African horse sickness Epidemiology: *Culicoides* spp. diversity, vectors and overwintering of the virus in Eastern Cape Province, South Africa

by

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I declare that the above dissertation is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

I further declare that I submitted the dissertation to originality checking software and that it falls within the accepted requirements for originality.

I further declare that I have not previously submitted this work, or part of it, for examination at Unisa for another qualification or at any other higher education institution.

_____ 28/02/2024_____

Signature Date

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Their unconditional love, sacrifices and encouragement shaped me from a young age and have been a driving force behind my success.

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TABLE OF CONTENTS

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

AHS – African horse sickness

AHSV – African horse sickness virus

AKAV – Akabane virus

ARC – OVR – Agricultural Research Council – Onderstepoort Veterinary Research

BEFV- Bovine ephemeral fever virus

BT – Bluetongue

Bti - *Baccilus thuringensis israelensis*

BTV – Bluetongue virus

DALRRD – Department of Agriculture, Land Reform and Rural Development

DR – Diagnostic Registration

EEV- Equine encephalosis virus

EHD – Epizootic haemorrhagic disease

EHDV – Epizootic haemorrhagic disease virus

ELISA – Enzyme-linked Immunosorbent Assay

GPS – Geographical Positioning System

KNP – Kruger National Park

OBP – Onderstepoort Biological Products

OIE – International Office of Epizootics

OROV – Oropouche virus

PCR – Polymerase Chain Reaction

RMSF – Rocky Mountain Spotted Fever

RNA – Ribonucleic Acid

SANAS – South Africa National Accreditation System

SAVC – South African Veterinary Council

UNISA – University of South Africa

WOAH – World Organisation for Animal Health

ABSTRACT

African horse sickness (AHS) is a devastating, non-contagious viral disease of equids. However, zebra are resistant to the disease. Previously it was accepted that outbreaks of AHS would start in the northern parts of South Africa and gradually move southwards as the season progressed. This stemmed from the belief that the large population of zebras in the Kruger National Park, acted as the source of the virus. In outbreaks that have occurred over the past 25 years, it has become evident that this was not true, as outbreaks would persist for two or more seasons in the south of the country, with the same AHS serotype causing the first outbreak being the only one recorded in that area in subsequent years. Therefore, it is possible that the virus overwinters in the area. However, the overwintering mechanism of the African horse sickness virus (AHSV) is not fully understood. It has been suggested that the AHSV overwinters in adult *Culicoides* populations, at low levels of viremia in natural hosts, or in other vertebrate or invertebrate hosts such as dogs and ticks, respectively. The aim of this study was to investigate how the AHSV overwinters in the study area, and the role dogs and ticks play in this process and to determine the diversity for both *Culicoides* and tick species in the study area. This study was undertaken in the Sarah Baartman district in the Eastern Cape Province of South Africa. A total of 28 sites were recruited for collection of *Culicoides* and tick species, and blood samples from dogs. A 220 V down draught-UV light trap was used to collect *Culicoides* specimens, while ticks were collected directly from live hosts. Blood samples were drawn from dogs using the cephalic vein. All these samples were sent to the Agricultural Research Council – Onderstepoort Veterinary Research (ARC-OVR) for analysis. Over the survey period 918 collections of *Culicoides* were made, and 44,850 *Culicoides* specimens, comprising of 49 *Culicoides* species were recovered. A total of 1,260 tick specimens comprising of 10 species from three genera were collected and identified during the survey. Over a period of one year, blood samples were collected from 100 dogs. Each dog was sampled four times, once every season. Blood samples were analysed using an AHS indirect ELISA to test for the presence of IgG antibodies. *Culicoides* and tick samples were first tested for the presence of AHSV using an inhouse PCR. All samples that tested positive on the inhouse PCR were subjected to the WOAH (accredited and validated) test. Only samples that tested positive with the WOAH test were considered AHSV positive. While five *Culicoides* pools tested positive with the inhouse PCR, only two pools of these pools tested positive for the virus on the WOAH

PCR test. Furthermore, of the two pools that tested positive: one was from Site 1 and consisted of two specimens of *C. bolitinos* and the other pool was from Site 7 and consisted of six specimens of *C. tuttifrutti*. This study provides an insight into the diversity of both *Culicoides* and tick species in the study area. Findings reported here demonstrated that dogs are not preferred hosts for *Culicoides* species given that none of the dogs tested positive for AHSV antibodies. Based on the results from this study, only *Culicoides* species have the potential to play a role in the overwintering of AHSV in the area. However, additional tick species and other possible vertebrate hosts should also be investigated. Further research is needed to investigate the role of *C. tuttifrutti* and other blood feeding flies in the transmission of AHSV and other orbiviruses.

Keywords: Transmission of African Horse Sickness, equids, AHS serotype, African Horse Sickness Virus, AHS, diversity of *Culicoides*

ABSTRAK

Afrika-perdesiekte (APS) is ’n verwoestende, nie-aansteeklike virale siekte van perdagtiges. Sebras is egter bestand teen die siekte. Voorheen is aanvaar dat uitbrekings van APS in die noordelike dele van Suid-Afrika sou begin en geleidelik suidwaarts sou beweeg soos die seisoen vorder. Dit het gespruit uit die oortuiging dat die groot bevolking sebras in die Krugerwildtuin die bron van die virus was. In uitbrekings die afgelope 25 jaar, het dit duidelik geword dat dit nie waar is nie, aangesien uitbrekings vir twee of meer seisoene in die suide van die land sou voortduur, met die APS-serotipe wat die eerste uitbreking veroorsaak het die enigste een wat in die daaropvolgende jare aangeteken is. Daarom is dit moontlik dat die virus in die gebied oorwinter. Daar is egter nie voldoende begrip van die oorwinteringsmeganisme van die Afrika-perdesiektevirus (APSV) nie. Daar is voorgestel dat die APSV oorwinter in volwasse *Culicoides*-bevolkings, teen lae vlakke van viremie in natuurlike gashere, of in ander werweldiere of ongewerwelde diere soos honde en bosluise, onderskeidelik. Die doel van hierdie studie was om te ondersoek hoe die APSV in die studiegebied oorwinter, die rol wat honde en bosluise in hierdie proses speel, en om die diversiteit van beide *Culicoides*- en bosluis spesies in die studiegebied te bepaal. Hierdie studie is in die Sarah Baartman distrik in die Oos-Kaap provinsie van Suid-Afrika onderneem. Altesaam 28 terreine is besoek vir die insameling van *Culicoides*- en bosluis spesies, en vir bloedmonsters van honde. ’n 220 V-intreklug-UV-ligval is gebruik om die *Culicoides*-monsters te versamel terwyl bosluise direk van lewende gashere ingesamel is. Bloedmonsters is uit die kefaliese aar by honde getrek. Al hierdie monsters is na die Landbounavorsingsraad-Onderstepoort Veterinêre Navorsing (LNR-OVN) gestuur vir analise. Tydens die opname tydperk is 918 insamelings van *Culicoides* gemaak, en 44,850 *Culicoides*-monsters bestaande uit 49 *Culicoides* spesies is opgespoor. ’n Totaal van 1,260 bosluis-monsters bestaande uit 10 spesies uit drie genera is tydens die opname ingesamel en geïdentifiseer. Tydens ’n tydperk van een jaar is bloedmonsters by 100 honde ingesamel. Bloedmonsters is vier keer, een keer per seisoen, by elke hond ingesamel. Bloedmonsters is ontleed met behulp van ’n APS indirekte ensiemgekoppelde immunosorbenttoets (ELISA) vir die teenwoordigheid van IgG-teenliggame. *Culicoides*- en bosluismonsters is eers getoets vir die teenwoordigheid van APSV met behulp van ’n interne PKR. Alle monsters wat positief getoets het met die interne PKR is aan die Wêreld organisasie vir Dieregesondheid (WOAH) se geakkrediteerde en geldige toets onderwerp. Slegs monsters wat positief getoets het met die WOAH-toets is as APSV-positief beskou. Alhoewel vyf *Culicoides*-poele positief getoets het met die interne Polimerase kettingreaksie (PKR), het slegs twee poele van hierdie poele positief getoets vir hierdie virus met die WOAH-PKR-toets. Verder, van die twee poele wat positief getoets het,

was een van Terrein 1 en het bestaan uit twee monsters van *C. bolitinos*, en die ander poel was van Terrein 7 en het bestaan uit ses monsters van *C. tuttifrutti*. Hierdie studie bied insig in die diversiteit van beide *Culicoides*- en bosluis spesies in die studiegebied. Die bevindinge wat hier gerapporteer is, het getoon dat honde nie voorkeur gashere vir *Culicoides*-spesies is nie, aangesien geen honde positief getoets het vir APSV-teenliggame nie. Op grond van die resultate van hierdie studie het slegs *Culicoides* spesies die potensiaal om 'n rol te speel in die oorwintering van APSV in die gebied. Addisionele bosluis spesies en ander moontlike gewerwelde gashere moet egter ook ondersoek word. Verdere navorsing is nodig om die rol van *C. tuttifrutti* en ander bloed voedende vlieë in die oordrag van APSV en ander orbivirusse te ondersoek.
Sleutelwoorde: Oordrag van Afrika-perdesiekte, perdagtiges, APS-serotipe, Afrika-perdesiektevirus, APS, diversiteit van *Culicoides*

ISISHWANKATHELO

Isifo samahashe iAfrican horse sickness (iAHS) sisifo esitshabalalisayo, esingosuleliyo. Nangona kunjalo, amaqwarhashe ayaxhathisa kwesi sifo. Ngaphambili kwakusamkelwa ukuba uqhambuko lweAHS luza kuqala kwiindawo ezisemantla oMzantsi Afrika luze ngokuthe chu lunwenwe ukuya emazantsi ngokuhamba kwexesha lonyaka. Oku kubangelwa yinkolelo yokuba uninzi lwamaqwarhashe eKruger National Park, lusebenza njengemvelaphi yale ntsholongwane. Kuqhambuko oluye lwenzeka kule minyaka ingama 25 adlulileyo, kuye kwacaca ukuba oku bekungeyonyani, nanjengoko ukuqhambuka bekuye kuqhubeke kangangezihlandlo ezibini okanye ngaphezulu ngexesha lonyaka kumazantsi eli lizwe, kunye neAHS efanayo ebangele ukuqhambuka kokuqala ibekuphela kwako ekubhalwe phantsi kule ndawo kwiminyaka elandelayo. Ngoko ke, kunokwenzeka ukuba le ntsholongwane inwenwa ubusika bonke kwindawo leyo. Nangona kunjalo, indlela yokunwenwa ubusika bonke kwentsholongwane yokugula kwamahashe iAfrika horse sickness virus (iAHSV) ayiqondwa ngokupheleleyo. Kuye kwacetyiswa ukuba iAHSV inwenwa ubusika bonke kwiimbuzane (*Culicoides*) ezindala, kumanqanaba aphantsi kokubakho kweentsholongwane egazini kumakhaya endalo, okanye kwamanye amakhaya ezilwanyane ezinethambo lomqolo okanye izilwanyana ezingenathambo lomqolo ezifana nezinja kunye namakhalane, ngokulandelanayo. Olu phando lwenziwe kwisithili iSarah Baartman kwiPhondo leMpuma Koloni eMzantsi Afrika. Kukhangelwe iindawo ezingama28 zizonke ukuze kuqokelelwe iintlobo zeembuzane, namakhalane, kunye neesampuli zegazi ezinjeni. Kusetyenziswe isibane sokuthiyisela/sokubamba izinambuzane i220 V down draught-UV ukuqokelela iisampuli zeembuzane, ngelixa amakhalane aqokelelwa ngokuthe ngqo kwizilwanyane eziphilayo. Iisampuli zegazi zitsalwe kwizinja kusetyenziswa umthambo osemkhonweni. Zonke ezi sampuli ziye zathunyelwa kwiAgricultural Research Council – Onderstepoort Veterinary Research (iARC-OVR) ukuze zixilongwe. Ngexesha lophando kwenziwa ingqokelela yeembuzane ezingama 918, kunye neesampuli zeembuzane ezingama 44,850, ezibandakanya iintlobo zeembuzane ezingama49 ezafunyanwayo. Zizonke ziisampuli zamakhalane ali 1,260 eziquka iintlobo ezili10 ezivela kumaqela amathathu afanayo ezithe zaqokelelwa zaze zachongwa ngexesha lovavanyo. Kwisithuba esingangonyaka, iisampuli zegazi zaqokelelwa kwizinja ezili100. Kwathathwa iisampuli zegazi kane kwinja nganye, kanye ngexesha lonyaka. Iisampuli zegazi zaxilongwa kusetyenziswa iAHS indirect ELISA ukuvavanya ubukho bezithintelintsholongwane zeIgG. Iisampuli

zeembuzane nezamakhalane zavavanywa kuqala ukujonga ubukho beAHSV kusetyenziswa iPCR yangaphakathi. Zonke iisampuli eziye zafunyaniswa zinentsholongwane kwiPCR yangaphakathi zifakwe kuvavanyo lweWOAH (oluvunyiweyo noluqinisekisiweyo). Ziisampuli kuphela eziye zafunyaniswa zinentsholongwane ngovavanyo lweWOAH eziye zathathwa ngokuba zineAHSV. Ngelixa amagcuntswana amahlanu eembuzane afunyaniswa enentsholongwane ngePCR yangaphakathi, ngamagcuntswana amabini kuphela kula magcuntswana afunyaniswe enentsholongwane kuvavanyo lweWOAH PCR. Ngaphaya koko, kumagcuntswana amabini afunyaniswe ukuba anayo le ntsholongwane: elinye belisuka kuSite 1 kwaye lineesampuli ezimbini ze *C. bolitinos*, elinye igcunstwana belisuka kuSite 7 kwaye lineesampuli ezintandathu ze *C. tuttifrutti*. Olu phando lunikeza ulwazi ngentlobo ntlobo zeembuzane kwakunye neentlobo ntlobo zamakhalane kwindawo uphando ebelubanjwe kuyo. Iziphumo ezixelwe apha zibonise ukuba izinja azikhethwa ziintlobo zeembuzane kuba akukho nanye kwizinja ezivavanyiweyo efunyaniswe inezithintelintsholongwane zeAHSV. Ngokusekelwe kwiziphumo zolu phando, ziintlobo zeembuzane kuphela ezinokuthi zidlale indima yokunwenwa ubusika bonke kweAHSV kwindawo. Nangona kunjalo, iintlobo zamakhalane ezongezelelweyo kunye nezinye izilwanyane ezinethambo lomqolo ezinokubakho kufuneka ziphandwe nazo. Uphando olongezelelweyo luyafuneka ukuphanda indima ye *C. tuttifrutti* nezinye izinambuzane ezifunxa igazi ekusasazeni iAHSV kunye nezinye iintsholongwane ezisuleleka ngenxa yezinambuzane (i-orbiviruses).

Amagama angundoqo: Usuleleko lweAfrican Horse Sickness, amahashe, uhlobo lweAHS, Intsholongwane yokugula kwamahashe (iAfrican Horse Sickness Virus), iAHS, iyantlukwano kwiimbuzane

KAKARETSO

Lefu la Dipere la Afrika (AHS) ke lefu le sithabetsang, le sa tshwaetsaneng la kokwana-hloko ya mefuta e anyesang ya dipere. Leha ho le jwalo, diqwaha ha di be le lefu lena. Pele ho ne ho amohelwa hore ho qhoma ha AHS ho tla qala dikarolong tse ka Leboya la Afrika Borwa mme butle-butle le lebe dikarolong tse borwa ha nako ya selemo e ntse e tswela pele. Sena se ne se bakwa ke tumelo ya hore palo e ngata ya diqwaha tse Kruger National Park, e sebetsa e le mohlodi wa kokwana-hloko. Ho qhoma ho etsahetseng dilemong tse 25 tse fetileng, ho bonahetse hore sena e ne e se nnete, kaha mafu a sewa a ne a tla tswela pele ka dinako tse pedi kapa ho feta tsa selemo karolong e borwa ho naha, ka mokgwa wa ho bokella disele kapa dikokwana-hloko, o tshwanang wa AHS e bakang sewa sa pele e le sona feela se tlalehilweng sebakeng seo dilemong tse latelang. Ka hona, ho ka etsahala hore kokwana-hloko e phele nakong yohle ya mariha sebakeng seo. Leha ho le jwalo, ho phela nakong yohle ya mariha ha kokwana-hloko ya lefu la dipere tsa Afrika (AHSV) ha ho so utlwisiswe ka botlalo. Ho ile ha etswa tlhahiso ya hore AHSV e phela nakong yohle ya mariha ho di-Culicoide tse kgolo, ka maemo a tlase a boteng ba vaerase mading ho baamohedi ba tlhaho, kapa ho dihlopha tse ding tsa diphoofolo tse nang le mokokotlo kapa tse se nang mokokotlo tse kang dintja le diboseleise, ka ho latellana. Sepheo sa phuputso ena e ne e le ho batlisisa hore na AHSV e phela mariha oohle jwang sebakeng sa phuputso, le karolo eo dintja le diboseleise di e bapalang tshebetsong ena le ho fumana hore na mefuta ya di-Culicoide le diboseleise e na le mofuta ofe sebakeng sa phuputso. Phuputso ena e entswe seterekeng sa Sarah Baartman Porofenseng ya Kapa Botjhabela Afrika Borwa. Kakaretso ya dibaka tse 28 e ile ya ngodiswa bakeng sa pokello ya mefuta ya di-Culicoide le diboseleise, le disampole tsa madi a dintja. Sefi sa lebone sa 220 V down draught-UV se ile sa sebediswa ho bokella mehlala ya di-Culicoide, ha diboseleise di ne di bokellwa ka ho toba ho baamohedi ba phelang. Ho ile ha nkuwa disampole tsa madi ho dintja ho sebediswa mothapo wa cephalic. Disampole tsena kaofela di ile tsa romellwa Lekgotleng la Dipatlisiso tsa Temo - Onderstepoort Veterinary Research (ARC-OVR) bakeng sa tlhahlobo. Nakong ya phuputsi ho ile ha etswa dipokello tse 918 tsa di-Culicoide, mme mefuta e 44,850 ya di-Culicoides, e nang le mefuta e 49 ya di-Culicoides e ile ya fumanwa. Kakaretso ya mehlala ya diboseleise tse 1,260 tse nang le mefuta e 10 ho tswa melokong e meraro e ile ya bokellwa le ho tsejwa nakong ya dipatlisiso. Ka nako ya selemo se le seng, ho ile ha bokellwa disampole tsa madi ho dintja tse 100. Ntja ka nngwe e ile ya etswa disampole

ka makgetlo a mane, hanngwe ka nako ya selemo. Disampole tsa madi di ile tsa hlahlojwa ho sebediswa ELISA e sa tobang ya AHS ho etsa tlhahlobo ya boteng ba disele tse sireletsang mmele mafung tsa IgG. Di-Culicoide le disampole tsa dibosoleise di ile tsa lekwa pele bakeng sa boteng ba AHSV ho sebediswa PCR e teng. Disampole tsohle tse fumanweng di na le AHSV ho PCR e teng di ile tsa etswa tlhahlobo ya WOAH (e amohelehang le e tiisitsweng). Ke feela disampole tse fumanweng di na le AHSV ka tlhahlobo ya WOAH di ileng tsa nkuwa di na le AHSV. Le hoja matamo a mahlano a di-Culicoide a ile a fumanwa a e na le PCR ya kahare, ke matamo a mabedi feela a matamo ana a ileng a fumanwa a e na le kokwana-hloko tekong ya WOAH PCR. Ho feta moo, ho matamo a mabedi a ileng a fumanwa a e na le AHSV: le leng le ne le tswa Setsing sa 1 mme le ne le na le mehlala e mmedi ya C. bolitinos le letamo le leng le ne le tswa Setsing sa 7 mme le ne le na le mehlala e tsheletseng ya C. tuttifrutti. Phuputso ena e fana ka temohisiso ka mefuta-futa ya mefuta ya di-Culicoide le diboseleise sebakeng sa phuputso. Diphumano tse tlalehilweng mona di bontshitse hore dintja ha di ratehe bakeng sa mefuta ya di-Culicoid kaha ha ho le e nngwe ya dintja tse fumanweng di na le disele tse sireletsang mmeleng mafung tsa AHSV. Ho ipapisitswe le diphetho ho tswa phuputsong ena, ke mefuta ya di-Culicoide feela e nang le monyetla wa ho bapala karolo ya ho phela mariha oohle wa AHSV sebakeng seo. Leha ho le jwalo, mefuta e meng ya diboseleise le diphoofolo tse ding tse nang le lesapo la mokokotlo le tsona di lokela ho etswa dipatlisiso. Ho hlokahala dipatlisiso tse ding ho batlisisa karolo ya C. tuttifrutti le ditshintsi tse ding tse fepang madi phetisong ya AHSV le dikokwana-hloko tse ding tsa orbivirus.

