Abstract

African horse sickness (AHS), a disease of equids caused by the African horse sickness virus (AHSV), is of major concern in South Africa. With mortality reaching up to 95% in susceptible horses and the apparent reoccurrence of cases in regions deemed non-endemic, most particularly the Eastern Cape, epidemiological research into factors contributing to the increase in the range of this economically important virus became imperative. The vectors, *Culicoides* (Diptera: Ceratopogonidae), are considered unable to proliferate during the unfavourable climatic conditions experienced in winter in the province, but annual occurrence of AHS suggests that the virus has become established and that vector activity continues throughout the year. Surveillance of *Culicoides* within the province is sparse, and little was known of the diversity of vector species or the abundance of known vectors, *Culicoides imicola* and *Culicoides bolitinos*. Surveillance was performed using light trapping methods at selected sites with varying equid species over two winter and two outbreak seasons to determine diversity, abundance and vector epidemiology of *Culicoides* within the province. The research provided an updated checklist of *Culicoides* species within the Eastern Cape and contributes to an increase in knowledge of AHS vector epidemiology contributing to prevention and control in southern Africa.

Key words: *Culicoides*, midges, African horse sickness, vector, epidemiology, light trapping, *Orbivirus*.

Introduction

Outbreaks of African horse sickness (AHS), caused by African horse sickness virus (AHSV), have occurred annually in the Eastern Cape since 2006 (AHS Trust, 2017), contrary to the established epidemiological model of this disease in South Africa (Guthrie, 1999; de Klerk, 2012). This is a concern because, with mortality exceeding 90% in susceptible horses, AHS is one of the most lethal viral diseases of equids listed by the OIE – World Organization for Animal Health (World Organization for Animal Health, 2019). The endemicity of this arthropod-borne, non-contagious, infectious disease in southern Africa greatly constrains the transport of horses within and from South Africa to Europe and elsewhere (MacLachlan & Guthrie, 2010; Grewar, 2016). With an estimated 103 710 horses, 17 226 donkeys and mules (26% of South Africa's equines) and an unknown but comparable number of plains and mountain zebras (de Klerk, 2012), the number of equids at risk for AHSV infection in the Eastern Cape is large. Records of 433 cases of AHS in 2007/6 were reported through citizen science-based reporting (AHS Trust, 2017) and 818 cases in 2001 and 310 cases in 2008 by the Directorate of Veterinary Science (Directorate Veterinary Services of South Africa, http://www.nda.agric.za/vetweb/epidemiology//Disease%20Database/OIEData/OIE_query_Criteria.asp) and Liebenberg-Weyers (2015), listed the Eastern Cape as the province with the highest incidence of AHSV in South Africa for the period 1993-2011.

African horse sickness virus (AHSV), an *Orbivirus* in the family *Reoviridae*, is transmitted almost exclusively by certain species of *Culicoides* biting midges (Meiswinkel *et al*., 2004). The geographical and seasonal prevalence of AHS therefore depends on the simultaneous presence of the virus, susceptible hosts and competent insect vectors (Venter *et al*., 2006).

With regard to susceptible hosts, the first records of AHS in South Africa date from 1652, soon after the introduction of horses from Europe and the Far East to the Cape of Good Hope (Henning, 1965). In 1854-1855 an AHS outbreak resulted in a loss of 70 000 animals which was ~40% of the population in the Cape of Good Hope (Mellor & Hamblin, 2004). From 1955 to 2006, AHS was more prevalent in the northeastern regions of South Africa than in the (current) Western Cape and Eastern Cape provinces (Bosman *et al*., 1995). Based on the assumption that the underlying mechanism preventing endemicity in the south was that adult *Culicoides* vectors of AHSV were inactive for so long in winter in the cooler central escarpment and southern regions of South Africa that AHSV did not survive winter there (Guthrie, 1999). The decline in outbreaks in the south was partly attributed to the elimination of large, free-ranging herds of zebra (*Equus burchellii*), considered a cycling host of the virus (Barnard, 1993; Mellor & Hamblin, 2004). The introduction of a polyvalent attenuated vaccine in 1974, which apparently created a geographical barrier of immune horses that impeded the southerly spread of AHSV, also contributed to this decline (Bosman *et al*., 1995). Based on incidence patterns between 1955 and 2006, it became accepted that AHS spread from the northern (endemic) areas to the southern regions annually in summer, if climatic conditions were favorable for proliferation of *Culicoides* (Guthrie, 1999; de Klerk, 2012). In support of the model, antibodies against AHSV were

detected in less than 15% of donkeys bled in the Eastern Cape between 1983 and 1995 (Venter *et al.*, 1999b).

Culicoides imicola Kieffer is considered the most important vector of AHSV throughout its distribution area, including South Africa (Meiswinkel *et al.*, 2004; Purse *et al*., 2015), based on its proven vector status, wide geographical distribution, and abundance around livestock. Long-term light trapping in South Africa indicates that *C. imicola* is the most abundant livestock-associated *Culicoides* species, especially in the tropical northern regions where AHS is most prevalent (Meiswinkel *et al*., 2004). It usually dominated trapped samples at outbreaks of AHS in South Africa (Venter *et al*., 2006). Due to the rarer historical occurrence of AHS in the Eastern Cape, the distribution and abundance of *Culicoides* vectors of AHSV were addressed only twice (Venter *et al*., 1996; Meiswinkel, 1997), at least ten years before the current upsurge in cases in that province. *Culicoides imicola* represented less than 2% of the trapped *Culicoides* sample in Middleburg (31.49°S, 25.01°E), 83.6% in Adelaide (32.70°S, 26.30°E) and 34% in Steytlerville (33.33°S, 24.34°E) (Venter *et al*., 1996). In 1993 *C. imicola* was not trapped in the sandy dune fields west of Port Elizabeth in the south of the Eastern Cape, and *Culicoides bolitinos* Meiswinkel accounted for more than 91% of the specimens collected (Meiswinkel, 1997).

The local persistence of AHS suggests that competent adult *Culicoides* may now overwinter in the area, sustaining AHSV year-round, contrary to the established epidemiological model of annual reinvasion from the north. This apparent change necessitates re-evaluation of the species composition and seasonal abundance of the *Culicoides* species in the Eastern Cape, so from 2014 to 2016, the abundance and species diversity of *Culicoides* around various equine species in the south-western Eastern Cape was monitored with light traps. During summer, *Culicoides* species were trapped at sites of suspected and confirmed cases of AHS. To explore the potential overwintering of AHSV in adult *Culicoides*, surveys were conducted near a variety of equine species during the colder winter months. The objective was to estimate the local abundance of *C. imicola* and to identify other *Culicoides* species that potentially might transmit AHSV in this region.