Mantswe a sehlooho: Phetiso ya Bolwetse ba Dipere tsa Afrika, di-equid, serotype ya AHS, Vaerase ya Bolwetse ba Dipere tsa Afrika, AHS, mefuta e fapaneng ya *di-Culicoide*

CHAPTER 1
GENERAL INTRODUCTION

1.1. BACKGROUND

Horse owners suffer severe losses, due to African horse sickness (AHS) every year. Though AHS is a controlled and notifiable animal disease in South Africa, and a World Organisation for Animal Health (WOAH) listed disease, the number of confirmed cases reported to DALRRD are low (Table 1.1). During the 1998 AHS outbreak in the cooler, mountainous, central region of South Africa an estimated 100 horses succumbed to the disease, yet no cases are reflected on the DALRRD database for this area (Meiswinkel & Paweska, 2003). Based on the number of AHS cases reported to DALRRD, the Eastern Cape Province has the second highest incident rate of AHS in South Africa after Gauteng (Table 1.1). Every year since 1993 (except for 1997), cases of AHS have been reported to DALRRD from Eastern Cape, with two major outbreaks in 2001 and 2008, with more than 900 and 500 cases respectively (DALRRD, 2021).

African horse sickness is an acute or subacute, non-contagious insect borne, viral disease of Equidae that is endemic to Africa. The AHS virus (AHSV) is an Orbivirus in the family Reoviridae (Coetzer & Guthrie, 2004). The mortality rate can be a high as 95% in naïve equine populations (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). Diagnosis of AHS can only be confirmed by identifying the virus in a laboratory. The clinical signs of AHS include fever, swelling of the head and eyes, oedema of the lungs, pleura and subcutaneous tissue, difficulty in breathing and frothy discharge from the nose, although it should be kept in mind that other equine disease will have similar symptoms (Blood & Henderson, 1974; Constable *et al.*, 2017). No treatment has been shown to have any effect on the course of the disease, but careful nursing and symptomatic treatment is not without value (Blood & Radostits, 1989). Horses should however be vaccinated yearly, and preventative measures such as the use of repellents, insecticides and stabling are recommended.

Table 1.1 African horse sickness confirmed cases reported to DALRRD since 1993

year	EC	OFS	GP	KZN	LIM	MP	NW	NC	WC	Total
1993	41	0	0	11	1	1	2	0	0	56
1994	225	0	0	8	0	0	12	0	0	245
1995	2	0	1	1	1	0	0	0	1	6
1996	12	26	36	15	21	18	5	0	18	151
1997	0	6	14	5	10	2	2	1	0	40
1998	21	0	0	28	15	5	15	0	0	84
1999	1	9	54	12	16	33	35	17	35	212
2000	41	2	83	90	43	12	39	41	2	353
2001	959	1	42	0	12	12	81	20	0	1,127
2002	90	0	77	33	16	28	32	9	1	286
2003	120	0	16	349	3	12	5	0	0	505
2004	78	4	112	18	13	28	51	12	20	336
2005	202	1	51	248	0	6	12	0	2	522
2006	23	5	213	82	10	82	32	70	45	562
2007	29	0	10	18	1	1	17	1	6	83
2008	513	5	241	11	4	10	100	6	13	903
2009	8	24	93	85	24	43	17	127	1	422
2010	39	2	77	46	4	26	7	7	0	208
2011	80	31	510	128	5	89	63	41	101	1,048
2012	7	1	54	9	2	27	4	0	0	104
2013	139	14	200	144	46	66	55	4	7	675
2014	32	1	147	50	1	2	31	18	107	389
2015	33	2	97	13	2	3	23	1	6	180
2016	3	2	86	21	1	6	15	1	21	156
2017	14	41	149	36	10	18	29	42	0	339
2018	22	15	21	13	0	1	3	36	0	111
2019	53	10	376	36	26	18	25	2	0	546
2020	7	11	168	17	3	11	16	2	2	237
2021	1	20	103	3	7	6	21	61	41	263
2022	5	26	105	31	1	3	10	12	1	194
Total	2,800	259	3,136	1,561	298	569	759	531	430	10,343

African horse sickness virus (AHSV) was already present in Southern Africa when the first horses were introduced in the 17th century, and in 1719 it was recorded that 1,700 horses succumbed to the disease in the Cape of Good Hope (Coetzer & Guthrie, 2004). The largest ever outbreak in South Africa was during 1854-1855 season, when more than 70,000 animals succumbed to the disease in the Southern parts of the country (Bayley, 1856).

In 1943, Rene Du Toit implicated *Culicoides* species in the transmission of AHS and bluetongue viruses to susceptible hosts (Du Toit, 1944). Until 1998, *C. imicola* Kieffer was considered to be the only vector species in South Africa. During an AHS outbreak in the high lying eastern Free State Province (Meiswinkel & Paweska, 2003), it was found that *C. bolitinos* Meiswinkel was also involved in the transmission of the disease. However, in the laboratory an additional 11 *Culicoides* species can became infected with virus, after being fed on a high titre of artificially infected blood, thus more species may be involved in the transmission of AHSV and other related viruses (Paweska *et al.,* 2002; 2003; Venter *et al.,* 1998; 2004, 2006; Venter & Paweska, 1999).

It is suspected that ticks may also play a role in the transmission of AHSV in Africa. The tick species, *Hyalomma dromedarrii* Koch has been shown to become infected with AHSV and transmit the virus between horses in Egypt (Awad *et al.,* 1981; Blood & Radostits, 1989); Salama *et al.,* 1981. As ticks can live for many years, it is possible that this is how the virus overwinters in an area, although the exact role played by ticks in the transmission of AHSV is still unclear (Thompson *et al.,* 2012; Wilson *et al.,* 2009). In view of this, this study investigated if ticks get infected with AHSV or not by analysing ticks from various farms in the Makana Municipality.

In 1906, Theiler showed that dogs were susceptible to AHSV, and since then several cases had been documented where dogs died, after eating infected horse meat (Braverman & Chizov-Ginzburg, 1996; Van Rensburg *et al.,* 1981; Van Sittert *et al.,* 2013). O'Dell (2017) reported that in 2006, the Section of Pathology, Faculty of Veterinary Science, University Pretoria, confirmed the first case of AHS in a dog with no history of consumption of AHS infected meat. Subsequently Van Sittert *et al.,* (2013) also reported that dogs had died at the Malelane Research Unit in 2012. The 57 dogs at the Malelane Research Unit were all fed only commercial, dry pet food. Further antibody tests of the remaining animals showed that 43% (out of 57 animals) had antibodies against AHS (Van Sittert *et al.,* 2013). It has been reported that there are increasing incidents of canine AHS mortality in endemic areas (Hanekom *et al.,* 2023; O'Dell, 2017; Van Sittert *et al.,* 2013). A study by Hanekom *et al.* (2023) in Gauteng showed a seroconversion rate of 6% in the 366 dog sera tested. In Egypt 21% of 187 dogs tested during a study, were seropositive for bluetongue virus, a related Orbivirus in the family Reoviridae (Oura & Harrak, 2011).

The rationale for this investigation, was that although clinically infected Equidae are the major source of the AHSV during an outbreak, there may be other vectors or cycling hosts besides *Culicoides* species involved. If other silent, non-equine cycling hosts are involved in transmission of the virus in enzootic areas, this may, prolong virus circulation, between seasons when no insects are present. It is possible that dogs may act as silent amplifying hosts of the virus (Blood & Henderson, 1974).

1.2. PROBLEM STATEMENT

African horse sickness (AHS) is a devastating viral disease of equines, with outbreaks reported every year in South Africa. Within naïve equine populations the mortality rate can be 95%. The incubation period of the disease is about 5 - 7 days with clinical signs characterised by pyrexia, oedema of the lungs, pleura and subcutaneous tissue (Blood & Henderson, 1974; Constable *et al.,* 2017). During outbreaks of AHS, the affected areas are placed under quarantine, with no equine movement into or out of that area. Additionally, an AHS outbreak within the AHS zones of the Western Cape Provines will lead to the suspension of direct horse exports from South Africa to the European Union and other countries. In many rural areas horses are used on farms to patrol large areas and herding of livestock. The loss of these animals severely affects the income of workers that are reliant on these animals for transport and work (Grewar *et al.,* 2013, Grewar 2016). Traditionally it was believed that cases would start in the frost-free areas of the northern and eastern parts of South Africa, and gradually move southwards as the season progressed. From outbreaks recorded during the past 25 years, it emerged that this is not true as many outbreaks persist for two or more seasons in the south of the country, with the same AHSV serotype causing the outbreak being the only one recorded in that area in subsequent years. This means that the virus is able to overwinter in these areas. However, the overwintering mechanism of the virus is not fully understood. It has been suggested that the virus overwinters in adult *Culicoides* populations, at low levels of viremia in the natural hosts, or in other invertebrate hosts (Losson *et al.,* 2007; Mellor *et al.,* 2000; Napp *et al.,* 2011; Venter *et al.,* 2014). In view of this, it is important to investigate if the AHSV overwinters in adult *Culicoides* populations, or in other vertebrate and invertebrate hosts like dogs and ticks respectively.

1.3. RESEARCH QUESTIONS

Cases of AHS are reported every year in the Eastern Cape Province with the outbreaks occurring regularly, and often continuing from one summer season to the next. Based on this, it has been suggested that the virus is able to overwinter in the area. However, the mechanism by which the AHSV is able to overwinter has not been fully investigated.

This study seeks to establish, where and how the virus overwinters by answering the following research questions:

1. Does the AHSV overwinter in adult *Culicoides* populations in the Eastern Cape Province of South Africa?
2. What is the diversity and abundance of *Culicoides* spp. in the study area?
3. Which tick species are found on various hosts in the study area?
4. Are ticks in the study area infected with AHSV?
5. Do dogs in the study area have antibodies against AHSV?

1.4. STUDY AIM AND OBJECTIVES

The aim of this study was to establish how the African horse sickness virus overwinters in the Eastern Cape Province of South Africa, and the role dogs and ticks play in this process.

The aim of this study was realised by achieving the following objectives:

- To determine whether African horse sickness virus overwinters in adult *Culicoides* populations in the Eastern Cape Province of South Africa.
- To determine the diversity of *Culicoides* species in the study area.
- To determine the diversity of tick species from various hosts in the study area.
- To determine whether ticks are infected with AHSV in the study area.
- To investigate the presence of AHSV antibodies in dogs.

1.5. ANTICIPATED BENEFITS OF THE STUDY

Due to climate change, there has been changes in the pattern of occurrence of vector-borne diseases, and this could explain the persistence of AHSV in certain parts of the country throughout the year. Findings of this study can be used by stakeholders in the horse industry and agriculture, to design disease control strategies in the study area. This

study explored the role vertebrate and invertebrate anaimals play in the overwintering mechanism of AHSV in the study area. This study highlighted the diversity of different *Culicoides* spp. in the study area. From the findings of this study, the question of AHSV overwintering in adult *Culicoides* populations in the study area is addressed. The outcome of this study highlights the diversity of tick species in the study area, as well as whether tick species in this area were infected with AHSV. Dogs as potential cycling or overwintering hosts of AHSV in the study area is elucidated. Data generated from this study is freely available to all interested parties, including horse owners, government officials and researchers.

1.6. ETHICAL CONSIDERATIONS

The details of the study were explained to horse owners and farmers prior to the commencement of the research. Consent forms were signed by the participating individuals indicating, that they agreed to voluntary participate in the study (Annexure: 1). Section 20 Permission under The Animal Diseases Act, 1984 (Act No 35 Of 1984) to perform research / study was granted by Department of Agriculture, Land Reform and Rural Development (DALRRD) (Annexure: 2).

Ethical clearance was obtained from UNISA's ethics committee for this study, before the collection of the blood samples commenced (Annexure 3). Doctor G Mutero, a qualified veterinarian supervised the collection of blood samples from dogs. He had more than 20 years of experience and was employed as a state veterinarian at Grahamstown State Veterinary Office. Qualified, and South African Veterinary Council (SAVC) registered, veterinarians and animal health technicians collected the blood samples from the dogs. Dogs were handled properly, and a muzzle was used to restrain dogs and to prevent them from biting anyone collecting blood and ticks. Dogs were identified by their names. Participating horse owners and farmers were allowed to voluntarily withdraw from the study at any time.

CHAPTER 2
LITERATURE REVIEW

2.1. WHAT IS AFRICAN HORSE SICKNESS

African horse sickness is an acute or subacute, insect borne, viral disease of Equidae and is endemic to sub-Saharan Africa, though, epidemics have been reported outside of the continent (Mellor & Hamblin, 2004). The disease is characterised by lesions associated with respiratory and blood circulation impairment (Kahn, 2005). There are 9 serotypes of the virus, and while some serotypes are cross-protective, i.e. 1 and 2, 3 and 7, 5 and 8, 6 and 9, serotype 4 is not cross-protected by any of the other serotypes (Mellor & Hamblin, 2004). The mortality rate can be as high as 95% in naïve horses (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Weyer *et al.,* 2013).

2.2. ETIOLOGY OF AFRICAN HORSE SICKNESS

African horse sickness virus is a double-stranded RNA Orbivirus in the family Reoviridae (Carpenter *et al.,* 2017; Kahn, 2005). The genome of this virus is composed of 10 double stranded RNA segments which encodes seven structural proteins (VP1-7) and four non-structural proteins (Constable *et al.,* 2017). The AHSV is similar in morphology and share properties with other Orbiviruses, such as equine encephalosis virus (EEV), bluetongue virus (BTV) and epizootic haemorrhagic disease virus (EHDV), and these three (n=3) viruses are associated with erythrocytes (red blood cells) and affect the ability of RBCs to absorb and transport oxygen to the tissues (Coetzer & Guthrie, 2004; Constable *et al.,* 2017).

2.3. CLINICAL SIGNS OF AFRICAN HORSE SICKNESS

African horse sickness is characterised by pyrexia, oedema of the lungs, pleura and subcutaneous tissue. The incubation period of the disease is about 5-7 days (Blood & Henderson, 1974; Constable *et al.,* 2017). During controlled studies, when infection is artificially produced, the incubation period can vary from 2 to 21 days (Blood & Henderson, 1974). The viremic period corresponds with the start of fever and can continue for, between 2 and 14 days (Weyer, 2016).

There are 4 clinical forms of AHS: an acute or pulmonary, cardiac or subacute, mixed and temperature (horse sickness fever) form (Carpenter *et al.*, 2017; Mellor & Hamblin, 20024; Weyer, 2016).

2.3.1 Pulmonary form

In South Africa this form is also known as 'dunkop' which means thin head (Weyer, 2016). This is the highly acute form, and disease progression is so rapid that an animal can die without prior indication of illness. The mortality rates commonly often exceed 95% in this form, and it is responsible for most deaths (Bruner & Gillespie, 1973; Mellor & Hamblin, 2004). This is the most frequent form observed during acute outbreaks (Blood & Henderson, 1974). The incubation period of this form is 3-5 days (Kahn, 2005). Clinical signs include fever, pyrexia, coughing, nasal discharge, congestion, dyspnoea, and death caused by severe pulmonary oedema (Blood & Henderson, 1974; Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Weyer, 2016). When sweating commences, the horse becomes very weak, and develops a staggering gate, with lastly recumbence (Blood & Henderson, 1974). The start of dyspnoea is usually very sudden, and death usually occurs within few hours of its appearance (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). The pulmonary form is usually the one that affects dogs (Coetzer & Guthrie, 2004).

2.3.2 Cardiac form

This form is also called 'Dikkop' which means thick head, this is because of the subcutaneous swelling of the head (Mellor & Hamblin, 2004; Weyer, 2016). This form is subacute with an incubation period of 1-2 weeks. Fever is usually observed for 5-7 days followed by the swelling of the supraorbital fossa which is very characteristic for this form (Kahn, 2005). Subcutaneous swelling of the neck and head, that may extend to the thorax, brisket and shoulders are common (Coetzer & Guthrie, 2004; Kahn, 2005; Mellor & Hamblin, 2004). Mortality rate are approximately 50% (Mellor & Hamblin, 2004; Weyer, 2016). Death usually occurs within one week after appearance of clinical signs.

2.3.3 Mixed form

Mixed form is the combination of cardiac and pulmonary form, which involves both the lungs and subcutaneous oedema, and supraorbital fosse swelling are observed (Mellor & Hamblin, 2004; Weyer, 2016). It is only on post-mortem that lesions of both pulmonary and cardiac forms are seen and thus diagnosis can be made (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). Horses affected by this form may show signs of respiratory

distress, followed by subcutaneous swelling. Mortality rate of this form is about 70%. Death usually occurs three to six days after the appearance of the febrile reaction (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004).