Materials and methods

Study area

Light trap surveys were conducted in the south west of the Eastern Cape where cases of AHS were prominent (Fig. 1). The rainfall in the area varied 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). The average rainfall across the study years varied from 548 mm in 2014, 682 mm in 2015 and to a very low 287 mm in 2016 (SAWS, 2016). The low total in 2016 was the lowest recorded average across the study site in over ten years as recorded by the South African Weather Service (SAWS, 2016). 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 9-18°C and 4-6°C, respectively (Palmer, 2004; Klages *et al*., 2011). The area is mostly frost-free with temperature minima seldom below freezing. This was shown to be consistent for the study years as the temperature ranged between 17.5°C-32.9°C in summer and 18.5°C-5.9°C during the winter months across the study region (SAWS, 2016). 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.

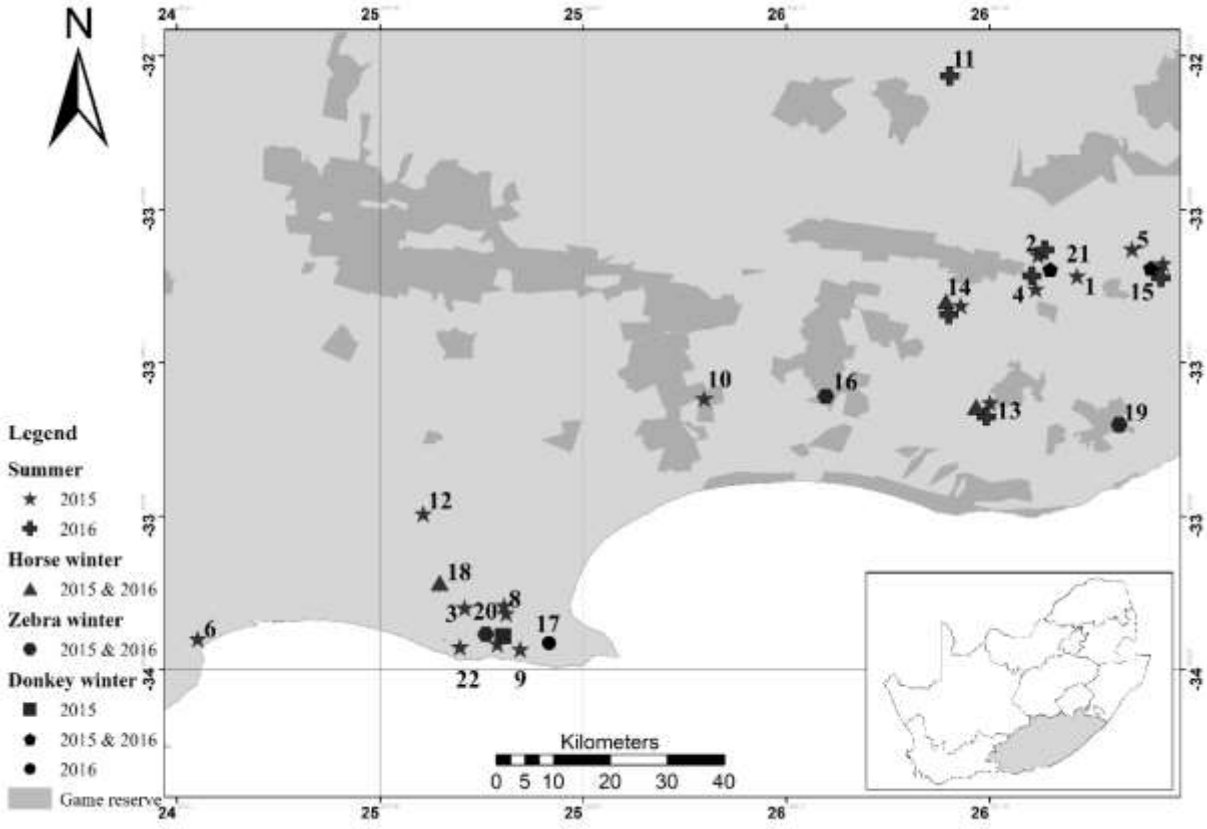

Fig. 1. Sites in the south-western region of the Eastern Cape Province of South Africa where light traps were operated for the collection *Culicoides* midges from 2014 to 2016. 1: Fairview Estate, 2: Grahamstown Riding Club, 3: Greenbushes, 4: Highlands, 5: Hoolton Park, 6: Jeffreys Bay, 7: Kaba Farm, 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), 18: Hopewell Horse Trails, 19: Kariega Reserve, 20: Kragga Kamma Game Park, 21: Society for the Prevention of Cruelty to Animals (SPCA) Grahamstown and 22: Theesecombe.

Culicoides *collection*

Light trapping for *Culicoides* ran from November 2014 to August 2016. During the 2014-2015 outbreak season, and subsequent 2015 winter season, 220 V bespoke Onderstepoort downdraft light traps (Venter *et al*., 2009) modified to hold two 8 W G5 fluorescent T5 UV blacklight blue (365 nm) tubes (288 mm) and a 12 V (0.34 A) fan driven by a lead battery, were used. During the 2015-2016 outbreak season and 2016 winter season, the fluorescent tubes were replaced by six 3W UV (315-400 nm) light-emitting diodes (LEDs) and the traps were fitted with day-night sensors (7.5-15 lx) (VR Electronics, Port Elizabeth, South Africa) to improve energy consumption. The probable difference in trap efficiency for the collection of *Culicoides* was taken into account during the analysis of results.

Depending on the size of the property, two or three traps were hung at each site per night. Traps were hung 1.5 m-2.0 m from the ground. Insects were collected in 200 ml of deionized water with 1 ml of Savlon® antiseptic (containing chlorhexidine gluconate 3g/1 and cetrimide 30g/l: Johnson & Johnson, South Africa) to break the surface tension of the water. The following morning the insects were transferred to 70% ethanol and stored at 4°C in the dark until analyzed. *Culicoides* specimens were sexed and identified to species level using the wing picture atlas of Afrotropical *Culicoides* (Meiswinkel, 1996; Labuschagne, 2016). Numbers are typically given to identify *Culicoides* species that have not been officially named and described. The numbering system devised by Rudy Meiswinkel (Meiswinkel 1995; Labuschagne, 2016) is still used in South Africa for yet undescribed species, e.g. C. sp. # 50. Females were sorted on abdominal pigmentation into nulliparous, parous, gravid and freshly blood-engorged (Dyce, 1969).