2.3.4 Horse sickness fever

There are minimal clinical signs, usually only mild fever is observed and thus this form may go unnoticed or unrecognised, and it usually occur in areas where the disease is endemic (Blood & Henderson, 1974). It is a common form of AHS in donkeys and zebra which are resistant to the development of clinical disease (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). The most common clinical characteristic is an increase in body temperature of about 39˚C to 40˚C or more, which lasts about 1-6 days then returns to normal (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004).

2.4. SPECIES SUSCEPTIBLE TO AFRICAN HORSE SICKNESS

African horse sickness primarily affects all Equidae (horses, donkeys, mules, zebra), though elephants, camels, sheep, goats, and scavenging carnivores may also be affected (Coetzer & Guthrie, 2004; Constable *et al.,* 2017). Zebra are very resistant to AHSV, and only show a mild fever during an experimental infection (Coetzer & Guthrie, 2004). Due to endemic immunity, zebra, as natural cycling or amplifying hosts for AHSV, have been known to be infected with AHS virus for up to 60 days without showing any symptoms or dying from the disease (Barnard, 1993). In the Kruger National Park, zebra foals lose their passive immunity against AHSV when they are 5-6 months old, but then within a few short months become seropositive against all AHSV serotypes (Barnard, 1993).

Mules are considered less susceptible and deaths in donkeys are rarely recorded in sub-Saharan Africa (Meiswinkel & Paweska, 2003). In the Middle East, donkeys appear to be more susceptible to the disease with mortality rates that can reach 10% (Coetzer & Guthrie, 2004). Theiler was unable to produce fatal AHS disease in donkeys, after injecting them with large quantities of virulent blood and only a mild fever reaction was produced (Coetzer & Guthrie, 2004). This indicates that donkeys are very resistant to the disease.

Although AHS rarely occur in dogs, it can affect dogs with no history of ingesting infected horse meat (Constable *et al.,* 2017). Dogs are the only other species that acquire the highly severe form of AHS (Coetzer & Guthrie, 2004; Hanekom *et al.*, 2023; O'Dell,

2017; Van Sittert *et al.,* 2013). There is a little evidence that AHSV antibodies can be detected in domestic ruminants apart from camels. Pigs, cats and monkeys are resistant to infection with AHSV (Coetzer & Guthrie, 2004).

2.5. DISTRIBUTION OF AFRICAN HORSE SICKNESS

African horse sickness is endemic to sub-Saharan Africa and possibly Yemen (Meiswinkel *et al.,* 2004). This disease was firstly recognised in South Africa after the introduction of domesticated horses in the 1700's. Regular outbreaks are reported in Southern Africa, Ethiopia, Ghana, Nigeria and Senegal. The disease makes occasional incursions in Pakistan, Iran, India, Turkey and eastern Mediterranean, Cyprus, the Middle East and Saudi Arabia (Coetzer & Guthrie, 2004). In 1987 the disease spread throughout Spain and Portugal, after importation of sub-clinically infected zebra, but it was eliminated in 1991. The outbreak continued for four years and by the end more than 430 equines had died of AHS (Ortega, 1995), although some authors estimate the number closer to 1,000 equines dead or slaughtered (Lubroth, 1987).

During 1959–1961 AHS expanded eastward from Iran to Afghanistan, Pakistan and India, and westward to Iraq, Syria, Lebanon, Turkey, Cyprus and Jordan. During this outbreak an estimated 300,000 equines died, most of which were associated with subsistence workers (Carpenter *et al.,* 2017).

In March 2020, there were sick horses in the Nakhon Ratchasima province of Thailand, blood samples were taken from 4 horses and submitted to Pirbright Institute, United Kingdom by a private veterinarian. On all tests performed, all the samples tested positive for AHSV, confirming the clinical diagnosis of AHS and was serotyped as serotype 1 (King *et al.,* 2020). The route of entry into Thailand by AHSV-1 is still being investigated. The low number of AHSV sequences from countries, other than South Africa, compounds the issue of identifying the original source of the outbreak, but the close relationship with the African strain of the virus, would indicate a recent introduction (King *et al.,* 2020).

The primary African and European vector of AHSV, *C. imicola* occurs in Thailand and surrounding countries (Wirth & Hubert, 1989). The wide geographical distribution of *Culicoides* species and particularly *C. imicola* in this region and the potential year-round seasonal activity as adults, pose and increases the risk of AHSV spreading to neighbouring countries (King *et al.,* 2020).

In a study, of an outbreak in Hua Hin District, Prachuap Khiri Khan Province, Thailand, 12 *Culicoides* species were recorded, with *C. oxystoma* Kieffer dominant with 71.9%, followed by *C. imicola* at 20.4% (Kamyingkird *et al.*, 2023). It is not known if *C. oxystoma* is a vector of AHSV, but previously three other viruses namely Akabane, Buyip Creek and Kasba had been isolated from this species (Bellis 2013, Labuschagne 2016, Meiswinkel *et al.*, 2004).

Control measures were implemented, in Thailand, in a bid to prevent further outbreaks of AHSV, which included vaccination of all susceptible animals with live attenuated vaccine, stabling of animals, treatment of stables and horses with insecticides and repellents (Lu *et al.*, 2020). In non-endemic areas these control measures should be implemented on any farm of stable yard to prevent the spread for AHS. Widespread surveillance and immediate reporting are very important in ensuring that AHS, does not spread into other provinces or neighbouring countries, as these outbreaks have an impact on the OIE disease free status of a country (King *et al.*, 2020). In endemic countries these control measures should be standard operating procedures on any farm, with immediate reporting of suspected cases of AHS, and this also applies to any other notifyable disease, to the State Veterinarian of that area.

Outbreaks of AHS occur every year in South Africa, but major epidemics before 1953, occurred at intervals of roughly 20-30 years (Coetzer & Guthrie, 2004). Severe losses were reported in 1780, 1801, 1839, 1855, 1862, 1891, 1914, 1918, 1923, 1940, 1946 and 1953 (Coetzer & Guthrie, 2004).

The outbreak in 1854-1855 is still considered to be the most severe in South Africa, with about 70,000 horses dying, which was an estimated 40% of the horse population in the Cape of Good Hope (Coetzer & Guthrie, 2004; Paweska, 2003). Another major outbreak was recorded in 1891 - 1892, in the same region and about 25,000 horses died (Paweska, 2003).

Cases of AHS were regularly recorded in the eastern district of the Cape Colony during late summer and autumn. The massive AHS outbreak in 1854-1855, created awareness with the general public and the government instituted an inquiry into the origin, treatment and all the factors that could be implemented to mitigate the disease (Bayley, 1856).

There are some records of AHS outbreaks in the former Transkei homeland. In the 1891 - 1892 an AHS outbreak in the former Transkei region was detected as far north as the

Umzimkhulu district, where it started very early in November and spread to Port St. Johns, Umtata, Centani, and Komga, although Butterworth and Idutywa were not infected (Paweska, 2003; Paweska & Meiswinkel, 2001). Outbreaks in Port St. Johns in, 1921, 1922 and 1924, was of great concern for the disease was reported over three consecutive seasons. Insufficient knowledge on strategies that can be implemented to control the disease and of the disease itself could have led to the long duration of this particular outbreak. The estimated death rate went up to about 75% in Port St. Johns (Paweska, 2003).

During the outbreak of 1923 in South Africa, 1,700 horses, 114 mules and 100 donkeys were recorded as having died from AHS, from December 1922 up to the end of May 2023. Due to the severity of the outbreak, it may be possible that donkeys died, though they are normally very resistant to infection with AHSV. The heavy rains in January 1923 can be correlated with this AHS outbreak (Du Toit, 1924).

In 1934 about 800 horses died of AHS in the Western Cape and in 1940 a further 1,000 horses died in the same area. These were the last outbreaks where large numbers of horses died in South Africa, after the introduction of the vaccine by Onderstepoort in the 1930's (Paweska, 2003; Paweska & Meiswinkel, 2001).

During the 1996 summer season in South Africa an estimate of 500 horses, died of AHS and 80% of death were due to AHS serotype 2 and 4. Almost all these cases occurred in the northern, north-eastern and central parts of South Africa (Meiswinkel, 1998). During this time, only 151 confirmed cases were reported to DALLRD (Table 1.1).

In an outbreak of AHS in the eastern Free State of South Africa between February and May 1998, approximately 100 horses died in relatively isolated population of 330 horses, yet no confirmed cases were reported to DALRRD (Table 1.1). Only 80 out of 330 horses were vaccinated annually with polyvalent attenuated Onderstepoort Biological Products (OBP) vaccine. This area is mountainous, with severe winters including snow and is regarded as AHS-free area. During a study in the middle of this outbreak, farmers or horse owners were interviewed to get the better understanding of the outbreak. The first fatality among horses occurred between the last week of January and the first week of February 1998. The last death was reported on 24 May 1998 after the first severe frosts of the season (Meiswinkel & Paweska, 2003). Over a 13-day period *Culicoides* spp. were collected on 14 of 16 farms affected. A total of 189 pools, comprising of 19,632

individuals of 19 *Culicoides* species was tested for the presence of virus. Out of these, 149 pools were *C. bolitinos* (16,500 parous females). From the pools of *C. bolitinos*, five isolations of AHSV-6, two isolations BTV (serotype unknown) and three isolations of unidentified viruses were made. Two pools of *C. imicola* representing 188 individuals and 38 pools of other species of *Culicoides* species representing 2,944 individuals did not yield any virus (Meiswinkel & Paweska, 2003). The numbers of *C. imicola* recorded during the outbreak was similar as what was collected from the same area in 1991 and 1993 (Venter & Meiswinkel, 1994). The possible origin of the infection, of this particular outbreak, might have been seventy ponies and two stallions that were imported from the Basotho kingdom (Meiswinkel & Paweska, 2003).

In this area, and in other areas where cattle are predominantly being farmed, *C. bolitinos* is often the dominant species collected. *Culicoides bolitinos* achieves dominance in the cooler wetter central uplands and in sandy coastal areas of South Africa. This shows that the AHSV circulates all over the country (Meiswinkel & Paweska, 2003). With *C. bolitinos* as a vector of AHSV there are important epidemiological implications, as the immatures stages of *C. bolitinos* inhabit cattle, buffalo and wildebeest dung (Meiswinkel, 1989; Nevill, 1968), the presence of these animals increases the risk of infection for non-immune equids. Disease risk is increased further if *C. imicola* is present as this species can become super abundant in ideal conditions, with more than 1 million specimens of *C. imicola* recorded in a single one-night collection during the 1996 outbreak (Meiswinkel, 1998; Meiswinkel & Paweska, 2003).

2.6. AFRICAN HORSE SICKNESS CONTROL ZONE

In general, AHS outbreaks start in December/January and peaks during early autumn (March/April) and disappear abruptly after the first severe frosts or snow in early May. Generally, it is believed that only the frost-free northern and eastern lowveld of South Africa is endemic for AHS and that the disease starts there each year and spread southwards (Barnard, 1993; Bosman *et al.,* 1995; Venter *et al.,* 2014). It is possible that AHSV is maintained by the large populations of zebra (*Equus burchelli*) in the Kruger National Park (KNP), where foals are born throughout the year. In the Kruger National Park zebra foals lose their passive immunity against AHSV when they are 5-6 months old but then within a few short months become seropositive against all AHSV serotypes (Barnard, 1993).

Cases of AHS in racehorses and other competition horses are very rare, and although these animals are frequently transported between various racing and competition events, and breeding centres, they are rigorously monitored daily and treated as soon as any problem or disease are picked up. Therefore, their movement play a limited role in the transmission of AHSV (Bosman *et al.,* 1995).

Though AHS vaccination is not compulsory by law in South Africa, the majority of horses in KwaZulu-Natal and the northern provinces of South Africa are vaccinated yearly due to the high prevalence of the disease in these provinces. Guidelines were set by the National Department of Agriculture Land Reform and Rural Development (DALRRD) for protecting equines from AHSV in the AHS infected zone (DALRRD, 2012). Annexure: 4 describes the vaccination protocol that should be followed. The Animal Diseases Act 1984 (Act no 35 of 84) regarding vaccination against AHS does state: "All equines in the Republic except equines in the African Horse sickness free zone and the African Horse sickness surveillance zone as described in Table 1, shall between the ages of 6 and 12 months, then between the ages of 12 and 18 months and then again once every year thereafter be immunized with an effective remedy by the responsible person."

The epidemiology of AHS in South Africa makes it impossible to obtain an AHS disease-free status for the whole country. To facilitate the export of horses from South Africa an AHS controlled area, was establish in the most south-western region of the country (Figure 1.1). This zone is divided into an AHS free zone surrounded by a surveillance zone and a protection zone with the rest of South Africa considered endemic. Movement of equines to the AHS controlled area are subjected to strict movement control and require certification and permits (DALRRD, 2015; Grewar, 2018).

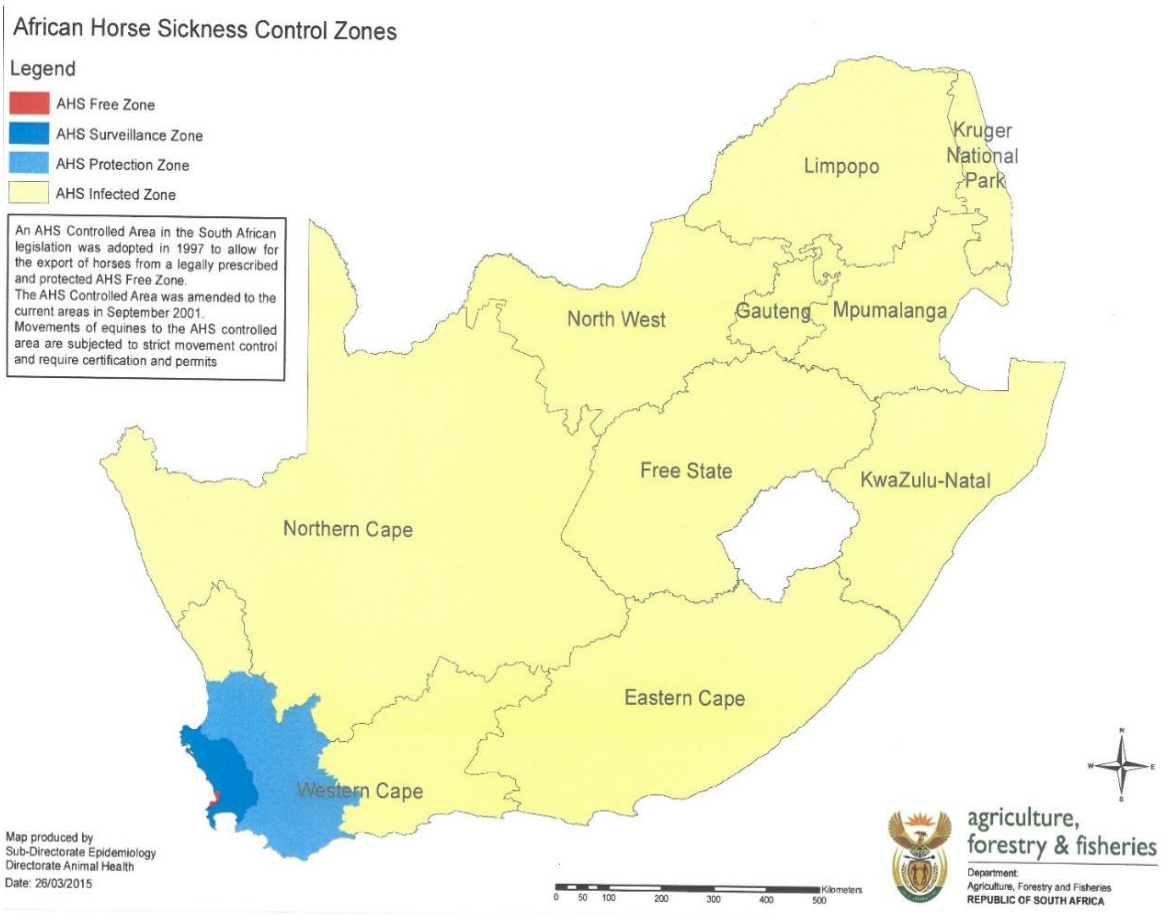


Figure 1.1: Map showing African horse sickness control zones (DALRRD, 2015a)

Since the establishment of the AHS zones in the Western Cape, various outbreaks have occurred within the AHS surveillance zone (Grewar *et al.*, 2013, Grewar *et al.*, 2019; Sinclair *et al.*, 2006; Venter *et al.*, 2006). During these outbreaks, field collections have indicated that *C. imicola* was the most abundant species (Venter *et al.*, 2006). Each outbreak has led to the suspension of direct horse exports from South Africa to the European Union and other countries (Grewar *et al.*, 2013).

Annually cases of AHS are reported from the Eastern Cape with more than 100 cases reported to DALRRD in 1994, 2001, 2003, 2005, 2008 and 2013 (DALRRD, 2021; Riddin *et al.*, 2019). The latest outbreak of AHS recorded in South Africa in 2023, started in February 2023 and spread across South Africa like a veld fire. More than 300 horses have since died of the disease. Horses died all over the country with the Eastern Cape recording the highest number of incidents. In the Eastern Cape 189 fatalities were recorded (Grocott's Mail, 2023).

Globally there are concerns that vector-borne diseases are spreading beyond the endemic areas. Climate change and increasing global trade may be driving this. The BT outbreaks that started in 2000 in the Mediterranean basin is a good example of this. Since the BT outbreaks started, it spread across Europe with most countries recording the disease and more concerningly is that multiple serotypes were recorded (Elbers *et al.,* 2008; Enserink, 2006; Gómez-Tejedor, 2004; Mehlhorn *et al.,* 2007; Mellor *et al.,* 2008; Purse *et al.,* 2008a; Rodríguez-Sánchez *et al.,* 2008; Saegerman *et al.,* 2008; Wilson & Mellor, 2008). Because BTV and AHSV are both orbiviruses and both are spread by *Culicoides* midges the concern is that if AHSV is introduced to an area where BT is already endemic the local vector species of *Culicoides* may spread the virus through that area (Faverjon *et al.,* 2015; Nelson *et al.,* 2022; Robin *et al.,* 2016; Thompson *et al.,* 2012).

2.7. TRANSMISSION OF AFRICAN HORSE SICKNESS

Laboratory and field trials have implicated *Culicoides* biting midges (Diptera: *Ceratopogonidae*) as the primary vectors of AHSV (Carpenter *et al.,* 2017). The virus is spread primarily by *C. imicola* and to a lesser extent by *C. bolitinos* in South Africa (Van Sittert *et al.,* 2013). Vector competence studies with both species indicate that *C. bolitinos* is a better vector for arboviral disease transmission than *C. imicola* (Paweska *et al.,* 2002; Venter *et al.,* 2000, 2006). Due to the different breeding media that *C. bolitions* and *C. imicola* utilises, the numbers of each species, breeding out differs significantly. *Culicoides bolitinos* breeds in cattle, buffalo and wildebeest dung while *C. imicola* prefers to breed in moist organically enriched soils. This means that *C. imicola* can become superabundant under ideal situations and huge numbers can be caught in light traps at night (Meiswinkel, 1998; Meiswinkel *et al.,* 2004). In a survey that was conducted in Germany during the outbreak of BT in 2007 to 2008, it was found, that *C. imicola,* the main vector in the Mediterranean and Africa, was absent and that the *Culicoides* species that were abundant in that area were the vectors of BTV (Mehlhorn *et al.,* 2009).

For transmission to take place, adult midges first have become infected after ingestion of a blood meal from an infected mammalian host, then after an intrinsic incubation period of 4-20 days, depending on the temperature, a small percentage to the midges can transmit the virus to a new host when they take a subsequent blood-meal (Purse *et al.,* 2006; Saegerman et al. 2008; Takamatsu *et al.,* 2004).

Midges acquire the virus from infected horses, donkeys, mules and zebra by taking blood meals from viremic animals. Virogenesis in the midge take about eight days after which AHSV localises in the salivary glands. It is then transmitted to the next host when the midge takes its next blood meal. Both infection rate and rate of virogenesis of AHSV within the midges are temperature dependent which means the lower the temperature, the lower the infection rate and the longer the period virogenesis (Coetzer & Guthrie, 2004; Constable *et al.,* 2017). Horses remain viraemic for 18-21days, donkeys for 12 days and zebra may remain viremic for up to 6 weeks. Therefore, zebras remain an important source of infection to horses, as they do not get the clinical disease but are able to cycle the virus for up to 60 days (Coetzer & Guthrie, 2004; Constable *et al.,* 2017).

A vector-free or disease-free period is defined, as the period of time that adult vectors are absent, provided that the period is longer than the maximum duration of viremia in the mammalian host. If this happens the newly emerged insects do not become infected, and thus the cycle of virus transmission is broken, and therefore no virus will be available to be transmitted (Takamatsu *et al.,* 2004).

The probability that elephants act as cycling hosts of AHSV are very low. For example, 100 elephants were tested in an endemic area in South Africa, and although they had high complement fixing titres for both AHS and BT viruses, neutralising antibodies to these viruses were not detected (Barnard, 1997; Barnard *et al.,* 2006; Coetzer & Guthrie, 2004)

During a study conducted to assess if ticks played a role in the transmission of bluetongue virus, *Ixodid* and *Argasid* ticks were tested as potential vectors. Bluetongue virus serotype 8 was taken up by four different species of *Ixodid* ticks and the virus were able to pass through the gut barrier and spread via the haemolymph into the saliva glands, ovaries and testes. Therefore, the presence of the virus in the saliva is very indicative, as it shows that the virus could be transmitted via the saliva when ticks feed on the next host (Bouwknegt *et al.,* 2010). Studies have shown that ticks (*Hyalomma dromadarii* Koch and the brown dog tick, *Rhipicephalus sanguineus* Latreille) and mosquitoes can become infected when artificially infected or fed on an infected host and subsequently allowed to feed on a susceptible host. Thus, ticks and mosquitoes can play a role in the transmission of AHS, though *Culicoides* species remain the major vector species involved (Constable *et al.,* 2017; Zientara *et al.,* 2015).

In a study by Salama *et al.* (1981) AfHSV serotype 9 was isolated from 17% of 2,089 field specimens of *Hyalomma dromadarii* that were collected in Egypt. Unfortunately, the authors did not indicate, if the viruses isolated was from freshly fed ticks, or from the gut or from tissues of the ticks tested. These same authors reported the presence of AHSV in newly emerged adult ticks and they succeeded in transmitting the virus via tick bites from infected animals to susceptible camels and horses (Mellor, 1993). Mellor (1993) reported that in a further study in Egypt conducted by Salama *et al.* in 1981 and reported in (Dardiri & Salama, 1988) showed that the brown dog tick (*Rhipicephalus sanguineus*) was able to transmit AHSV experimentally from infected dogs and horses to healthy dogs and horses (Mellor, 1993). In another study by Awad *et al.* (1981), the authors also succeeded in transmitting AHSV-serotype 9 from infected animal to a susceptible host via tick bites of adult *Hyalomma dromadarii* (Mellor, 1993)

The question remains, is: how the virus arrives or introduced into an area, where AHS has been absent for a long time. For an outbreak to occur, there needs to be; susceptible hosts, an infected host moving into the area, infected vectors transported into the area, via the wind or even a horsebox moving through, and competent vectors present in the area. There is also a possibility of mechanical transmission of the virus on contaminated syringes, surgical instruments, or mechanical vectors (Constable *et al.*, 2017).

Bloodmeal studies worldwide have revealed that *Culicoides* species feed on a wide variety of hosts. These hosts include horses, cattle, dogs, pigs, various wildlife species, birds and even humans (Braverman *et al.*, 1971, 1976; England *et al.*, 2020; Kamyingkird *et al.*, 2023; Kar *et al.*, 2022; Nevill & Anderson 1972; Slama *et al.*, 2015; Snyman *et al.*, 2021; Tomazatos *et al.*, 2020).

2.8. CONDITIONS THAT FAVOUR OUTBREAKS OF AFRICAN HORSE SICKNESS

African horse sickness occurs mainly during late summer and autumn in South Africa. During the 1922-1923 outbreak, prolonged and heavy rains created favourable conditions for propagation of the vectors of AHS (Du Toit, 1924). Low-lying, inland valleys, marshes and riverine areas create suitable breeding sites for *C. imicola* that is conducive for AHS outbreaks, if the virus is present in the area (Coetzer & Guthrie, 2004). The occurrence of outbreaks of AHS in South Africa have been associated with the

combination of drought and heavy rainfall caused by the warm phase of ENSO (Van Den Bossche & Coetzer, 2008).

2.9. ECONOMIC IMPORTANCE OF AFRICAN HORSE SICKNESS

Until the late 1950's South Africa exported some 350,000 horses to other parts of the world, largely in support of the first and second world wars. The outbreak of AHS in the Middle East in 1959 raised global fears of AHS, and an embargo on the movement of horses out of Africa was enforced for the next four decades. The exception was the USA, which was still importing horses from Africa, on condition, that upon arrival a 60-day post arrival vector proof quarantine was implemented (Coetzer & Guthrie, 2004).

The European union introduced the South African horse export protocol in 1997 and since then South Africa exported close to 1,000 horses from the Kenilworth quarantine station, in the AHS free zone in Cape Town, worth an estimated R250 million per annum (Coetzer & Guthrie, 2004, Grewar 2018; Grewar *et al.*, 2013, 2019).

In the Eastern Cape Province, horses are still a vital mode of transportation, of adults to cultural events. Communities have introduced horse racing and other events, to attract tourists and to provide the youth with activities and sources of income, thus ultimately reducing drug abuse and trafficking. Horse racing in the poor communities is a rapidly developing sport. Horses are also used by shepherds, looking after livestock and are used by community police forums in the mountainous area for stock theft prevention (Rhodes University, 2013).

The provincial department of agriculture and other local municipalities support traditional horse racing, by supporting and sponsoring some of these events. For example, the Amatole district municipality selects jockeys to send to a professional jockey academy for training (Rhodes University, 2013).

Outbreaks of AHS affect food security and poverty alleviation as these animals are not only used as means of transport, but they are also used as draught animals, for racing and for enjoyment by both young and old (Paweska & Meiswinkel, 2001). Therefore, AHS is a major threat to equine sport and companion animal industries which involves, the production of high-performance animals. Export of horses is a major economic boost (Carpenter *et al.*, 2017).

2.10. VECTORS OF AFRICAN HORSE SICKNESS

2.10.1. Culicoides spp and other insects

Culicoides spp. (biting midges) are regarded as the most important vectors, in the transmission of AHSV. Although other insects have been incriminated as possible vectors of AHSV, none of them have been conclusively shown to play a role under the natural conditions (Coetzer & Guthrie, 2004).

2.10.2. Mosquitoes

As early as 1903, Pitchford-Watkins and Theiler suggested that *Anopheles* mosquitoes could be involved in the transmission of AHSV. Researchers have shown experimentally that *Anopheles stephensi* Liston, *Culex pipiens* Linnaeus and *Aedes aegypti* Linnaeus could transmit the virus but failed to infect *A. aegypti* and *Culex quinquefasciatus* Say (new name for *Culex pipiens fatigans* Wiedemann) by feeding them on virus suspensions or infected horse (Coetzer & Guthrie, 2004; Mellor, 1993).

In 1934, it was shown that mosquitoes of the genus *Aedes* could acquire and replicate AHSV for a period of approximately week but could not transmit the virus to susceptible host (Coetzer & Guthrie, 2004). Initial studies on AHSV, at Onderstepoort Veterinary Institute failed to elicit any evidence of AHSV transmission by mosquitoes between viraemic and naïve horses (Carpenter *et al.,* 2017).

2.10.3. Biting flies

Biting flies may play a minor role in the mechanical transmission of AHS, but as the virus is susceptible to desiccation and high temperature, and viremia in horses is relatively low, this method of transmission is likely to be inefficient (Coetzer & Guthrie, 2004).

2.10.4. Ticks

Studies in Egypt, isolated AHSV from *Hyalomma dromedarii*. Therefore it is possible that the virus may be transmitted between horses by this tick species (Awad *et al.,* 1981; Carpenter *et al.,* 2017; Salama *et al.,* 1981; Wilson *et al.,* 2009).

2.11. ROLE OF INSECTS IN AHS

2.11.1 Discovery of Culicoides species as vectors of AHSV

During the 1854-1855 AHS outbreak one farmer suspected that an insect was the vector of the disease, and that possibly the direction of the wind played a role in blowing the

vectors into an area. On the other hand, a veterinarian from England postulated that AHS was a type of malaria arising from ground after sunset (Bayley, 1856). From these two viewpoints, it is clear nobody knew what or how AHS was transmitted. In the end both the farmer and the veterinarian were on the right tract, as AHSV is transmitted by an insect and malaria was shown to be transmitted by mosquitoes in 1898 (Cox *et al.*,2010), additionally both *Culicoides* and mosquito species are very active from sunset until sunrise.

Various researchers have expressed views regarding the vectors of AHSV as, mosquitoes, ticks, and other biting flies. In Sudan during an AHS outbreak, it was postulated that horn flies (*Lyperosia minuta* Bezzi) were involved as they were observed around the animals and there was a remarkable absence of other blood sucking arthropods observed in the area (Du Toit, 1944). Researchers utilised various methods for collection of biting insects including sweeping of grass, collection of biting insects from bait animals at night, flashlights to illuminate insects in the act of feeding, all these methods failed to yield the required results as few insects were captured. Then during the latter part of 1941-1942 season light traps were obtained and brought into operation. Insects collected with these traps yielded the first positive results (Du Toit, 1944).

Positive results were obtained by injecting ground up wild *Culicoides* caught, in these traps, into susceptible hosts. The results indicated that biting midges of the genus *Culicoides* of the family Ceratopogonidae (the family was incorrectly stated as Chironomidae by Du Toit) harboured BTV and AHSV. This investigation into the transmission of BT and AHS was carried out at Onderstepoort during 1942 to 1943 (Du Toit 1944). It was concluded that certain species of the genus *Culicoides* were capable of being infected with and transmit BTV to susceptible animals by biting. Based on this research Du Toit anticipated that the transmitter or transmitters of AHSV would also be found within genus *Culicoides* (Du Toit 1944). Du Toit successfully transmitted AHSV by *Culicoides* bite from an infected to a susceptible horse, 12 days after feeding the insects infecting blood meal. This was the first definitive demonstration by Du Toit of the biological transmission of AHSV by any species of arthropod (Mellor, 1993).

Though *Culicoides* species play a very important role in the transmission of AHSV and 1,340 *Culicoides* species have been described, it is vital to remember that not all species are vectors of disease. To date it has been shown worldwide that, *Culicoides* spp. may

transmit up to 76 viruses, 48 protozoa as well as filarial nematodes to man, livestock and wildlife. There are several human pathogens that are transmitted by *Culicoides* species including Rift Valley fever, vesicular stomatitis, Mitchel River, and Eastern equine encephalomyelitis. These viruses are primarily mosquito-borne but can also be transmitted by *Culicoides* species. The most important human pathogen transmitted by *Culicoides* is Oropouche virus (OROV). The most important non-human viral pathogens transmitted by *Culicoides* are African horse sickness virus (AHSV), Bluetongue virus (BTV), Epizootic haemorrhage disease virus (EHDV), Equine encephalosis virus (EEV), Akabane virus (AKAV), Bovine emphemeral fever virus (BEFV) and the Palyam viruses (Thompson *et al.,* 2012). Pathogen isolations from 28 Afrotropical *Culicoides* species yielded 42 viruses (including several unidentified viruses), 15 protozoa as well as filarial nematodes (Bellis 2013, Labuschagne 2016; Meiswinkel *et al.* 2004).

During an outbreak of bovine ephemeral fever (BEF) in cattle in Australia from 1967 to 1968, various attempts were made to identify the vector, which was suspected to be the biting midges. Various collection methods were explored, including the use of light traps, truck traps, burrow egress traps and animal baits (Dyce *et al.,* 1972). All the different types of collections were placed in separate plastic containers and sent to the laboratory. At the laboratory mosquito and *Culicoides* species were separated from the other insects, the *Culicoides* species were then also sexed, age graded and identified based on wing characters (Dyce, 1969; Dyce *et al.,* 1972). Although reserachers were unsuccessful to isolate BEF from any of the arthropods tested, they did succeed in isolating six other strains of arboviruses from the collected *Culicoides* and mosquito species (Doherty *et al.,* 1972; Dyce *et al.,* 1972).

In a study undertaken in Sudan, laboratory experiments with *C. imicola* (*C. pallidipennis* Carter, Ingram and Macfie in older publications), confirmed its ability to transmit both BTV and EHD group viruses. The study also concluded that *Culicoides kingi* Austen should be considered as a potential vector of BTV (Mellor *et al.,* 1984).

2.11.2. Different Culicoides species

Culicoides are tiny biting flies belonging to the family Ceratopogonidae. In certain areas they are also called sandflies. The family Ceratopogonidae contains about 125 genera with approximately 5,500 species. Out of these 125 genera, 4 are known to contain species that are blood suckers. These genera are *Austroconops*, *Culicoides*, *Forcepomyia*

subgenus *Lasiohelea* and *Leptoconops* (Mellor *et al.,* 2000). *Culicoides* are easily differentiated from other genera by the wing characters. *Culicoides* species are found almost all over the world except the extreme polar regions, New Zealand, Patagonia and Hawaiian Islands. There are approximately 1,340 recognised *Culicoides* species worldwide, and 96% are obligate blood feeders (Bellis 2013, Labuschagne 2016; Meiswinkel *et al.* 2004; Thompson *et al.,* 2012). These tiny creatures measure between 1-3 mm, making them amongst the smallest heamatophagous flies in the world. These insects are active between sunset and sunrise and are very seldom during the day (Bellis 2013, Labuschagne 2016; Meiswinkel *et al.* 2004; Nevill, 1967).

Culicoides imicola, is the major old-world vector of BTV and AHSV and is an Afro-Asiatic species preferring warmer areas than those found in Europe (Capela *et al.,* 2003; Mellor & Boorman, 1995). In recent years *C. imicola* has expanded its range into Europe and have now even been recorded up to Switzerland (Cagienard *et al.,* 2006). In 1968 in Kenya an extensive survey was conducted to establish the larval habitat of the two dominant BT vector *Culicoides* species namely *C. cornutus* de Meillon, and *C. pallidipennis* (*C. imicola*). It was discovered that they bred in large numbers in fine mud and dung surrounding cattle pens (Walker & Davies, 1971). Blood meal preferences studies in Kenya revealed that *C. pallidipennis* (*C. imicola*) and *C. schultzei* (Enderlein) preferred feeding on cattle (Walker & Boreham, 1976).

2.11.3. Ticks as vectors

There are three families of ticks, these are: *Ixodidae* (hard ticks), *Argasidae* (soft ticks) and *Nuttalliellidae*. Worldwide approximately 700 hard ticks, 200 soft ticks and 1 species of *Nuttalliellidae* have been described. In South Africa, there are 6 genera of hard ticks: *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus* (Makwarela *et al.,* 2023).

i) Lifecycle of ticks

The life stages of ticks are the eggs, from the eggs six-legged larvae hatches, and after moulting it becomes an eight-legged nymph which will become an adult after moulting. Ticks are either one-host, two-host or three-host parasites. A one-host tick will remain on the same host for all its life stages and only leave the host to lay eggs. Transovarial transmission of *Babesia* species has been demonstrated for one-host tick species. In two-host ticks the larvae seek out and attach to the first host, usually a rodent or lagomorph.

The larvae molt into nymphs on the first host and the engorged nymphs drop off the first host, usually in the late summer or fall and overwinter in the nymphal stage. Nymphs molt into adults the following spring. The adults will then seek out the second host usually a larger herbivore (bovids, cervids, etc). Adults feed on the second host during the summer and mate. In the fall, females drop off the second host to continue the cycle. Females may reattach and feed multiple times. Humans may serve as first or second hosts for ticks with this life cycle. Also, the second host does not necessarily have to be a separate species, or even a separate individual, as the first host.

In three-host ticks the larval, nymphal and adult stages each feed on a different host. Each stage attaches and drops of to molt after feeding, the next stage will seek out a new host. The adults feed and mate on the third host during the summer. Females drop off the host in the fall to continue the cycle. Females may reattach and feed multiple times. The three hosts do not necessarily have to be different species, or even different individuals. In addition, humans may serve as first, second or third hosts. Three-host ticks leave the host between each of the life stages. With two- and three-host ticks, the second or third host species that the larvae or nymph feed on are not necessarily same host species as what the adult feeds on. This have serious implications for disease transmission, as the host that the larvae or nymph feed on may act as reservoir or cycling hosts for a disease that may then be transmitted by the adult or in the case of three-host ticks by the nymph to a new host (Walker *et al.,* 2003).

ii) Ticks as vectors of pathogens

Ticks have longer lifespan than *Culicoides* species and may also act as biological vectors of BTV and the vectorial capacity of the American soft tick *Ornithodoros coriaceus* has was reported in 1985 (Bouwknegt *et al.,* 2010). Experimentally ticks also become infected after feeding on viremic sheep blood and soft ticks could also transmit BTV cell cultured BTV. The feeding of ticks on vireamic animals and the lengthy survival of ticks off the host, makes ticks excellent candidates to serve as pathogen reservoirs (Bouwknegt *et al.,* 2010).