Summer diversity

The study was divided in a summer and a winter survey.

During summer (November 2014 to June 2015 and December 2015 to May 2016) light trap collections, trapping was conducted for one night at each site with an active suspected or confirmed case of AHS at a total of 15 sites (Fig. 1). To maximize the variety and number of *Culicoides* collected, three traps were operated at each case, representing different degrees of exposure to the weather, namely open, semi-protected and protected. Protected positions were usually within the stabling of the equids; open positions were usually under an isolated tree. At each site, the three traps were deployed > 30 m (Venter *et al*., 2012) from each other to ensure no mutual interference. Trapping was performed immediately following the detection of a suspected case unless weather conditions were deemed unfavourable for collection (i.e. severe rain and wind). Trapping would then be performed in the within 24 hours or as soon as collection was viable.

Winter diversity

Winter collections occurred from June to August in 2015 and 2016, for two nights per month at sites near either horses, donkeys or zebras. Site selection was based on the outbreak radius of the preceding outbreak season. Two traps, one in an open and one in a protected from weather, were operated at each of the three equid species concurrently, producing six trap collections per night made under comparable weather and moonlight conditions to avoid bias. The traps at the open and protected positions at each site were > 30 m apart to minimize mutual interference.

Statistical analyses

Differences in trapping effort between sites was accounted for in the analysis of collections by dividing the total number of *Culicoides* specimens collected at the site by the total number of traps used there. This was then divided by the total number of trapping nights to give a mean *Culicoides* species count per trap, per night. Data was analysed in Statistica 12.0 (Statsoft, USA). The diversity of *Culicoides* species among and between suspected outbreak sites for the 2014-2015 outbreak season were assessed using alpha (α), beta (β) and gamma (γ) diversity indices using an R-Cran package for the measurement of partitioned diversity.

Results

Overall 18 465 *Culicoides* specimens were collected in 249 light trap collections made at 22 sites from November 2014 to August 2016. The number of collections made at each site varied from 33 (Hogwallow Farm (site 15)) to two (Kaba Farm (site 7) and Uitenhage (site 12) (Tables 1, 2). The mean collection size was 74.2 midges/night/trap and ranged from 0 midges/night/trap for 2 collections made at Kaba Farm (site 7) during outbreak season (Table 2) to 3363.0 midges/night/trap for nine collections made at Hogwallow (site 15) (Table 1). The biggest collection recorded in a single trapping event was 2829 specimens at a suspected case of AHS at Greenbushes (site 3) (Table 1).

Comparison of the collections made in open, semi-protected and protected positions indicated that the degree of site exposure did not have a significant effect on the mean number of individuals collected ($F_{54, 188} = 1.3601$; $p = 0.56$). Although somewhat higher mean numbers of *C. bolitinos, C. imicola* and *C. zuluensis* were collected in protected positions, the standard errors were large and may indicate too small a sample size to detect a difference.

Table 1. *Culicoides* species composition shown as relative abundance (%) at 15 sites with suspected cases of AHS in the south of the Eastern Cape Province of South Africa, as determined with light trap collection from November to June (2015/2016). Site numbers refer to Fig. 1. *Culicoides* species denoted by numbers (#) refer to as yet undescribed species.

Site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	Mean	No. of sites present	%
No of collections made	3	5	3	3	3	6	2	6	7	3	3	2	7	8	9			
C. bolitinos	15.4	33.8	64.7	2.2	64.9	37.0		29.7	58.1	66.2	0.5	5.6	43.0	53.8	40.1	78.3	14	41.5
C. imicola	7.7	5.0	27.3		0.7	22.0		26.9	31.8	1.0	0.5	71.8		1.8	40.4	50.7	12	26.9
C. zuluensis	15.4	45.0	7.1	23.8	13.2	16.7		24.2	4.8	1.5		22.5		1.2	3.5	17.3	12	9.2
C. pycnostictus		7.5	<0.1	43.7	11.5	14.1		5.5	2.0	10.6	2.9		22.6	16.7	0.7	13.4	12	7.1
C. cornutus											84.2					7.1	1	3.8
C. sp. #54 df				0.5	0.2	0.4		4.4		0.5			1.1	0.4	12.1	6.2	8	3.3
C. leucostictus				10.1	5.4	0.6		7.1	0.7	0.3	0.5		12.9	6.8	0.4	3.5	10	1.9
C. nivosus			<0.1	0.6	0.2	2.2			0.3	1.2	0.7		3.2	6.3	0.3	1.9	10	1.0
C. gulbenkiani	7.7	1.3	0.5	2.1	2.7			1.1	0.3	5.2			3.2	3.0	0.3	1.7	11	0.9
C. magnus	53.9	6.3	0.2	12.1		0.2		1.1						0.2	0.1	1.8	8	1.0
C. similis						1.2				0.5	2.0		7.5	5.0	0.1	1.4	6	0.7
C. tuttifrutti		1.3	0.1	0.2						8.2	0.9		3.2	1.2	0.5	1.1	8	0.6
C. isioloensis										3.7			1.1	0.4	1.2	0.9	4	0.5
C. neavei				0.6	0.8	0.6								2.2	0.1	0.7	5	0.4
C. sp. #90						2.9									<0.1	0.5	2	0.3
C. enderleini											4.2		1.1		<0.1	0.4	3	0.2
C. bedfordi					0.2	0.8			0.5	0.5	0.7					0.3	5	0.2
C. onderstepoortensis			<0.1	2.1											<0.1	0.3	3	0.1
C. sp. #33						1.3										0.2	1	0.1
C. subschultzei											1.9			0.2	0.1	0.2	3	0.1
C. brucei				0.5										0.2	0.1	0.1	3	0.1
C. glabripennis									0.7	0.3				0.1		0.1	3	0.1
C. exspectator				0.5										0.1		0.1	2	<0.1
C. olyslageri				0.5					0.3							0.1	2	0.1
C. nr angolensis				0.4		0.1								0.1		0.1	3	<0.1
C. ravus						0.1					0.9					0.1	2	0.1
C. tropicalis											0.2			0.2	<0.1	0.1	3	<0.1
C. huambensis				0.2										0.2		0.1	2	<0.1
C. sp. #50										0.5					0.1	0.1	2	<0.1
C. kibatiensis					0.3										<0.1	<0.1	2	<0.1
C. nr albopunctatus									0.2						<0.1	<0.1	2	<0.1
C. nigripennis									0.3							<0.1	1	<0.1
C. engubandei												0.1				<0.1	1	<0.1
C. eriodendroni													1.1			<0.1	1	<0.1
Total collected	13	80	2836	828	592	1295	0	182	589	405	590	1200	93	1142	3363	13208		
Mean	4.3	16	945.3	276.0	197.3	215.8	0	30.3	84.1	135.0	196.7	600	13.3	142.8	373.7	188.7		
Max	13	74	2829	448	335	1002	0	81	265	230	573	1007	60	281	1743	2829		
No. of species collected	5	7	9	16	11	15	0	8	12	14	13	4	11	20	22	34		

Table 2. *Culicoides* species composition shown as relative abundance (%) at 10 sites during winter (July – August) in the south of the Eastern Cape, as determined with light trap collections during 2015 and 2016. Site numbers refer to Fig. 1. *Culicoides* species denoted by numbers (#) refer to yet undescribed species.