2.11.4 Ticks as vectors of AHSV

In the Aswan region of southern Egypt where AHSV is endemic, the virus was isolated from the blood of healthy street dogs, from *Rhipicephalus sanguineus*, and from *Hyalomma dromedarii* during winter. Infected ticks were able to retain the virus for up

to 10 weeks. Experimenatally AHSV serotype 9 was transmitted through the bites of adult *Hyalomma dromedarii* from an infected to susceptible horse (Blood & Radostits, 1989; Coetzer & Guthrie, 2004; Dardiri & Salama, 1988; Kahn, 2005; Mellor, 1993). The two tick species *R. sanguineus* and *H. dromedarii* are discussed below as they form part of the possible vectors of AHSV as was shown in a study conducted in Egypt (Dardiri & Salama, 1988).

Rhipicephalus sanguineus

It is also known as the kennel tick or brown dog tick. The conscutum and the scutum are yellowish to reddish brown and mostly appear smooth. This tick's eyes are distinct and slightly convex. This tick is the most widespread tick species throughout the tropics and subtropics, because of its specialised feeding on domestic dogs (Walker *et al.,* 2003).

Host preference

Domestic dogs are the preferred host for *R. sanguineus* although they can also be found on cattle. *Rhipicephalus sanguineus* is monotropic, meaning that all developmental stages i.e., the larvae, nymphs and adult ticks a single host species. The adult ticks usually attach to the ears, neck and shoulders, the nymphs attach to ears and shoulders, and larvae are found particular on the belly and flanks (Madder *et al.,* 2014).

Life cycle

Rhipicephalus sanguineus is a three-host tick and the engorged female detaches to lay eggs about 3, 200 eggs within 7 to 28 days after detaching from the dog (Walker, 2003). The eggs hatch within 3 to 10 weeks and then a larvae emerge, attaches to a host and is engorged within 3 to 8 days, then moult within 2 to 6 weeks, and the nymphs is engorged within 4 to 10 days and moult within 2 to 26 weeks. The life cycle is completed within 10 weeks under normal conditions (Walker, 2003).

Distribution of *Rhipicephalus sanguineus*

The distribution of *R. sanguineus* occurs primarily in areas between the latitude 50˚N and 30˚S. This tick is distributed throughout all climatic regions and countries of Africa, because of its feeding preference to domestic dogs. This tick species is well adapted to staying inand surviving in kennels, houses in sheltered moist cool areas (Madder *et al.,* 2014; Walker, 2003).

Rhipicephalus sanguineus as a vector

The Veterinary importance of this tick species is its ability to transmit the causative agent of canine tick fever and Babesiosis in dogs, *Babesia canis* and *Babesia gibsoni* is primary found in the Far East and Africa. *Babesia canis* infections are found in most, if not all, of the African continent and is endemic to U.S.A. *Babesia canis* can be transmitted both transovarially and transtadially. *Rhipicephalus sanguineus* was mistakenly associated with the transmission of *Babesia caballi* and *Babesia equi* of horses whereas *R. turanicum* are probably the vector species for these pathogens (Walker *et al.,* 2000).

This tick also transmitts of the causative agent *Ehrlichia canis,* which cause canine ehrlichiosis (Walker *et al.,* 2000). Canine ehrlichiosis is transmitted by all stages of this tick and is common in USA, where it is present in all areas where *R. sanguineus* is present. *Rhipicephalus sanguineus* has also been implicated in the transmission of *Rickettsia rickettsia,* which cause canine Rocky Mountain spotted fever (RMSF), but the role it plays as a vector is unknown. Canine Rocky Mountain spotted fever mainly affect pure bred dog breeds and these are more likely to suffer and display clinical symptoms of the disease (Walker *et al.,* 2000).

Additionally, two other minor minor diseases transmitted by *R. sanguineus* are *Haemobartonella canis* which is a rickettsial parasite and *Hepatozoon canis* which is transmitted to dogs when they ingest or bite an infected tick infected with these parasites (Walker *et al.,* 2000). In a study by Olmeda-Garcìa *et al.,* (1993) it was demonstrated experimentally that *R. sanguineus* can transmit the filarial nematode, *Dipetalonema dracunculoides* from nymph to adult tick.

Hyalomma dromedarii

This tick is also known as the camel tick (Walker, 2003). Adult *Hyalomma dromedarii* has long mouth parts and are generally large, are yellow-brown to nearly black in colour, and legs are paler than the scutum and the legs may be ringed by paler bands (Madder *et al.,* 2014)

Host preference

Hyalomma dromedarii prefer camels (*Camelus dromedaries*) although cattle, sheep and goats can also be infested. The adult ticks are found mostly attached to the inner thighs, udder and genital areas and outer nostrils of camels (Walker, 2003). The larvae and the

nymphs prefer feeding on small burrowing animals and hares, although nymphs can also infest camels, cattle and horses (Madder *et al.*, 2014)

Life cycle

Hyalomma dromedarii has a two or a three-host life cycle and as two host tick, the larvae feeds and moult to nymphs on small animals or hares, whilst the adult ticks feed on large herbivores. As a three host tick the larvae feed on small animals, detach and moult to nymphs which can then either attach to another small mammals or feed on the same large animal as adult. This life cycle of *H. dromedarii* looks to be continuous throughout the year.

Distribution of *Hyalomma dromedarii*

Hyalomma dromedarii is commonly found in the Mediterranean, steppe and desert climates that are north of the equator in Africa. This tick has adapted to extreme dry habitats and to camels as their preferred hosts (Walker, 2003). This tick is found in the arid areas of North Africa from Mauritania in the west to Egypt in the east. It is present in Sudan, Ethiopia and Kenya but is also found in Namibia, where it was introduced through the importation of infected camels (Madder *et al.*, 2014).

Hyalomma dromedarii as vector

African horse sickness virus was isolated from *Hyalomma dromedarii* in Egypt (Dardiri & Salama, 1988; el-Husseini *et al.*, 1986). This tick transmits *Theirelia annulata* to cattle which causes the tropical theireliosis and is also mechanical vector of camel pox. It has been implicated in cases of tick paralysis in children in Egypt (Madder *et al.*, 2014). This species can cause lesions on the posterior, lower abdomens of goats (Onyiche & MacLeod, 2023).

2.12. OVERWINTERING OF AHSV

The exact overwintering mechanism of AHSV is not known. Various authors have postulated that the virus survives in an invertebrate or vertebrate host (Blood & Radostits, 1989; Hanekom *et al.*, 2023; Takamatsu *et al.*, 2004; Thompson *et al.*, 2012; Wilson *et al.*, 2009). Transovarial transmission of AHSV from the female *Culicoides* to their eggs, may be possible as there is evidence that vertical transmission of Orbiviruses occurs in

mosquitoes and sandflies, but up to date, no evidence of live AHSV has been found in any of the immature stages of *Culicoides* species (Thompson *et al.,* 2012).

It is known that dogs may be come infected and even die after eating meat from and animal that had died from AHS. There have also been cases of dogs that had dies from AHS without eating infected meat. Antibodies against AHSV has been isolated from dogs and researchers have postulated that dogs may play a role in circulating AHSV in endemic areas and act as a source of the virus during cold periods (Braverman & Chizov-Ginzburg, 1996; Coetzer & Guthrie, 2004; Hanekom *et al.,* 2023; O'Dell, 2017; Salama *et. al.,* 1981; Van Rensburg *et al.,* 1981; Van Sittert *et al.,* 2013). Where and how AHSV overwinters or persist in areas for more than one season is not known, though it has been suggested that the virus overwinters in adult *Culicoides* populations, at low levels of viremia in the natural hosts, or in other invertebrate hosts (Losson *et al.,* 2007; Mellor *et al.,* 2000; Napp *et al.,* 2011; Venter *et al.,* 2014)

The outbreak of Bluetongue serotype 8 in 2006, in the North-west Europe, spread rapidly through local vectors (Meiswinkel *et al.,* 2008). The re-emergence of the BT a few months after the initial outbreak shows the BTV8 survived the winter. Transmission of bluetongue in South Africa is broken when both *Culicoides* activity and virus replication in the vector midges stops in cold temperatures (Purse *et al.,* 2008; Saegerman *et al.,* 2008).

These outbreaks of BTV8 did not stop even during the cold temperatures in north–west Europe but continued. It was then suspected that ticks might be playing a significant role in transmission of BTV8 in that region. A study was initiated to establish if ticks are involved in these transmissions of BTV8 in North-West Europe, both hard (*Ixodidae*) and soft (*Argasidae*) ticks were tested. BTV8 was picked up by PCR in 20 out of 24 female ticks infected by capillary feeding and it was found that BTV8 could survive in hard ticks for at least 21 days and up to 26 days in soft ticks (Bouwknegt *et al.,* 2010).

2.13. CONTROL OF AFRICAN HORSE SICKNESS

African horse sickness is a noncontagious disease that can only be transmitted by bites of *Culicoides* spp. (Mellor & Hamblin, 2004). In light of this, there are three approaches to the control of AHS, namely vaccination, vector control and movement control.

There is no treatment for AHS apart from resting and good animal husbandry and appropriate treatment of any complications and secondary infections during the recovery period (Mellor & Hamblin, 2004). Different approaches exist for endemic and AHS free zones or countries. Vector control measures should be implemented in endemic areas (Weyer, 2016).

2.13.1. Vaccination

Vaccination for African horse sickness remains the most important control measure for outbreaks especially in endemic areas and/or countries (Weyer, 2016; Zientara *et al.*, 2015) The vaccination protocol is attached as Annexure 4.

In endemic areas and in areas that AHS outbreaks occur every year that is most parts of Africa, south of the Sahara, annual vaccination is the very practical means of control (Coetzer & Guthrie, 2004). Polyvalent, attenuated vaccines are commercially available at Onderstepoort Biological Products (OBP), Onderstepoort, South Africa. Early vaccines were based on virus strains attenuated by multiple passage in suckling mouse brain (Alexander *et al.*, 1936). As all serotypes of AHSV exist in South Africa and most parts of Sub-Saharan Africa, the use of these polyvalent vaccine is very important in these areas (Coetzer & Guthrie, 2004).

Those early vaccines introduced solid immunity but occasionally resulted in serious side effects which included fatal cases of encephalitis in horses and donkeys (Zientara *et al.*, 2015). These cell-cultured adapted viruses still form the basis of the current available Onderstepoort Biological Products (OBP) vaccines (Lubroth, 1988). During an outbreak in Iran in 1960 a massive vaccination strategy was adopted and a total of 60,000 horses were immunised yielding very good results (Howell, 1960).

The use of live polyvalent attenuated vaccine is advocated in South Africa, with a regulated vaccination period that starts from June until end of October every year. This is the time before the peak of AHS outbreaks, which allows immunised horse to have enough time to respond to the vaccine before they are challenged by natural exposure. The Onderstepoort (OBP) AHS vaccine currently used in South Africa is supplied in two polyvalent vials containing AHSV types 1, 3 and 4 and 2, 6, 7 and 8 respectively (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Zientara *et al.*, 2015).

African horse sickness virus serotype 5 was withdrawn in October 1993 from the vaccine because of the severe reactions and death in some of the vaccinated test animals. Each

bach of vaccine is tested prior to release. African horse sickness virus serotype 9 is not included in the vaccine because type 6 offers strong cross-protection against serotype 9, which in addition is rarely reported in South Africa and is therefore, considered of low virulence. Animals should be vaccinated every six months in their first and second years of life and thereafter annually (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004).

2.13.2. Vector control

It is impossible to eliminate populations of *Culicoides* species completely (Mellor & Hamblin, 2004). The focus is to reduce the number of potential infected midges from biting susceptible hosts. Infection of horse can, to a large extent, be prevented by stabling horses some hours before sunset and letting them out a few hours after sunrise, as most *Culicoides* species are active at night and are not inclined to enter buildings at night (Coetzer & Guthrie, 2004). The presence of animals in stables may increase vector abundance (Barnard 1997). *Culicoides imicola* usually enters stables housing horses, voluntarily in search for animals to feed on or to hide (Barnard 1997). In addition, this behaviour may be enhanced by odours and even the bad smell associated with dirty stables (Barnard 1997; Baylis *et al.,* 2010; Meiswinkel *et al.,* 2000). A stabling trial, in the Clarens area of the Free State Province, in 1998 found that the numbers of *C. bolitinos* entering the stable were reduced 14-fold using mesh screens over the windows to prevent the midges from entering (Meiswinkel *et al.,* 2000).

It is very important to know the breeding sites of vector species of *Culicoides*. Breeding media utilised by *Culicoides* species can be classed as: ground water (containing a varying degree of soil), animal dung, tree and rock holes, and rotting fruit and plants (Bellis 2013; Labuschagne 2016; Meiswinkel *et al.* 2004). Ground water habitats may range from running water; mud from fresh or salt marshes; mud from river-, pool-, or stream edges or soil mixed with leaf litter. Dung habitats may be pure dung or mud contaminated with dung (Bellis 2013; Labuschagne 2016; Meiswinkel *et al.* 2004). *Culicoides imicola* usually breeds in organically enriched moist but not waterlogged soils e.g., irrigated pastures. These areas should remain moist for sufficient time for the *Culicoides* spp. to finish its developmental stages (7 to 10 days) (Meiswinkel *et al.*, 2004; Nevill 1967; Pajor 1990).

When potential breeding sites for *Culicoides* spp. are few and small in size their elimination can be achieved easily through habitat modification. This can be achieved by

replacing leaking taps, closing off all leaks, draining damp areas although this may be impossible or may not be economically feasible in other situations (Mellor & Hamblin, 2004).

Application of larvicides, such as Abate, to suspected *Culicoides* spp. breeding sites provides a slow but effective release of the insecticide and may be effective for a period of up to 30 days. The control of larval *Culicoides* species using biological agents like *Baccilus thuringensis israelensis* (*Bti*) has not proved to be successful. Diethyltoluamide (DEET) seems to be the only commercially available repellent that has been shown to have a significant deterrent effect against *Culicoides* species for a period of up to four hours (Mellor & Hamblin, 2004).

Application of synthetic pyrethroids in and around stables, and directly to equids themselves can be efficacious against *Culicoides* species and more environmentally friendly. Any repellent and or insecticide applied directly to the animal, need to be registered as safe for use on that breed and the manufacturers' instructions must be followed. Application of ivermectin may also be effective in killing *Culicoides* species (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004).

2.13.3. Movement control

Infection can be introduced into a disease-free area by infected hosts (Constable *et al.*, 2017). Therefore, there should be a reliable system for the prevention of the introduction of infection to disease free areas, which include movement control and quarantine (Bosman *et al*., 1995).The infected hosts can be horses incubating the disease, clinically ill animals, animals showing no clinical signs including donkeys and zebra that are infected and viremic (Constable *et al.,* 2017). Appropriate control measures for the movement of equids should be instituted (Constable *et al.*, 2017). A complete vaccination protocol, which covers all nine AHSV serotypes, 42 – 60 days before introduction of the horse (Constable *et al.,* 2017). All horses need to be positively identified through microchipping and a passport detailing vaccination status and a veterinary certificate confirming health and issued not more than 48 hours before introduction (Constable *et al.*, 2017). All equids imported from areas where the disease is enzootic should be housed in isolation in insect –proof enclosures for a period of 60 days (Constable *et al.,* 2017). During an outbreak of AHS in Paarl, Western Cape Province, movement control measure was put in place to control the movement of equines in out and within the containment

zone without the required permit from the state veterinarian (Grewar, 2018; Grewar *et al.*, 2019).

From previous studies into the possible vectors of AHSV it is known that *Culicoides* species are the main vectors of the virus, though ticks and other blood feeding diptera may also play a minor role in the transmission of the virus (Awad *et al.*, 1981; Blood & Radostits, 1989; Du Toit, 1944, Meiswinkel *et. al.*, 2004; Meiswinkel & Paweska, 2003; Mellor *et al.*, 1984; Paweska J.T. *et al.*, 2002; 2003; Salama *et al.*, 1981; Venter *et al.*, 1998, 2004, 2006; Venter & Paweska, 1999).

This study set out to investigate the species diversity, and distribution of *Culicoides* and tick species in the study area. This study investigated the possible overwinter mechanisms that included attempting to isolate AHSV from *Culicoides* and tick species during winter. The role of dogs as a possible source of AHSV was investigated by attemping to test for antibodies against AHSV in the blood of the dogs. The presence of antibodies would indicate that these dogs had been exposed to AHSV at some stage of its life.

CHAPTER 3

RESEARCH METHODOLOGY

3.1. MATERIAL AND METHODS

In this section, the study area, sampling, PCR and ELISA methods and protocols used in this study are described.

3.1.1 Study area

Eastern Cape Province is one of the nine provinces of South Africa; the study took place in Sarah Baartman district municipality (Figure 3.1). Sarah Baartman district is one of the six district municipalities in the Eastern Cape Province. It is the largest district in the province making up approximately third of the geographical area. It comprises of seven local municipalities and surrounds Nelson Mandela Bay Metropolitan (Port Elizabeth), covers 58,245 km^2, has a population of 520,480 with an estimated annual population growth of 1.14%.

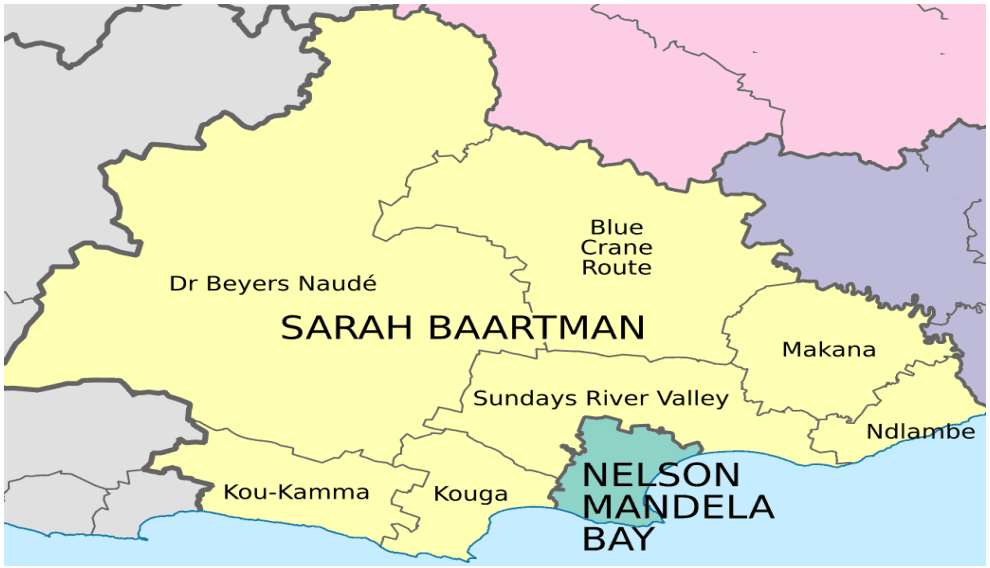


Figure 3 1: Map of the study site, showing the district and local municipalities (Municipalities of South Africa, 2023)

Tourism and farming industries generate the most revenue for this district. In addition, and the Grahamstown national arts festival attracts tourists from all over the world. There are also several beaches and game parks, that attract thousands of tourists every year (Hamann & Tuinder, 2012).

Agriculture in the Eastern Cape is dominated by beef farming and fruit farming in the southwestern parts and subsistence farming in the northeastern regions (Hamann & Tuinder, 2012). The Sarah Baartman municipality is a summer rainfall area that receives approximately 300 mm during the growing season (September to March) (Auerbach, 2020). The climate is highly varied with summer or winter rainfall occurring in the province depending on the area. The west is dry with rain during winter or summer, frosty winters and hot summers. The coastal areas have mild temperatures and Gqeberha has more days of sunshine than any other coastal town in the country. Strong westerly winds are common in this district. Summer temperatures on average are 27 ˚C, though some inland areas can record temperatures as high as 42 ˚C in summer. In winters the average temperature along the coast is 8 ˚C, though it can drop down to -1 ˚C inland.

Due to changes in climate, it has been proposed that the fruit farming be moved from the Langkloof to Gqeberha (formerly Port Elizabeth) (Auerbach, 2020). Makhanda and Alexandria chicory and dairy farms are abundant (Hamann & Tuinder, 2012). There are numerous game reserves and game farms throughout the Eastern Cape Province. As a result, several collection sites in this study were adjacent or within a few kilometres of a game reserve of farm. Climate change is causing havoc in the farming industry, with long periods of drought. Due to droughts many dams and rivers have dried up and consequently, Makana local municipality is supplies water to many areas within the district. Table 3.1 presents the sites that receive water from the municipality, the site description, the type of sample, and the host(s) present at each site.

Table 3 1: Collection sites used in the study, with site number, type of sampling, site description and animnal species present at the site.

Site no	Sample collected	Site description	Domestic animals present	Wildlife animals present
1	*Culicoides*	Sandy areas of Gqeberha (Port Elizabeth)	Cattle, horses and dogs	
2	*Culicoides*	Grahamstown area	Horses	
3	*Culicoides* Ticks blood samples	Grahamstown area highland mountains AHS yearly	Horses, cattle, sheep and dogs	
4	*Culicoides*	Western side of Gqeberha Sandy area Very few AHS cases	Horses	
5	*Culicoides*	Grahamstown area	Angora goats, sheep, cattle, chicken, dogs and horses	Warthog and other wildlife
6	*Culicoides* Ticks blood samples	Grahamstown area eastern side of Makhanda wooded farm small river 3 dams and 2 boreholes that supply water to both wildlife and domestic animals	Horses and dogs	Blesbok and zebra
7	*Culicoides* blood samples	Grahamstown area Close to game reserve AHS reported yearly fruit trees and vegetables Dams dry Horses are stabled at night rocky topography	Horses, dogs, cats and cattle	
8	*Culicoides* blood samples	Grahamstown area Summer: temperature can reach 42˚C. AHS regularly Small river bottom end of farm	Dogs, horses and cattle	Impala, duiker and warthog. Plains Zebra and Cape Mountain Zebra

9	*Culicoides* blood samples	Grahamstown area small dam approximately 500 meters from house	Horses dogs cats chickens, peacocks, sheep, goats and cattle	wild birds, tortoise, kudus and impala
10	*Culicoides* Ticks blood samples	Grahamstown area Winters very cold with severe frost No AHS reported	Cattle, sheep, goats, pigs, chickens and dogs	monkeys, warthogs and kudu
11	Ticks blood samples	Grahamstown area Makana local municipality is supplying water to this property. Dams dry for the last 3 years	Chickens, doves, dogs, goats, pigs and cattle	
12	Ticks	Grahamstown area high temperatures, that can go to 38˚C. Water is sourced through the Great Fish River irrigation system lucerne fields are under irrigation.	Pigs, goats, sheep and cattle	
13	Ticks blood samples	Grahamstown area northeast Grahamstown Water supply is from Makana municipality water reservoirs. part of the Makhanda township		
14	Ticks	Grahamstown area Very dry area: dams dry +4 years Water from borehole	Dogs, chickens and cattle	Wild birds impala, springbok, kudus, warthogs and nyala
15	Ticks blood samples	Grahamstown area borehole two small dams adjacent to the cattle kraals.	Donkeys, cattle, cats and dogs.	
16	Ticks blood samples	Grahamstown area dry area on the banks of Great Fish River	Cats, dogs, goats, sheep, cattle and pigs	ostrich kudus, warthogs and impala.

		Water for camps from the Great Fish River. borehole Dam dry + 3 years		
17	Ticks	Grahamstown area East of Grahamstown access road to farm bad		
18	Ticks	Grahamstown area West of Grahamstown Vegetables summer rainfall	Chickens, cats, dogs and cattle.	
19	Ticks blood samples	Grahamstown area South of Grahamstown surrounded game reserve	Cattle, goats, pigs, dogs and cats and cattle	Buffalo, giraffe andzebra
20	blood samples	Grahamstown area dry area, though on banks of Great Fish River. poorly developed area water supplied by Makana municipality site is neighbouring game reserves people casual employment reserve.	Cats, dogs, chickens, sheep, goats, cattle and donkeys	Big 5 plus plenty of zebra and wildlife
21	blood samples	Grahamstown area Northeast of Grahamstown	Cats, dogs, pigs, goats and cattle	
22	blood samples	Grahamstown area Next to game reserve Dry	Sheep, goats, cattle horse, dogs	
23	blood samples	Grahamstown area borehole the Great Fish River cultivated pastures	Cattle, sheep and goats, dogs horses	Kudu, warthogs, monkeys and impala.
24	blood samples	Grahamstown area Dry Goat farming stream on property water pumped to camps borehole that supply house	Goats, pigs, chickens	Baboons, monkeys, kudu, impala, warthogs.
25	blood samples	Riebeek East area very dry area	Cattle	

		borehole water summer rainfall beef production and small stock farming poor grazing and bush encroachment stock capacity low	
26	blood samples	Grahamstown area East of Grahamstown Water is supplied by Makana municipality as it is the part of the township.	Cats, dogs, goats, pigs and cattle
27	blood samples	Grahamstown area sheep production dams dry borehole water.	Dogs, sheep, cattle and horses, swans
28	Ticks	Grahamstown area	Cattle and dogs

3.2. RESEARCH DESIGN

A cross-sectional study design was used to investigate vectors that are likely to be involved in the overwintering of AHSV in the study area. A cross-sectional survey provides a snapshot of occurrence from a selected sample that represent a larger area and it is useful to estimate outbreaks of disease (Owens, 2002). Cross-sectional studies are observational in nature and if descriptive in natute, it is limited in that it can't predict for example disease (Thomas & Taylor, 1990). When cross-sectional data are used for analytic purposes, authors and readers should be vigilant not to make causal inference, unless the exposure may safely be assumed to be stable over time and not influenced (Kesmodel, 2018).

This type of research is used to describe characteristics that exist within a community, but not to determine cause and effect relationship between different variables (Owens, 2002). This method is often used to make inferences about possible relationships or to gather preliminary data to support further research and experimentation (Thomas & Taylor, 1990). Based on this, a cross-sectional study was considered suitable for this study.

3.3. COLLECTION SITES

Twenty-eight (n=28) collection sites were recruited for collection of *Culicoides* species, tick species and blood samples from the dogs. However, not all sites were used for all three types of samples. All 28 sites were in the magisterial districts of Albany (Makana) and Port Elizabeth (Nelson Mandela Bay). Figure 3.2 depicts the 28 sampling sites used during the study. The sites were number coded to avoid the unethical use of farm names in the public domain (Table 3.1).

Not all the horse farms in the area were chosen to participate in this study. Farms were chosen based on their known history of AHS cases and one farm was chosen as a negative control due to the absence of AHS on this farm. Some of these sites were used to collect ticks, *Culicoides* spp. and blood samples from dogs, depending on the number of AHS cases reported from that site. Ten (n=10) sites were recruited for the collection of *Culicoides* species. Thirteen (n=13) sites were recruited for the collection of tick species in the Makana municipality. A further, 19 sites were used for the collection of blood samples from dogs. The dog blood samples were collected from November 2022 to September 2023 (Table 3.1).

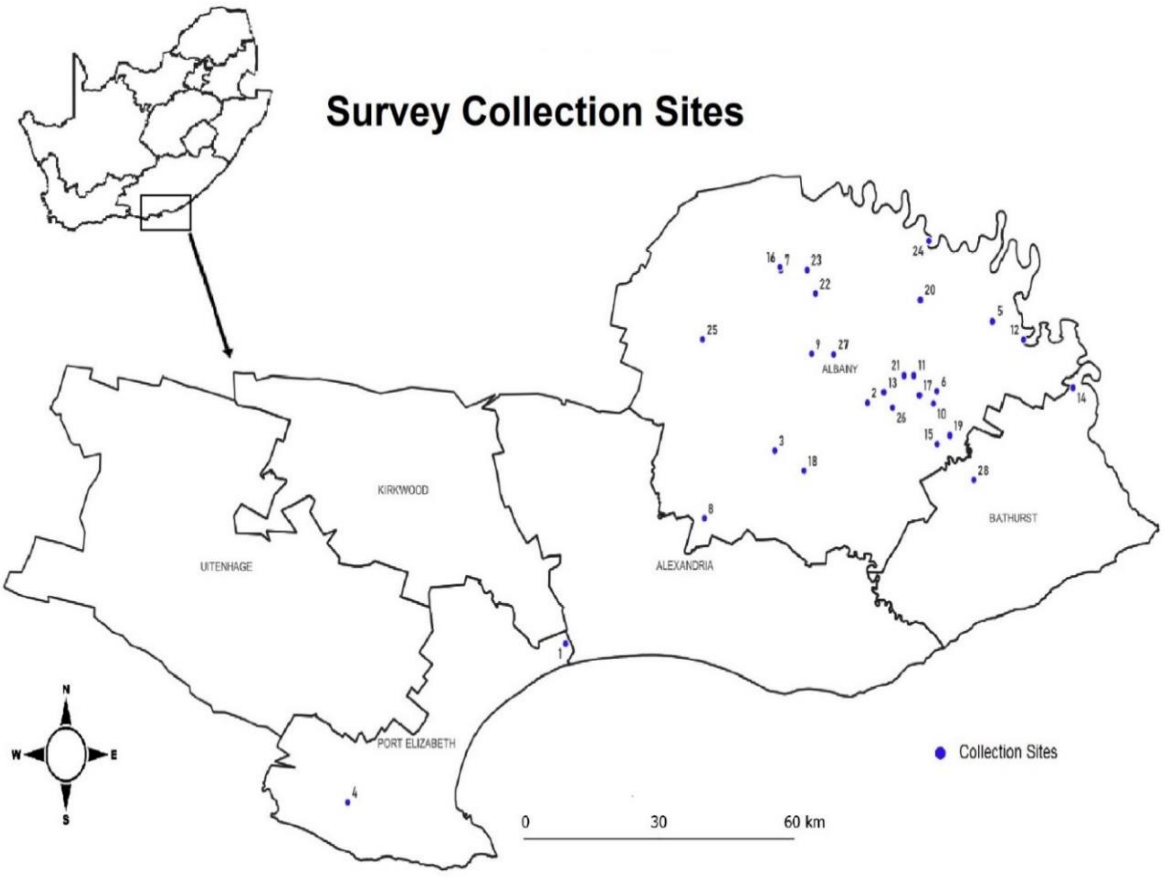


Figure 3 2: Map depicting all 28 collection sites of the survey (Labuschagne 2023)

During a study by Meiswinkel (1997) it was found that the main vector of AHS *C. imicola* was very rare to absent in these western sandy coastal areas. Sites 1 and 4 were specifically chosen as these are both on the sandy coastal areas of Port Elizabeth, though Site 1 is on the western side and Site 4 on the eastern side of the town. Site 10 was chosen as there was no records of AHS occurring at this farm. It was important to establish if *Culicoides* vector species were present on these farms or not.

3.4. SAMPLING AND PROCESSING METHODS

The outbreak of COVID 19 in Wuhan, China that started in December 2019 affected the pace and the plan of this study, as deadlines regarding the collection of samples could not be met. Due to lockdown measures to curb the spread of COVID 19, no travel was allowed and thus samples could not be collected. Even when the restrictions were relaxed, and some travel could take place farmers were reluctant to allow collection of samples as COVID 19 is a very contagious viral disease. The study used three different sampling methods, and these methods are described below.

3.4.1 Sampling and Collection of *Culicoides* species

Collection of *Culicoides* species started in 2017 and continued to 2022. There were two horse farms in the Port Elizabeth area that consented to participate in this study. This was a very important part of the research because, in a study by Meiswinkel in 1997 he found that, the main vector that was incriminated in the transmission of AHSV, *C. imicola* was absent in the sandy coastal areas of Port Elizabeth, which meant that the probability of AHS outbreaks in this area was very low, but that was before *C. bolitinos* shown to be a vector of AHSV as well (Meiswinkel 1997; Meiswinkel & Paweska 2003).

The area of Port Elizabeth was used as the negative control. In contrast, Grahamstown horse owners regularly report outbreaks of AHS. For this reason, the study focused more on the Grahamstown area with the hope of answering the question of where and how, do AHSV overwinter in the area. Thus, eight out of ten farms in this study were in the Grahamstown area. If the virus overwinters in adult *Culicoides* species, it should be evident in this area that reports cases of AHS yearly.

As part of the ARC Climate Change Collaboration Centre (ARC CCCC) project on: The impact of climate change on identified priority agricultural pests and diseases *Culicoides* sample collection was undertaken in the Eastern Cape. From 2017 samples were collected at five sites, with additional sites being added as farmers agreed to participate, and by 2022 eight farms were surveyed, after two farms withdrew from the study. Over the six-year period from 2017 to 2022, 10 farms were surveyed and *Culicoides* data from all 10 were included in this study.

During the survey 220V UV-light down-draught light traps were used for the collection of *Culicoides* species (Plate 3.1). A coarse top net made from tulle was used to prevent the collection of larger insects and moths, thus minimising the collection of non-target species (Meiswinkel. *et al*, 2004). The traps were operated from mains electricity, and hung close to the animals, normally beneath the eves of the stable at approximately 2m from ground level and out of the reach of the animals. The traps were switched on in the evening of collection and switched of the next morning. The midges were collected into a 1% water/Savlon* solution which reduced surface tension in collecting beakers suspended beneath the light trap, allowing the insects to sink to the bottom of the collection beaker. The contents of the beaker were then poured into a collection jar and labelled (Plate 3.2). The site name, date of collection and co-ordinates were written in

pencil on the labels on the outside of the collection beaker/jar (Plate 3.2). A pencil was used for the labels, as ethanol can wash away any writing in pen. Back at the laboratory, the insects collected were transferred to 80% ethanol and stored until the samples were sent to ARC-Onderstepoort Veterinary Research (ARC-OVR) institute for analysis.

*containing Clorhexidane gluconate 0.3 g/100 ml and Cetrimide 3.0 g/100 ml [Johnson & Johnson, SA]

At the ARC-OVR the received samples were cleaned by separating the *Culicoides* from the other insects. The number of *Culicoides* were counted while cleaning the sample and all the collections containing more than 500 *Culicoides* specimens were sub-sampled using the method of Van Ark & Meiswinkel (1992).

Plate 3.1: A 220 volt UV light down-draught light trap with collection beaker/jar

Plate 3.2: Labelled sample bottle (Photo by researcher)

At the ARC-OVR the received samples were cleaned by separating the *Culicoides* from the other insects. The number of *Culicoides* were counted while cleaning the sample and all the collections containing more than 500 *Culicoides* specimens were sub-sampled using the method of Van Ark & Meiswinkel (1992).

The samples were stored until they could be identified. *Culicoides* spp. were identified to species level by examination of the light and dark markings on the wings, age-graded and sexed (Dyce, 1969; Labuschagne, 2016; Meiswinkel, 1996).

3.4.2 Collecting tick species

Tick species were collected once a month for the duration of the study. Ticks were collected from 2019 until 2022. There was a break in 2020 due to the outbreak of COVID 19 that led to movement restrictions in South Africa, to curb the spread of the disease.

Different tick species were collected from cattle, horses, sheep, goats and dogs in farms that had AHS outbreaks in the past 5 years and from farms that had no AHS outbreaks for more than 5 years. Adults and nymphs were collected from the above hosts for testing for the presence of AHSV. Forceps were used to remove ticks from live hosts.

The most common places to look for ticks on an animal are mostly the bare parts such as the udders, under the tail root, between the legs, around and in the ears and the groin area (Bouattour *et al.*, 1999). An effective way to detect adult ticks, especially when they are

engorging, it is to feel the hair coat of the host with the palm of the hand (Walker *et. al.,* 2003). To find immature ticks or unfed adults the hair can be parted systematically using a forceps (Walker *et al.,* 2003). In severe tick infestation area, ticks were found all over the body. Calves are often less infected by adult ticks than older cattle (Bouattourr *et al.,* 1999). Care needs to be taken when collecting ticks from live hosts, to prevent ticks from attaching to the collector as ticks are the vectors of various pathogens (Walker *et al.,* 2003).

Ticks were placed into sample containers containing 70% alcohol, with the ticks from separate host species going into different sample containers. The specimen containers were closed tightly to prevent any leakage occuring during transportation. The specimen containers were labelled with a lid pencil as ethanol wash away any ink writing on the label. On each label the site name, date of collection, host species and GPS co-ordinates are written (Walker *et al.,* 2003).

All *Culicoides* and tick samples were packed in resealable plastic bags and placed in a box for shipping to the ARC-OVR. The box was clearly labelled with the address of ARC-OVR and with the name of the relevant laboratory.

3.4.3 Collecting blood samples from dogs

To confirm if dogs in the study area were exposed to AHSV naturally, dogs were bled to determine whether they had antibodies against AHSV. The blood samples from dogs were collected four times, once every season over a period of a year. Samples were only taken from the dogs whose owners were willing to participate in the study. Blood samples from dogs were collected by qualified veterinarians and animal health technicians (i.e., registered with the South African Veterinary Council to practice).