Dominant equine	Horse				Donkey			Zebra			Mean	No. of sites present	%
Site	**13**	**14**	**18**	**22**	**15**	**17**	**21**	**16**	**19**	**20**			
No of collections made	23	20	16	5	24	12	19	20	24	16			
C. bolitinos	45.5	64.9	9.4		45.3	10.0	14.7	5.3	18.5	4.8	9.6	9	32.6
C. pycnostictus	28.7	12.9	30.8		4.1	44.0	1.8	21.1	41.7	54.9	5.7	9	19.5
C. zuluensis	1.2	3.5	6.2	60.0	21.2	2.0	7.3		0.4	0.2	3.9	9	13.2
C. imicola	0.6	1.5	32.1	25.5	8.5				0.8	6.0	2.5	7	8.3
C. sp. #54 df	1.2	0.5			8,5			73.7	3.8	0.2	1.6	6	5.4
C. magnus	0.6	0.5	17.7	7.3	1.7		61.5			10.6	1.6	7	5.3
C. nivosus	4.8	2.0	2.9	1.8	1.7	36.0	1.8		11.1	13.6	1.5	9	5.1
C. leucostictus	4.8	3.0		1.8	2.6	6.0	2.8		9.8	1.8	0.9	8	3.1
C. isioloensis	1.2	0.5			2.0	2.0			0.8		0.4	5	1.2
C. onderstepoortensis	1.8	0.5			0.2		1.8		0.8	5.3	0.3	6	1.1
C. huambensis	6.0	5.9			1.1		1.8				0.3	4	1.1
C. gulbenkiani	2.4	1.5	0.8	1.8	0.6		5.5		1.7	0.1	0.3	8	0.8
C. tuttifrutti		1.5			0.8		0.9		2.1		0.2	4	0.7
C. sp. #50				1.8	<0.1				2.3	1.9	0.2	4	0.6
C. similis		1.0			<0.1				1.9		0.1	3	0.3
C. kibatiensis					0.5						0.1	1	0.3
C. sp. #94					0.4						0.1	1	0.2
C. neavei		0.5	0.3		<0.1				0.6	0.5	0.1	5	0.2
C. subschultzei					0.3						0.1	1	0.2
C. bedfordi					<0.1				1.5		0.1	2	0.2
C. brucei					0.2				0.4		0.1	2	0.2
C. sp. #90									1.3	0.1	<0.1	2	0.2
C. enderleini					0.1						<0.1	1	0.1
C. exspectator	1.2								0.4		<0.1	2	0.1
C. sp. #107									0.4		<0.1	1	<0.1
C. engubandei					0.1						<0.1	1	<0.1
C. trifasciellus					<0.1						<0.1	1	<0.1
C. micheli									0.2		<0.1	1	<0.1
C. hortensis					<0.1						<0.1	1	<0.1
C. glabripennis										0.1	<0.1	1	<0.1
Total collected	167	202	374	55	2895	50	109	19	530	856	5257		
Mean	7.3	10.1	23.4	11	120.6	4.2	5.7	0.95	22.1	53.5	29.4		
Max	56	43	125	48	777	29	54	10	226	234	777		
No. of species collected	13	15	8	7	25	6	10	3	20	14	30		

Culicoides population composition

- 39 species of *Culicoides* were collected at equines during this survey (Tables 1, 2). Females (n = 16 692) represented 90.4% of all specimens collected. Sex ratios (female:male) varied between species: 27.7:1 for *C. imicola*, 23.5:1 for *C. bolitinos*, 10.1:1 for *C. zuluensis* and 4.4:1 for *C. pycnostictus*. While summer collections of *C. bolitinos* and *C. imicola* were largely female-dominated, sex ratios were slightly more male-biased in winter, when some smaller collections consisted entirely of males. In contrast, *C. pycnostictus* not only exhibited largely male-dominated collections in winter, but also in some summer collections, e.g. March and early June of 2016.

Older or parous/pigmented *Culicoides* females (7524) were less abundant than younger nulliparous/non-pigmented females (9 169) with a parous: nulliparous ratio of 0.82:1. In the absence of a clear seasonal trend, there was an increase in the relative abundance of parous females of *C. bolitinos*, *C. imicola* and *C. zuluensis* in late summer (May - June) and throughout winter. Gravid *C. pycnostictus* females were present throughout the year in larger numbers than other parity stages, most notably in winter. Only 50 (0.3%) freshly blood-engorged females of all species were collected throughout the study (Riddin, 2017).

Culicoides bolitinos, representing 39% of all species collected, was the most abundant; *C. imicola*, representing 21.6%, was the next most abundant (Tables 1, 2). These two species were widespread, being present at 20 of the 22 collection sites (Fig. 1).

A total of 13208 specimens of *Culicoides* representing 34 species were sampled in 70 collections made at confirmed or suspected outbreak cases of AHS from November to June (Table 1), a mean of 188.7 midges/trap. The dominant species, forming 41.5% of the collection, was *C. bolitinos* (Table 1). The next most dominant species was *C. imicola* (26.9%) followed by *C. zuluensis* (9.2%) and *C. pycnostictus* (7.1%) (Table 1). While *C. bolitinos* was present at all but one sites, *C. imicola*, *C. zuluensis* and *C. pycnostictus* was absent at three of the 15 outbreak sites. Other species which were found at 10 (66%) or more sites included *C. gulbenkiani* (11), *C. nivosus* (10) and *C. leucostictus* (10) (Table 1).