The selection of sites for the collection of blood samples from dogs, was done based on the exposure risk to AHS at each site. Farms that had lost horses in the past five years, due to AHS were prioritized for the blood sampling of dogs. These dogs were bled once in each season to monitor if they would seroconvert to AHS or not. The geographical co-ordinates of the sites were recorded (Annexure 5).

A muzzle was used to restrain dogs during blood collection and to prevent dogs from biting the handler. The cephalic vein was used to bleed the dogs. The area of the vein was shaved and disinfected using methylated spirits or 70% alcohol. A 21-gauge needles and a 3 ml syringe was used to draw blood and to control the amount of blood taken from

each animal. Cleaning the shaved area before inserting the needle into the vein prevented any infection that might happen at the bleeding site.

Pressure was applied above the shaved area to allow the vein to bulge before inserting the needle (Plate 3.3). Blood was drawn very slowly to avoid any shock to the dog. When the desired amount of blood was achieved (1 to 1,5ml), the needle was gently pulled out. After removing the needle, pressure was applied over the venepuncture site for 30 seconds to stop the bleeding, and disinfectant applied (Zubair Shabbir *et al.*, 2013).

Plate 3. 3: Showing collecting blood from a dog (Photo by researcher)

The blood was then transferred into the serum vacutainer (red top) as these were the best for antibody testing (Plate 3.4). All blood tubes were labelled with the name of the site and the name of the dog. All blood samples were packed into in a cooler box and transported to the State Veterinary Offices, in Grahamstown. At the workstation, the submission form for ARC- OVR was filled in for each site. The blood samples were submitted to Grahamstown Provincial Veterinary Laboratory, which then sent the blood

to Onderstepoort for testing. Upon arrival at ARC-OVR a confirmation email was received indicating that the samples had been received at ARC-OVR.

The Indirect and competitive blocking ELISAs, a serological assay, using either soluble AHSV antigen or a recombinant protein VP7 have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especial for large scale investigations (Mellor *et al.*, 1990), and these tests are recognised by the European Commission (2002). At the Virology laboratory all the blood samples were tested with an AHS indirect ELISA to detect IgG antibodies in the serum or plasma.

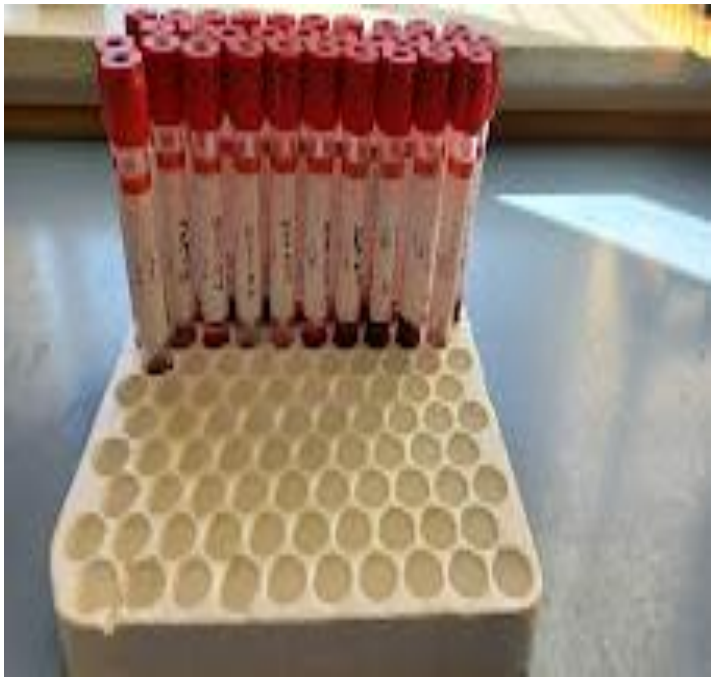


Plate 3. 4: Red top tubes with dog blood (Photo by researcher)

3.4.4. Procedure at ARC-OVR

Once the samples arrived at diagnostic registration, (DR), the information on the submission form was entered into an electronic database. Each submission form, also called a job, received a unique barcoded identity (D-number). The samples were then be dispatched to Virology where they were registered again, as part of quality assurance procedures. Copies of the submission forms were made, and these accompanied the samples to the designated laboratory within the virology section and the copy from DR were filed in the administration office. These samples were kept at 4˚C prior to testing.

The results of the tests were recorded on a laboratory worksheet indicating the following:

- Name of the laboratory

- Registration number (The D number)
- Sender and Province
- Sender Reference
- Species
- Name of the person who registered the sample
- Date received
- Number of samples
- The storage shelf
- Results: Sample IDs, Name of test and results i.e. S/P or PP values
- Name of technician who tested the samples
- Name of technician who verified the results
- Date tested
- Date verified
- Kit used (ELISA)
- Kit batch
- Kit expiry date
- Positive cut-off value

The laboratory work sheet with the results written by hand were attached to a copy of the submission form and ELISA printout (applicable to ELISA laboratory) indicating where the job number was on the printout for verification of the hand transcript.All completed jobs (D-numbers) and the number of samples per job were written in a book when results bundles were submitted to the administration office for results capturing on the system and report printing. The administration assistant signed in the book that they had received the results. The results were captured on an electronic system and a report printed. A technician verified the report. The technical signatory did second verification and authorised the report by signing it. The administration assistant then emailed the report. The reports were filed accordingly and retained for a period stipulated in ARC-OVR quality manual. The AHS ELISA is SANAS accredited.

3.4.5 ELISA Protocol

The AHS indirect ELISA is a serological assay which can be used for the detection of IgG antibodies raised against African horse sickness virus in serum or plasma. The assay

can be utilized for the detection of anti AHSV IgG antibodies in infected or vaccinated equines. Wild animals and canines can be infected with AHSV and seroconvert. Even though the assay was not validated for these species due to the scarcity of species specific AHSV antibody positive sera, the HRP conjugate binds to mammalian IgG, making the test suitable for detection of AHSV antibodies in mammalian species other than domestic equines.

AHSV infected or vaccinated animals produce IgG antibodies, which bind to the rVP7 antigen in the ELISA plate. The binding is revealed through addition of a protein G-horseradish peroxidase conjugate. The unbound conjugate is washed away, and a substrate/chromogen solution is added. In the presence of enzyme, the substrate is converted into a product which reacts with the chromogen to generate a blue color. Upon addition of the stop solution, a yellow color is generated. The absorbance at a single wavelength of 450 nm [A (450)] is measured using an ELISA plate reader (BIOTEK ELx 808). Color development indicates the presence of AHSV antibodies in the sample (positive result). ELISA technique is a qualitative method.

Prior to using the AHS indirect ELISA, the conjugate (Protein G-Horseradish Peroxidase), the positive and negative controls and the serum or plasma samples were prepared. The conjugate (Protein G-Horseradish Peroxidase) was diluted in StabilZyme HRP conjugate Stabilizer or in a diluent buffer, with reference to the specific batch data sheet. The Conjugate can be stored for one week at 2-8 ˚C if prepared with a conjugate stabilizer. The positive and negative control sera were diluted 1:100 in diluent-buffer (e.g., 5 µl of control serum plus 495 µl of diluent-buffer) into appropriate wells on the double-well dilution plate according to the planning sheet. Use a separate pipette tip for different control sera. Mix by gentle swirling or vortex mixer. Lastly serum or plasma samples were diluted 1:100 in diluent-buffer (e.g., 5 µl of serum or plasma sample plus 495 µl of diluent buffer) into appropriate wells on the double-well dilution plate according to the planning sheet. Separate pipette tips were used for different sera samples andthese were mixed by gentle swirling or use a vortex mixer.

ELISA Test Procedure

- Transfer 100 µl of diluted Positive and Negative Controls in duplicates (multi-channel pipette can be used) into appropriate wells on the AHSV rVP7 coated-plate according to the planning sheet.
- Transfer 100 µl of diluted samples into remaining wells in duplicates (multi-channel pipette can be used) using a separate pipette tip for each sample, into appropriate wells on the AHSV rVP7 coated plate according to the planning sheet. Mix the plates by gently swirling or firmly tapping the loaded plate 7 times to make sure the samples completely coat the bottom of the wells. Take care not to spill samples from well to well.
- Incubate for 1 hour (±5 minutes) at 35-39 °C.
- Wash each well with a diluted wash solution three times.
- Aspirate liquid contents of all wells after each wash. Automated Well-Washer can be used
- Following the final aspiration, firmly tap residual wash fluid from each plate onto absorbent material. Avoid plate drying between washes and prior to the addition of the next reagent.
- Dispense 100 µl of diluted conjugate to each well. Mix the plates by gently swirling or firmly tapping the loaded plate 7 times to make sure the conjugates completely coat the bottom of the wells. Take care not to spill samples from well to well.
- Incubate for 1 hour (±5 minutes) at 35 - 39 °C.
- Repeat step 6.4 – 6.6
- Dispense 100 µl of the substrate, TMB ready-to-use to each well. Mix the plates by gently swirling or firmly tapping the loaded plate 7 times to make sure the samples completely coat the bottom of the wells. Take care not to spill substrate from well to well.
- Incubate for 10 minutes (±2 min) at room temperature (18-25 ºC).
- Stop the reaction with 100 µl of Stop solution.
- Measure the absorbance of the samples and controls at 450 nm on a microplate reader BIOTEK ELx 808 on Software system (Gen 5.1)

ELISA Test validation

- For the assay to be valid, the Negative Control means (NCx) must have an OD less than 0.15.
- In addition, the Positive Control means (PCx) must have an OD of between 0.5-1.9
- If the controls are out of control limit ranges, test were invalid and were repeated.

Decision rule and interpretation of Results

- The results were rounded off to the nearest digit where applicable.
- Sample Percentage Positive (PP) values less than 5.0 were classified as negative for AHSV Ab.
- Sample Percentage Positive (PP) values greater than or equal to 5.0, but less than 10.0 were considered suspect for AHSV Ab. The animal was retested in a few weeks.
- Sample Percentage Positive (PP) values greater than 10.0 were classified as positive for AHSV Ab.

3.4.6. RNA extraction

Total ribonucleic acid (RNA) extracts were obtained from individual *Culicoides* and tick specimens using the Direct-zol RNA Miniprep kit from Zymo Research (#R2051). Extraction protocol for RNA extraction in a 20 µl reaction with 0.25 µM of each primer, at an annealing temperature of 58 °C, for 45 cycles. A 2µl aliquot of the resulting amplicons were analysed on a 1% agarose gel. A sample was assigned to a virus if an amplicon was present in the RT-PCR with the corresponding primer set.

3.4.7. PCR protocol

Both the PCR test for AHSV and LSDV use the one one-step real-time RT-PCR method. The advantage of this test is that it is faster to set up, cheaper and involves less handling of the samples thus minimising contamination and handling errors of the sample. In the one-step method, gene-specific primers are used, and both cDNA synthesis (RT) and PCR occured in one reaction tube. The RNA and primers were added to the tube and denatured at 95°C for 5 min, after 5 min the tube was quickly plunged into ice to stop the denaturing process. The enzyme was then added, and cDNA synthesis starts and annealing of the

primers take place usually at 52˚C for 50 cycles, but temperatures and duration of each cycle vary, depending on the PCR test performed for different viruses. After cycling, a 2µl aliquot of the resulting amplicons were taken and analysed on a 1% agarose gel.

For the AHSV PCR, the following unpublished primers specific to amplify a 255bp region of Segment-3 of AHSV (encoding VP3) was used:

VP3-F: ACGTTGAGTATTCAAATACGCCAGATA (Final: 0.5 mM concentration)

VP3-R: GCCCGCTAGAACGATTCACCAG (Final: 0.5 mM concentration)

For the LSDV PCR the following unpublished primers specific to amplify a 442bp region of the LSDV genome, as well as EmeraldAmp® GT PCR 2X Master Mix 2X (Takara) was used in this PCR method:

OP3: CACCAGAGCCGATAAC (Final: 0.5 mM concentration)

OP49: ATAGCTGCACTAGATAGCAC (Final: 0.5 mM concentration)

CHAPTER 4

RESULTS AND DISCUSSION *CULICOIDES* SPP.

4.1. *CULICOIDES* SPP. SURVEY

Light traps were operated at 10 collection sites within the study area from 2017-2022. Trapping started in 2017 as part of the ARC Climate Change Collaboration Centre (ARC CCCC) project on: The impact of climate change on identified priority agricultural pests and diseases. Over the six-year period, 918 collections were made and identified. In total 44,850 *Culicoides* specimens comprising of 49 *Culicoides* species were identified (Table 4.1). Since the focus of the study was to look at the overwintering of AHSV in *Culicoides* species, the priority was to analyse the winter and spring collections first. The remainder of the collections were identified later.

Six species were collected at all 10 collection sites, a further three species were present at 9 sites and three at 8 sites. Eight species were only collected at a single site. Ten of the 49 species collected during this study have not yet been described and named, these are indicated by numbers or as nr, this system was devised by Rudy Meiswinkel to indicate a new species until such a time that these species could be described and named. Site 3 recorded the most *Culicoides* species at 34 species, Sites 1, 5 and 6 all recorded 29 species and Sites 2 and 8 had 28 species while Site 9 had the least number of species at 15 species recorded (Table 4.1).

The diversity and distribution of *Culicoides* species within the Sarah Baartman municipality of the Eastern Cape Province, compares well with the results by Rawling *et al.,* (2003). Port Elizabeth were placed in cluster 1, where 48 species could be collected within this cluster. Cluster 8 had the greatest species diversity at 54 species and Port Edward, Mtunzini and Hell's gate made up this cluster (Rawlings *et al*., 2003). During the study by Rawlings *et al*., (2003) monthly light trap collections were made at 30 sites across South Africa over a two-year period, collecting over 3,000,000 *Culicoides* specimens comprising of 86 species. The different sites were grouped into clusters according to set criteria. This clustering system revealed that no area was free from *C. imicola* and *C. bolitinos,* the main vectors of AHSV (Rawlings *et al*., 2003).

Culicoides bolitinos was the dominant species collected, followed by *C. pycnostictus* Ingram and Macfie, and the third most abundant species collected was *C. imicola.* All

three these species were present at all 10 the collection sites. At Sites 1, 2, 3, 7 and 10 *C. bolitinos* was the dominant species collected and at Site 6 it was the second most abundant and at Site 4 the third most abundant species. *Culicoides imicola* on the other hand was the dominant species collected at Sites 4 and 9, the second most abundant at Site 5 and the third most abundant at site 7. At Site 5 *C. onderstepoortensis* Fiedler, was the dominant species collected, while at Sites 6 and 8 *C. pycnostictus* was the dominant (Table 4.1). The vector status of both *C. onderstepoortensis* and *C. pycnostictus* are not clear as both are considered ornithophilic species that prefer to feed on birds. Although Venter *et al.* (2009) did isolate AHSV from a single specimen of *C. pyconostictus* inoculated with AHSV serotype 3 and Paweska *et al.* (2002) isolated BTV from this species, but then other studies failed to retrieve virus from this species (Venter *et al.*, 2007).

The presence of both *C. bolitinos* and *C. imicola* increases the risk of AHS outbreaks in the area. Simple vector control measures, such as stabling, screening of stables and the use of repellents can be implemented, to reduce the risk of exposure to the bites of *Culicoides* midges if that will be economical viable to the owner of the property.

Weather patterns influence the distribution and occurrence of vector-borne diseases. A colonization area of 200 to 300 km of *C imicola* was observed in Europe in 1990 (Purse *et al.,* 2006). It is known that both *C. imicola* and *C. bolitinos* is widely distributed across South Africa and has been recorded at nearly every site surveyed (Labuschagne 2016). This means that not only are the study area are at risk for outbreaks of AHS, but the entire Eastern Cape Province and the rest of the country should be prepared for outbreaks under ideal conditions.

Table 4 1 Percentages of *Culicoides* species per site and overall percentage per species that was collected during the survey from 2017-2022 at the 10 collection sites. Number of Culicoides species collected per site and number of sites each species was present

Culicoides species	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Site 9	Site 10	Survey species %	Present No sites
C. sp. #33	22,06	-	-	-	-	-	-	0,15	-	-	5,593	2
C. sp. #50	0,10	0,07	-	-	-	0,03	0,14	0,07	-	-	0,053	5
C. sp. #54 (d/f)	1,05	1,30	4,04	0,10	-	0,39	11,13	13,57	-	0,50	2,690	8
C. sp. #54 (p/f)	-	-	0,03	-	-	-	-	-	-	-	0,004	1
C. sp. #61	-	-	0,03	-	-	-	-	0,07	-	-	0,009	1
C. sp. #66	0,02	0,07	-	-	-	0,16	-	-	-	0,50	0,040	3
C. sp. #75	0,07	-	-	-	-	-	-	-	-	-	0,018	1
C. sp. #90	0,21	-	0,05	0,05	0,49	-	0,07	4,47	0,75	--	0,385	8
C. sp. #94	-	0,69	0,16	-	0,10	4,61	0,69	-	-	-	0,792	5
Accraensis group	0,03	-	-	-	0,05	-	-	0,22	-	-	0,027	3
C. albopunctatus	-	-	-	-	-	-	-	0,07	-	-	0,004	1
C. bedfordi	0,09	1,55	0,13	0,15	1,67	0,10	-	0,07	0,75	9,00	0,500	9
C. bolitinos	55,92	37,32	64,01	13,95	2,79	24,69	53,84	12,16	7,46	34,00	38,916	10
C. brucei	0,02	0,29	1,00	-	0,29	0,19	-	-	-	0,50	0,265	6
C. dekeyseri	0,02	-	-	-	-	-	-	-	-	-	0,004	1
C. dutoiti	-	-	0,03	-	-	-	-	0,07	-	-	0,009	2
C. enderleini	-	0,04	0,05	-	0,05	0,10	0,14	-	0,75	-	0,044	6
C. engubandei	0,02	0,04	-	-	0,05	-	-	-	-	-	0,013	3
C. eriodendroni	-	-	-	0,15	-	-	-	-	-	-	0,013	1
C. exspectator	-	0,11	0,03	0,39	0,05	0,03	-	0,07	0,75	-	0,071	7
C. glabripennis	-	-	-	-	-	0,03	-	0,15	-	-	0,013	2
C. gulbenkiani	0,16	1,88	1,68	0,10	0,05	2,99	3,94	2,01	-	-	1,345	8
C. herero	-	-	-	-	0,10	-	-	-	-	-	0,009	1
C. hortensis	0,02	-	0,11	-	0,05	0,03	0,07	0,15	-	-	0,044	6