In the collections made at specific hosts (horse, zebra and donkey) during July to August, 5257 midges, representing 30 species were sampled (Table 2). As during outbreak season, *C. bolitinos* was the dominant species (32.6% of total specimens) (Table 2). The second-most abundant species was *C. pycnostictus* (19.5%), followed by *C. zuluensis* (13.2%). Although *C. imicola* was present at seven (70%) of the sites, it was only the fourth most abundant species representing 8.3% of the species collected. *C. bolitinos* was however, present at nine of 10 sites. Other species found at nine sites were *C. pycnostictus, C. zuluensis* and *C. nivosus.* Ten other species were found at more than half of the sites (Table 2).

Nine species, *C.* sp. #33, C. nr angolensis, *C. ravus*, *C. cornutus*, *C. tropicalis*, *C. nigripennis*, *C. olyslageri*, *C. eriodendroni* and C. nr albopunctatus were only collected at outbreak cases and not during

the colder winter months. Species that were collected during only during winter were *C.* sp. #107, C. sp. #94, *C. trifasciellus*, *C. micheli* and *C. hortensis*.

Since the 8 W G5 fluorescent T5 UV black light blue tubes were replaced by 6 UV LEDs during the 2015-2016 outbreak and 2016 winter seasons, these results were analyzed separately from those of the 2014-2015 outbreak and 2015 winter seasons. The conversion factor relative to *Culicoides* capture was a ~4.5 times higher collection by the modified Onderstepoort trap versus the energy conservative UV LED and day-night switch trap. However, this value does not consider other contributing factors (i.e. climate, land use *etc*) within the study years.

Culicoides diversity in summer/outbreaks

During the 2014-2015 outbreak season, 26 (but negative) and 62 collections were made at suspected or confirmed AHS cases respectively. In the subsequent outbreak season, 2015-2016, there were only three confirmed and 10 suspected (but negative) cases of AHS.

During both outbreak seasons some of the suspected cases of AHS were misidentified and found to be infections with the closely related equine encephalosis virus (EEV) (*Orbivirus*: *Reoviridae*) (Table 3). The mean number of *Culicoides* specimens collected at sites with suspected cases varied from less than one (Kragga Kamma (site 8) and Grahamstown Riding Club (2)) to 740.7 (Hogwallow Farm (site 15)) per trap (Table 3). *Culicoides bolitinos*, comprising from 29.8% to 100% of the collections dominant species, was dominant at two AHS, two EE and three coinfection confirmed cases (Table 3). It was the second-most abundant species at two more cases (Table 3). *Culicoides imicola* was the second most abundant at four (1 AHS, 1 coinfection, two EE) cases where *C. bolitinos* was the dominant species (Table 3). *Culicoides imicola* dominated at only three cases ranging from 0.3 to 600.0, of *Culicoides* collected (Table 3). Species like *C. leucostictus* (AHS), *C. magnus* (AHS) and *C. zuluensis* (EE) were dominant at one case each, but with five or fewer individuals collected (Table 3). *Culicoides pycnostictus* was the most abundant species at one (EE) three further confirmed orbiviral cases (1 AHS, 2 coinfection) (Table 3).

Sites with suspected cases at which neither AHSV nor EEV could be confirmed (Negative) lacked a trend in species composition (Table 3). Unlike at confirmed cases, *C. bolitinos* was dominant at only four sites. *Culicoides imicola* dominated two sites and *C.* sp. # 54df, *C. cornutus* and *C. pycnostictus* dominated one site each (Table 3). The second-most dominant species varied greatly (Table 3).

Confirmed cases of AHS occurred as early as November and continued up to June. Although the mean numbers of *Culicoides* collected dropped drastically during June, when average night temperatures were relatively low, no clear correlation was seen between night temperatures and mean numbers collected (Table 3). During the 2014-2015 outbreak season, collections were dominated by *C. bolitinos* from late January 2015 to early June 2015. Collections at outbreaks earlier in the season, i.e. November and December 2014, were dominated by *C. zuluensis* and *C. pycnostictus*. Notable numbers

Table 3. Case site *Culicoides* species composition as determined by light trap collections made at confirmed AHS, EE and negative cases occurring from November 2014 to February 2016 in the Eastern Cape. Site numbers refer to Fig. 1.

Site No.	Collection date	Avg night temp (°C)	Mean no/trap in one night (no of traps)	No of species collected	Dominant species (%)	Second most dominant species (%)
AHS						
8	2014/04/24	16.3	60.3 (3)	8	*C. bolitinos* (29.8)	*C. imicola* (27.1)
10	2014/11/10	15.5	135.0 (3)	14	*C. bolitinos* (66.2)	*C. pycnostictus* (10.6)
12	2015/04/01	19.1	600.0 (2)	4	*C. imicola* (71.8)	*C. zuluensis* (22.5)
4	2016/02/17	17.6	8 (1)	4	*C. leucostictus* (37.5)	*C. zuluensis* (25.0) C. nr angolensis (25.0)
2	2015/06/10	9.2	0.3 (3)	1	*C. imicola* (100)	NA
1	2015/06/18	7.6	4.3 (3)	5	*C. magnus* (53.9)	*C. bolitinos* (15.4) *C. zuluensis* (15.4)
AHS & EE						
13	2015/05/06	13.4	9.3 (3)	7	*C. bolitinos* (60.7)	*C. pycnostictus* (14.3)
15	2015/05/16	14.5	740.7 (3)	16	*C. bolitinos* (48.1)	*C. imicola* (37.4)
14	2015/01/16	19.2	153.0 (3)	17	*C. bolitinos* (51.6)	*C. pycnostictus* (14.8) *C. nivosus* (14.8)
EE						
8	2015/01/30	20	0.3 (3)	1	*C. zuluensis* (100)	NA
9	2015/02/13	21.2	71.8 (4)	11	*C. imicola* (59.6)	*C. bolitinos* (24.4)
9	2015/02/24	21.3	100.7 (3)	6	*C. bolitinos* (90.1)	*C. imicola* (5.3)
6	2015/03/04	16.3	94.5 (2)	13	*C. pycnostictus* (38.6)	*C.* sp. #90 (11.1)
6	2014/03/19	15.9	276.5 (4)	14	*C. bolitinos* (42.0)	*C. imicola* (24.1)
Negative						
4	2014/11/28	14.5	410 (2)	16	*C. pycnostictus* (44.0)	*C. zuluensis* (23.8)
5	2015/02/19	17.1	197.3 (3)	11	*C. bolitinos* (64.9)	*C. zuluensis* (13.2)
14	2015/03/04	20.6	188.3 (3)	14	*C. bolitinos* (53.3)	C. *pycnostictus* (19.3)
15	2015/03/22	16.1	88 (1)	4	C. sp. #54df (38.6)	*C. imicola* (37.5)
3	2015/04/01	19.6	945.3 (3)	9	*C. bolitinos* (64.7)	*C. inicola* (27.3)
15	2015/05/22	13.9	464 (2)	12	*C. imicola* (51.1)	*C. bolitinos* (22.4)
15	2016/02/25	23.2	29 (1)	10	*C. imicola* (31.0)	*C. bolitinos* (13.8)
11	2016/04/08	11.3	196.7 (3)	13	*C. cornutus* (84.2)	*C. enderleini* (4.2)
13	2016/06/08	16.0	30 (2)	9	*C. bolitinos* (36.7)	*C. pycnostictus* (26.7)

of *C. magnus* and C. sp. #54 df was collected from late 2014 to early 2015, late May and early June 2015, respectively.

During the 2015-2016 outbreak season the mean numbers of *Culicoides* collected were at least 10 times lower than in the 2014-2015 season (Fig. 2). These collections were dominated by *C. imicola* in February and March 2015, followed by *C. enderleini* during early April 2016. *Culicoides leucostictus* and *C. bolitinos* were also present during the 2015-2016 outbreak season (Fig. 2).

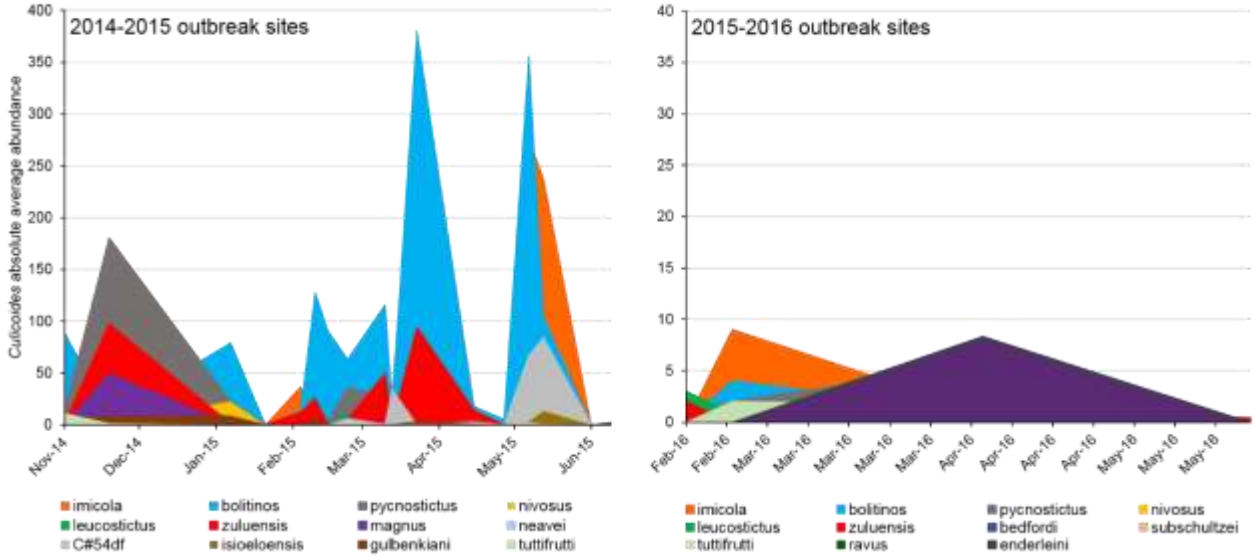

Fig. 2. Diversity of *Culicoides* species collected in light traps during the AHS outbreak seasons of 2014-2015 (scale X10); and 2015-2016 (scale X1) in the Eastern Cape.

Culicoides diversity in winter

Site selection and therefore *Culicoides* species diversity for the 2015 winter surveillance was dictated by the presence of equid species (horse, donkey or zebra). Diversity in sites for 2015 winter season did not show congruence for any of the equid species (Table 2).

Culicoides species diversity at the equid sites during the winter differed between equid specific-sites. The diversity profile between the 2015 winter equid sites showed smaller alpha diversity index differences with horse sites at 8.29, donkey sites at 6.24 and zebra sites at 5.95, and an overall alpha diversity index of 6.32. Beta diversity was surprisingly low at 1.17 average species difference between equid specific sites, however, the diversity profile showed high regions of possible error. The 2016 equid-specific sites revealed zebra sites had the overall highest alpha diversity at 5.25, followed by horse sites (4.43) and donkey sites (4.39). Diversity between equid specific sites was the lowest in comparison with a beta diversity of 0.97 species. Horse sites exhibited relative low numbers in 2015, with small peaks in *C. zuluensis* numbers in late July that were replaced by *C. bolitinos* in late August (Fig. 3). A greater diversity was seen at the horse sites in the 2016 winter season, dominated by *C.*

bolitinos, *C. magnus* and *C. imicola* in the initial trapping months followed by *C. bolitinos* and *C. pycnostictus* towards the end of the season (Fig. 3).

Fig. 3. Diversity of *Culicoides* species collected in light traps at equid sites: horse; donkey (scale x 4); and zebra, during winter 2015 and winter 2016.

Similarly, species diversity in donkey sites was lower in 2015 in comparison to the 2016 winter season with relatively large collections of *C. bolitinos* and *C.* sp #54 df initially to be replaced by *C. magnus* and *C. zuluensis* towards the end of the season (Fig. 3). In 2016, donkey sites initially had small peaks in *C. bolitinos* and *C.* sp. #54 df numbers followed by a stark increase in abundance in *C. bolitinos* and higher numbers of *C. imicola, C. zuluensis* and *C. isioloensis* in mid-season (Fig. 3). Towards the end of the 2016 winter season, *C. leucostictus* and *C. nivosus* numbers peaked at donkey sites. Zebra sites were dominated by *C. pycnostictus* throughout the 2015 and 2016 winter seasons (Fig. 3). Small populations of *C. magnus* and *C. nivosus* were seen during both seasons while winter 2016 had a larger diversity which included *C. onderstepoortensis, C. bolitinos, C. nivosus, C. magnus* and C. sp. #50 (Fig. 3).

Discussion

Light trap efficacy for collection of Culicoides

The mean number of midges collected per trapping event was low overall, with only four collections exceeding 1000 individuals. These mean numbers were considerably lower than those in the north of the country where more than one million midges can be collected near livestock in a single night (Meiswinkel *et al*., 2004), or during outbreaks of AHS (Venter *et al*., 2006) using the more powerful 220 V Onderstepoort trap. Although a high incidence of wind in the area could have influenced flight activity, these lower mean numbers may have been the result of the use of less powerful 12 V traps (Venter *et al*., 2009; Probst *et al*., 2015). Similarly, the overall lower numbers of *Culicoides* collected during 2015-2016 may be ascribed to the use of LEDs which was recently shown to be at least four times less powerful than conventional fluorescent lights in attracting European *Culicoides* species (Hope *et al*., 2015; Wakefield *et al*., 2016). These more energy-conservative 12 V traps did allow sampling over a wider and more diverse geographical area independent of 220 V electricity. Although light traps may not truly reflect the biting rates on hosts due to their inherently un-hostlike mechanism of attraction (Gerry *et al*., 2009; Viennet *et al*., 2011; Scheffer et *al*., 2012), they will acquire adequate diversity for estimation of community structure and species diversity (Venter *et al*., 2009; 2018).

Culicoides species composition

39 *Culicoides* species, including *C. imicola* and *C. bolitinos*, proven vectors of AHSV, were collected in the present study. More than half of these species were collected rarely and at few of the sites (Table 1, 2), which may limit their potential as efficient vectors of AHSV. The number of species collected was, however, nearly double that of 12-24 species previously reported in the Eastern Cape (Venter *et al*., 1996). Despite the lower overall numbers, the species diversity was higher than the 26 found over a whole year in a recent study (Venter *et al*., 2014), however, collections for the study were only collected at a single site in the north of the country in comparison to the 22 used within this study. The

greater species diversity may be a reflection of the greater diversity of sites sampled. As the study was restricted to equines in a relatively small part of the province, and outbreak site trapping was not replicated, 39 species may be an underestimation of the overall diversity in the area.

Characteristic of light trap results obtained in the vicinity of mammal hosts (Carpenter *et al*., 2008; Viennet *et al*., 2011), the collections were dominated by females of the abundant livestock-associated *Culicoides* species. Males usually do not disperse from larval development sites and the small but male-dominated collections of *C. pycnostictus* during the 2015 winter and throughout 2016, may indicate abundant and widespread larval developmental sites for this species in the area (Bidlingmayer, 1961). Notwithstanding the known larval habitats of the dung breeders in the present study, the "precise/optimum" and range thereof for most of the species collected are not known. The dispersal capacity of the various species from the larval habitats are also unknown and it was therefore not possible to determine the extent of the presence of larval habitats on the present results.

In contradiction to the notion that parous females peak in the late summer (Venter *et al*., 1997; Meiswinkel *et al*., 2008), nulliparous females were dominant throughout the year, with only a slight decline in nulliparous:parous female ratios in late summer in the present study. The continual abundance of nulliparous females may indicate overlapping generations and continuous breeding (Walker, 1977). In the absence of transovarial transmission (Osborne *et al*., 2015), an above average proportion of parous females may indicate an older population with a potentially higher infection rate (Venter *et al*., 1997). If orbivirus infected *Culicoides* vectors potentially become light-averse (McDermott *et al*., 2015), light traps may be nulliparity-biased (Braverman & Linley, 1993), leading to an underestimation of the possible infection rate and risk for virus transmission.

Species contributing to epidemiology of AHS

Some of the suspected cases of AHS were confirmed as EEV. Although mostly asymptomatic, the symptoms of EE sometimes resemble AHSV fever (Howell *et al*., 2004). AHSV and EEV can be transmitted by the same species of *Culicoides* midges (Venter *et al.*, 1999a). The abundance of *C. bolitinos* at confirmed cases of AHS and EE, their dominance at 54.5% of the sites and their continual presence at equids during winter, highlights the importance of this species as a potential vector of AHSV in the Eastern Cape. The present study supported Meiswinkel's (1997) observation that *C. bolitinos* is the most abundant species in the sandy dune area located in the south. While Meiswinkel (1997) found *C. imicola* to be absent, it was found in low numbers in this area in the present study. Vector abundance and isolations of AHSV during an outbreak in the cooler mountainous eastern Free State Province in South Africa combined with laboratory oral susceptibility comparable to that of *C. imicola* to AHSV, underline the vector status of *C. bolitinos* (Meiswinkel & Paweska, 1998; Mellor *et al*., 2000; Venter *et al.*, 2000).

The presence of a variety of livestock-associated *Culicoides* species with *C. imicola,* considered the most important vector in South Africa (Meiswinkel *et al*., 2004), but being the second-most abundant species, indicates that multiple species of *Culicoides* may potentially be involved in the epidemiology of AHSV in the Eastern Cape. Dominant species at confirmed cases of AHSV included *C. bolitinos, C.imicola, C. zuluensis, C. magnus,* and *C. leucostictus,* which Nevill *et al*. (1992b) rated as having high vector capacities for transmitting livestock viruses in South Africa, based on their relative abundance at livestock, geographical distribution and host preference. Although *C. zuluensis* was the only species to be collected at a confirmed outbreak of EEV, only one specimen was collected in three collections made (Table 3). However, Nevill *et al.* (1992b), rated this species, based on its abundance and host preference, as having a relative high overall orbivirus rating, and with *C. zuluensis* being present at most of the collection sites and 3rd most abundant species during both the summer and winter collections in the current study, it highlights the possible vectorial contribution of this species. *Culicoides magnus* was present in low numbers at confirmed cases of AHSV and throughout the winter at donkey and horse sites.

Although *C. pycnostictus* is considered to be a bird-feeder (Nevill & Anderson, 1972; Nevill *et al*., 1987), it will opportunistically feed on mammals (Meiswinkel *et al*., 2004). Its second most dominant status at AHS/EE/coinfection cases, wide distribution, presence throughout the trapping period, dominance at zebra and oral susceptibility to AHSV (Paweska *et al*., 2003) raise its potential vector capacity. Bluetongue virus (*Orbivirus; Reoviridae*) was previously isolated from field collected specimens (Nevill *et al*., 1992a), which confirms that this species will feed on bigger mammals.

All of the abundant and widespread species in the Eastern Cape were shown to be orally susceptible to one or more serotypes or isolates of AHSV in the laboratory (Venter, 2016). Since vector status is not solely dictated by abundance, it will be essential to compare the vector competence, host preferences and biting rate of these species to that of *C. imicola* and *C. bolitinos* to clarify their role in the epidemiology of AHSV in the area.

Over-wintering of adult Culicoides

Despite the smaller mean numbers collected from July to August, it was still possible to collect up to 777 midges in a single light trap at Hogwallow farm (site 15). In addition to *C. bolitinos* and *C. imicola,* a number of species including C. sp. #54 df, *C. pycnostictus* and *C. zuluensis* in the 2015 winter and *C. zuluensis* and *C. pycnostictus* in the 2016 winter were collected in moderate numbers. The presence of confirmed vectors may allow the overwintering of AHSV in adult midges (Rawlings & Mellor, 1994; Welby *et al*., 1996; Mellor & Hamblin, 2004; Becker *et al*., 2012; Venter *et al*., 2014). The presence of freshly blood-engorged and nulliparous females and males during winter support feeding and emergence throughout the coldest months.

Carpenter *et al*. (2011) determined that the minimum temperature necessary for replication of orbiviruses in a variety of midge species was 11°C to 13°C. With minimum temperatures in the Eastern Cape seldom dropping below 10°C and maximum temperatures often as high as 20°C to 25°C during winter, competent *Culicoides* species can potentially sustain viral replication and onward transmission throughout winter, especially if they retire to thermal refugia such as buildings. Although adult *C. imicola* rarely survive winters with average maximum daily temperatures below 12.5°C (Sellers & Mellor, 1993), they have been shown to be active above 12°C and to survive in an inactive state below 12°C (Mellor & Hamblin, 2004). Recent research has been shown to be in agreement with Sellers & Mellor (1993), with peak *C. imicola* activity at between 16°C to 18°C, and flight impairment at above 20°C (Venter *et al*., 2019). Further contribution to understanding of activity under field conditions showed peak capture immediately following sunset and 74% of *Culicoides* collected within the following 2-3 hours in mean nocturnal temperatures below 19°C (Venter *et al*., 2019). When mean nocturnal temperatures were above 19°C, peak numbers were sustained until after midnight, and temperatures below 10°C resulted in low collection numbers of *C. imicola* despite presence of cattle (Venter *et al*., 2019). Predominant activity for the present study appeared to occur on nights averaging between 13°C-20°C, supporting the overwintering potential of a number of *Culicoides* species in the absence of a vertebrate reservoir (Mellor & Hamblin, 2004). With minimum night temperatures rarely below 10°C and given the presence of this species in light traps during peak 2015 and 2016 winter periods, adults of this species could be active year-round in the Eastern Cape.

At least 15 species of *Culicoides* were present around all three equine species (Table 2). A further 15 species were found in one to two equine sites each. The overlap of community membership increases the opportunity for virus transmission between these equine species. *Culicoides* #54df, which breeds in zebra dung (Nevil *et al*., 2009), was found at all of the zebra sites, but was not restricted to those sites, and was relatively ubiquitous. Conversely, C. sp. #107 also breeds in zebra and horse dung (Nevill *et al*., 2009) but was collected only at one of the zebra site. Although the *Culicoides* communities at the horse sites were overall more diverse than at the donkey or zebra sites during the winter of 2015, the numbers of mammal-feeding species, including *C. bolitinos* and *C. zuluensis*, were high. This may be due to the greater diversity of livestock at the donkey sites compared to horse sites, which were most often dominated by horses. This higher diversity may allow a broad host specificity and an increase in *Culicoides* numbers at livestock (Barnard, 1997; Garcia-Saenz *et al*., 2011).

Within limits, *Culicoides* collections in light traps decrease linearly with a decrease in livestock numbers in the immediate vicinity of the trap (Garcia-Saenz *et al*., 2011). This, and light traps' limited ranges of attraction (Venter *et al*., 2012), may explain the relatively low numbers and species diversity observed at zebra sites. In contrast to horse and donkey sites, where the livestock hosts were within a few meters of the trap, trapping at zebra sites was conducted on large properties (203 ha to 9000 ha). Due to the mobility of the zebras, they did not remain close to the trap all night. The numbers and

species composition of *Culicoides* collected near caged zebras i.e. zoos, closely can resemble those of samples from near livestock and other bigger mammals in the same area (Labuschagne *et al*., 2007).

A potential shortcoming of the present study is that the collected midges were not subjected to virus detection. Detection of the virus during the winter months may have been able to supply valuable information on the potential overwintering of the virus in the vector. However, we emphasise that the infection prevalence in field-collected vectors is normally extremely low. In an overwintering study in north of the country, AHSV was only detected in pools of midges containing more than 1000 individuals (Venter *et al.*, 2014). Even during outbreaks, it can be lower than 0.005% (Venter *et al*., 2006). Taking into account the mean collection size (x = 74.2) in the present study, virus yield would have been too low to justify the cost of viral screening. Detection of the virus in field-collected midges, especially so during the winter months, would need an intensive and dedicated effort. The present study has, however, revealed sites (i.e. Hogwallow (15)) with above average midges numbers which that can be surveyed in future studies.

Conclusion

The diversity of *Culicoides* found in the present study indicated that their diversity and distribution in the Eastern Cape have changed or have previously been underestimated. Although proven vectors, i.e. *C. bolitinos* and *C. imicola*, were widespread and abundant in the area, their lower representation compared to the north of the country, and the continual presence of other orally-susceptible species, suggest that further vectors contribute to the persistence of AHSV in the south. Based on its abundance at equines *C. bolitinos* appears to be the biggest contributor to vector epidemiology. The numbers of *Culicoides* collected, and their species diversity during the coldest time of the year, implies that AHSV no longer needs to be introduced from the warmer, frost-free north of the country. AHSV can potentially be present in the Eastern Cape in adult *Culicoides* midges all year and outbreaks of AHS can therefore start as soon as populations reach an adequate level of activity.

Acknowledgements

We warmly thank the many landowners who permitted sampling around their animals. The Department of Zoology and Entomology, Rhodes University, and the Agricultural Research Council - Onderstepoort Veterinary Research (ARC-OVR) were greatly supportive and assisted with identification and methodology. The National Research Foundation and the African Horse Sickness Trust provided funding. We further acknowledge the support of the ARC Climate Change Collaboration Centre.

Permits for aspects of the research were obtained from the National Director of Animal Health, South Africa (reference: 12/11/1/6/2: permission to work on AHS under Section 20 of South Africa's Animal Diseases Act 35 of 1984); Rhodes University (ethical clearance number: ZOOL-05-2014); and the Cacadu District Department of Economic Development, Environmental Affairs and Tourism (permit

no. CRO 23/15CR to collect biological samples in terms of Section 63 of the Nature and Environmental Conservation Ordinance, 1974 (Ordinance 19 of 1974), Sections 24 and 25 of Environmental Conservation Decree 1992 (Decree No. 9 of 1992, former Transkei) and Sections 20 and 21 of the Nature Conservation Act. 1987 (Act No. 10 of 1987, former Ciskei)). 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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