C. huambensis	2,24	0,90	0,24	0,44	-	4,48	0,55	0,07	-	-	1,407	7
C. imicola	2,12	5,42	4,44	48,79	18,48	2,14	7,53	1,27	25,37	5,00	9,102	10
C. isioloensis	0,33	0,11	0,58	0,73	2,84	0,29	4,49	5,07	-	-	1,146	7
C. kanagai	-	-	0,03	-	-	-	-	-	-	-	0,004	1
C. kibatiensis	-	-	-	-	-	0,06	-	-	-	-	0,009	2
C. leucostictus	0,77	5,17	2,60	-	3,58	17,29	-	3,58	6,72	11,50	4,301	10
C. macintoshi	0,07	-	-	-	-	-	-	-	-	-	0,018	1
C. magnus	0,17	7,95	1,21	3,94	-	1,78	-	-	-	2,00	1,841	6
C. micheli	-	-	0,03	-	0,15	-	-	-	-	-	0,018	2
C. neavei	-	0,25	0,18	-	0,20	0,16	0,07	-	0,75	1,50	0,124	7
Nigripennis group	-	0,04	0,03	-	-	-	-	-	-	-	0,009	2
C. nivosus	1,56	2,24	0,95	8,07	4,26	0,13	0,28	3,95	12,69	3,50	2,323	10
C. sp. nr angolensis	-	0,18	0,13	0,05	0,05	0,13	-	-	-	1,50	0,084	6
C. olyslageri	-	-	0,03	-	0,05	-	-	1,12	-	-	0,075	3
C. onderstepoortensis	-	0,47	0,08	0,68	29,66	0,16	0,07	-	-	1,00	2,845	7
C. pycnostictus	2,59	8,24	7,27	13,99	9,80	34,04	2,63	17,75	11,19	7,00	11,040	10
C. ravus	-	0,04	0,03	-	0,25	0,03	-	-	-	-	0,035	4
C. rhizophorensis	0,58	-	-	-	-	-	0,07	0,30	-	-	0,168	3
C. schultzei	0,24	-	-	-	0,49	-	0,07	0,07	-	-	0,115	4
C. similis	0,19	0,47	0,53	0,92	4,07	0,58	0,62	12,01	6,72	3,50	1,549	10
C. subschultzei	0,12	0,47	0,34	-	16,57	-	4,56	4,77	19,40	-	2,332	7
C. trifasciellus	0,03	0,04	0,03	-	-	0,03	-	0,37	-	-	0,044	6
C. tropicalis	-	-	0,03	-	0,93	0,36	0,07	0,07	0,75	-	0,150	6
C. tuttifrutti	8,86	2,35	3,91	-	0,78	0,81	4,08	16,26	2,24	0,50	4,615	9
C. zuluensis	0,35	22,33	6,04	7,53	2,11	4,15	4,91	-	3,73	18,50	5,783	9
No species/ site	29	28	34	17	29	29	22	28	15	16	49	

Figure 4.1 below depicts the seven top *Culicoides* species collected during the survey. Of the total number of *Culicoides* specimens identified during this study each of these seven species represent more than four percent of the total identified. Figures 4.2-4.8 depict the percentage range collected of each species at the different sites.

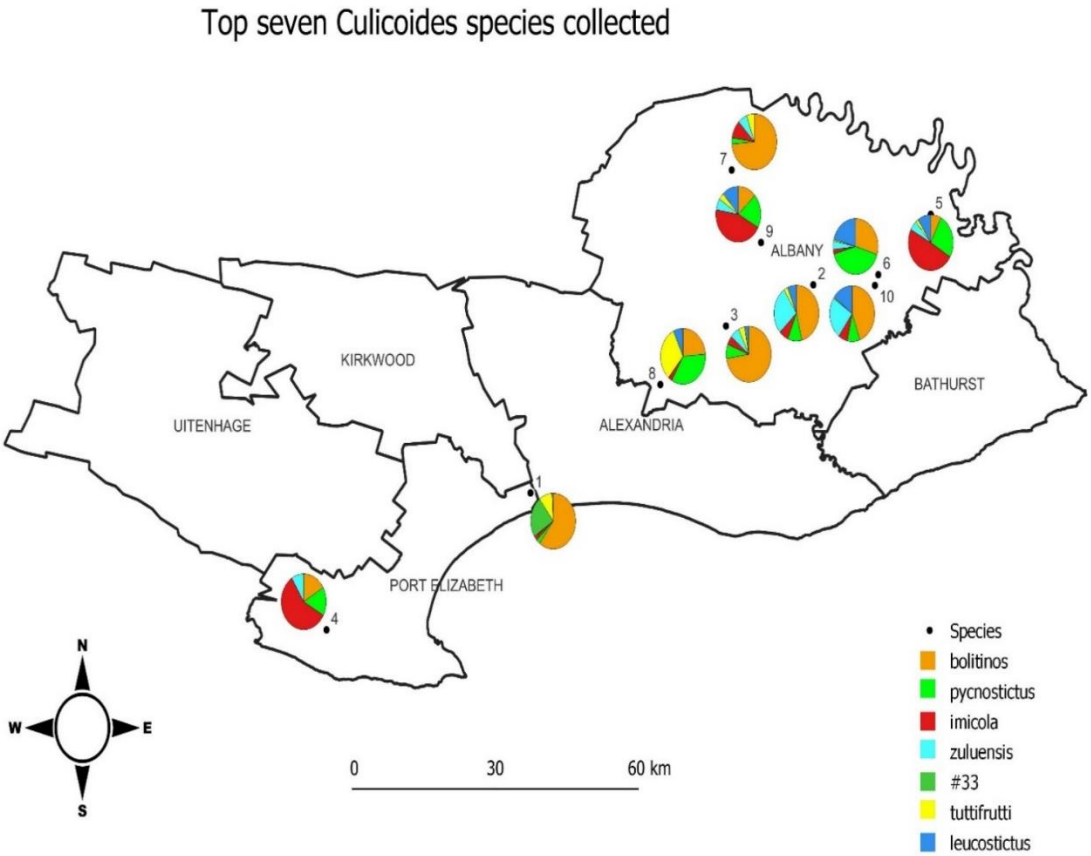


Figure 4 1: Map with pie charts reflecting the to seven *Culicoides* species collected at the collection sites (Labuschagne 2023)

During 1993, Rudy Meiswinkel collected *Culicoides* species in the sandy dune fields west of Port Elizabeth weather also played a major role in the number for *Culicoides* specimens collected. During this short survey, he collected midges at eight horse stables and dairies over a ten-day period with a single collection per site. The numbers he collected varied from 3 to 2,925 depending on whether it was a very windy night or a warm calm night. He did not collect a single specimen of *C. imicola* and concluded that due to the sandy soils, the area was free of *C. imicola.* The dominant species collected during the study by Meiswinkel was *C. bolitinos* at 91.7 % (Meiswinkel, 1997). Five years after this study it was shown that *C. bolitinos* was also a vector of AHSV and BTV (Meiswinkel & Paweska, 2003). Only one site from the study by Meiswinkel (1997) and this current study were the same site in this sandy dune fields of Port Elizabeth. Where Meiswinkel

collected no *C. imicola* at this site we collected this species and in fact it was the dominant species collected at 48.8%. He did however collect *C. bolitinos* at this site, though this was one of the sites where he only collected 24 specimens of *Culicoides* due to a very windy night. This site has changed during the approximately 25 years from when Meiswinkel did his study and when the current study started. A very large new stable block was added, where we collected in comparison with the old block where Meiswinkel collected. More irrigated paddocks were added and due to the new stable block, the density of horses increased. Unfortunately, this was one of the sites that withdrew from the study.

A recent survey at 22 sites in 2015 and 2016 in the Port Elizabeth and Grahamstown area recorded 39 species with *C. bolitinos* the dominant species, followed by *C. imicola* and *C. pycnostictus* as the third most abundant species (Riddin, 2017; Riddin *et al.,* 2019). Only two sites in the Grahamstown area were resampled during this current study. Both surveys were similar in that neither *C. bolitinos* nor *C. imicola* was the dominant species collected. The current study was conducted over a six-year period, with one of the sites participating for the duration of the study and the main difference was the number of species collected. The current study collected 28 and 29 species for these two sites while the 2015-2016 study only collected 4 and 11 species.

Both *C. imicola* and *C. bolitinos*, known vectors of AHSV were collected at all 10 collection sites. At Sites 4 and 9, *C. imicola* was the dominant species collected, and at site 5 *C. imicola* was the second most common species after *C. onderstepoortensis* (not reflected on this top seven species collected map as very low numbers were collected at the other collection sites). On the other hand, *C. bolitinos* was the dominant species collected at Sites 1, 2, 3, 7 and 10. At Site 6 *C. bolitinos* was the second most common species collected after *C. pycnostictus* and at Site 8 it was the third most common species after *C. pycnostictus* and *C. leucostictus.*

4.1.1 *Culicoides bolitinos*

Culicoides bolitinos has successfully moved from buffalo to cattle dung and that is why it has managed to be an abundant species in areas where cattle are kept (Nevill *et al.,* 2007). *Culicoides bolitinos* was collected at all 10 sites (Figure 4.2). At Sites 4, 5, 8 and 9 less than 20% of the total *Culicoides* specimens collected at each site was of this species.

However, at Site 4 there was no cattle though in the adjacent farms cattle were present. At Site 9 there were also no cattle present. There were cattle present at Sites 5 and 8.

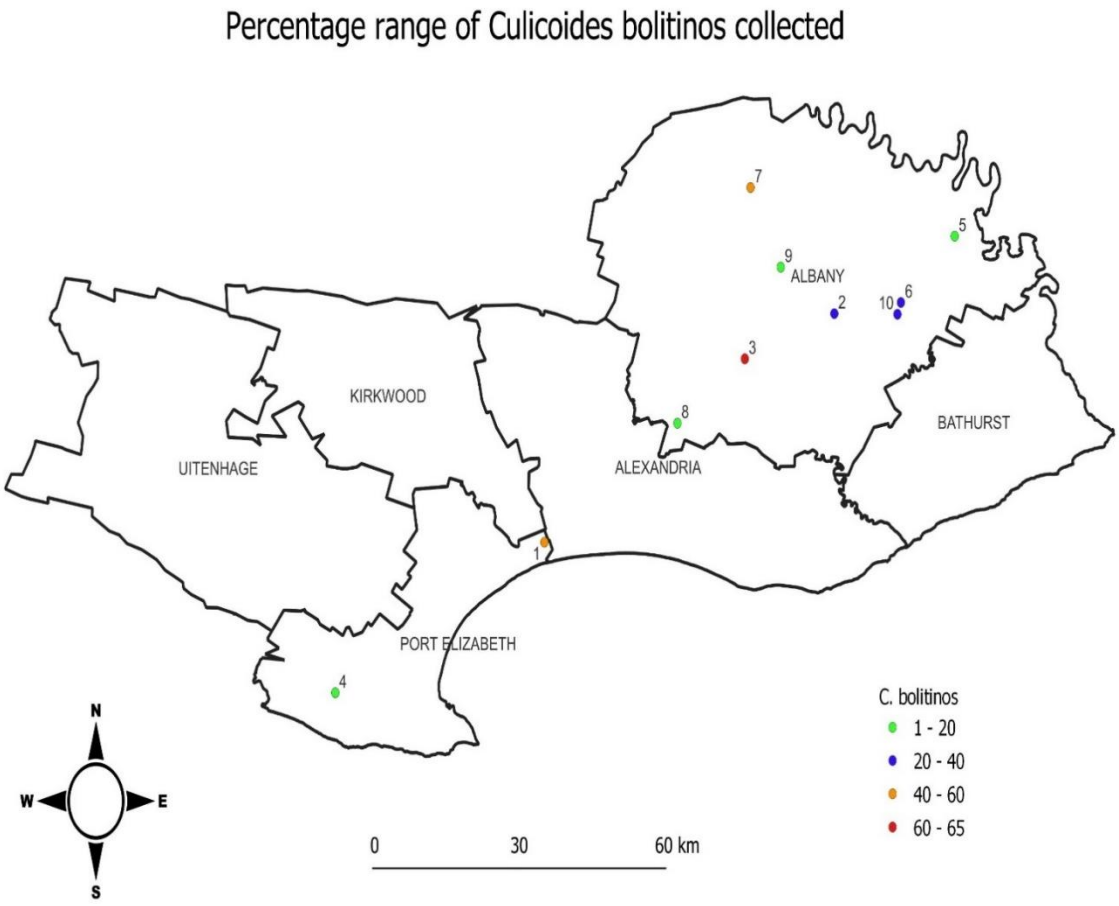


Figure 4 2: Map with percentage range of *Culicoides bolitinos* collected at the collection sites (Labuschagne 2023)

PCR was done on 4 pools of *C. bolitinos* from Site 1 in Port Elizabeth area and on 3 pools from Site 3 in the Grahamstown area to test for the presence of AHSV. Some pools tested positive for AHSV with the inhouse PCR test. With the WOAH accredited and validated PCR test one of these again tested positive. Both sites were having cattle, and the immatures were utilising the dung as breeding media. In areas where both cattle and horses are kept together, the chance of AHS outbreaks will be predominant.

Culicoides bolitinos has been collect in the following countries across Africa: Ivory Coast, Kenya, Lesotho, Malawi, Mauritius, Nigeria, South Africa and Zimbabwe. This species has also been recorded in all nine Biomes of South Africa. The biomes are Fynbos, Succulent Karoo, Desert, Nama-Karoo, Grassland, Savanna, Albany Thicket, Indian Ocean Coastal Belt and Forests. *Culicoides bolitinos* has been implicated as a vector of

both AHSV and BTV. This species breeds in the dung of cattle (Labuschagne *et al.*, 2007), buffalo and wildebeest dung (Braverman & Boorman, 1978; Lubega & Khamala, 1976; Meiswinkel, 1989, 1995, 1996; Nevill, 1968; Nevill *et al.*, 2007).

4.1.2 *Culicoides pycnostictus*

Culicoides pycnostictus is a widespread and common species that can become locally abundant and was collected at all 10 sites Figure 4.3). At Sites 6 and 8 this was the dominant species collected. Only at Site 6 more than 20% of the total *Culicoides* specimens collected at this site was of this species. Although *C. pycnostictus* was the dominant species at Site 8 it must be noted that overall low numbers of *Culicoides* specimens were collect at this site. Nevill *et al.* (1992) isolated BT virus from this species during a six-year survey conducted from 1979-1985 across South Africa.

Culicoides pycnostictus has been collect in the following countries across Africa: Ethiopia, Gambia, Kenya, Mozambique, Nigeria, Senegal, South Africa, Uganda and Zimbabwe. This species has also been recorded in all the nine Biomes of South Africa. This species breeds in mud collected from river, dam, lake edges, rainwater, seepage pools, drainage and artificial channels and from mud mixed with bird excreta or rotting vegetation (Khamala, 1975; Lubega & Khamala, 1976). Precipitin tests have shown that this species feeds on both larger mammals and birds, though it is considered to feed mainly on birds (Nevill & Anderson, 1972).

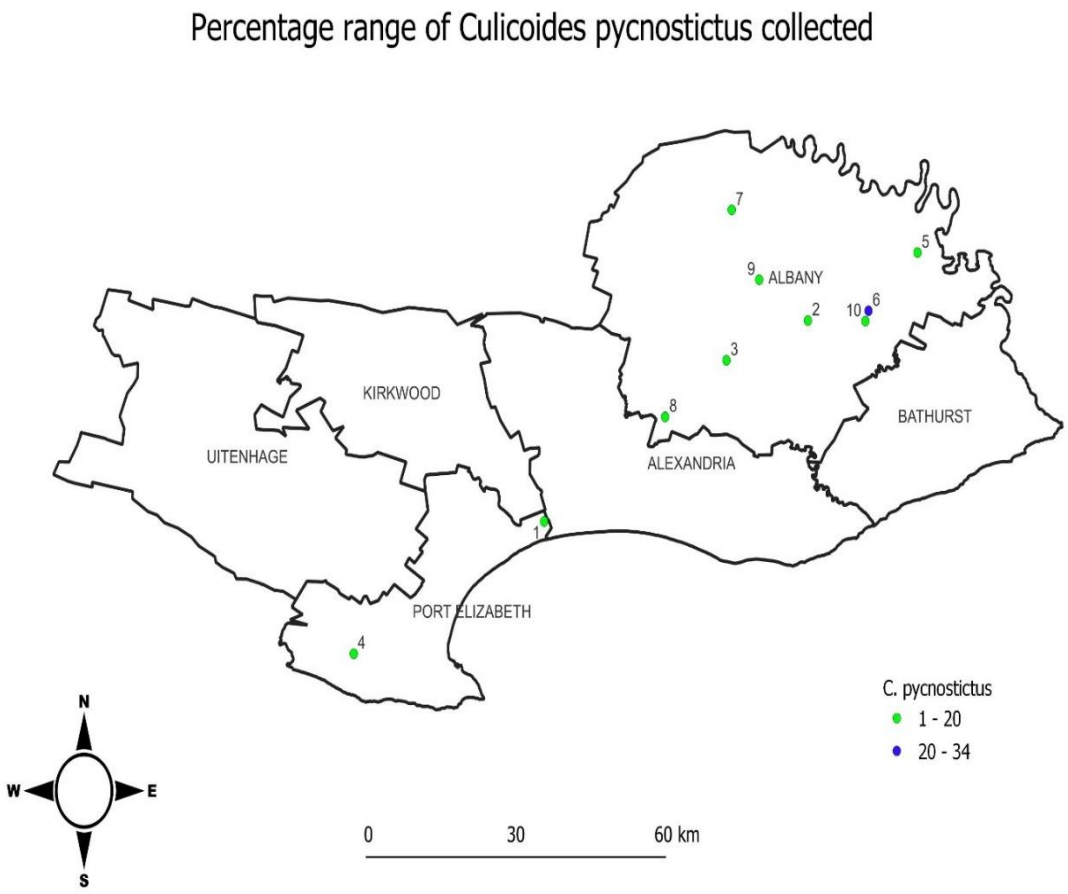


Figure 4. 3: Map with percentage ranges of *Culicoides pycnostictus* collected at the collection sites (Labuschagne 2023)

4.1.3 *Culicoides imicola*

Culicoides imicola is a very widely distributed and common species that can become super abundant under ideal conditions (highest collection ever made in a single trapping night with a single light trap was +/- 1.5 million at Vastfontein, Onderstepoort, South Africa, on the night of 10 February 2008) and was collected at all 10 sites. At Sites 4 and 9 where *C. imicola* was the dominant species collected, the only animals present at these sites were horses (Figure 4.4). At the other 8 sites less than 20% of the total *Culicoides* specimens collected were of this species. Nevill *et al.* (1992) isolated AHS, BT, EE, Letsitele and Simbu viruses from this species during a six-year survey conducted from 1979-1985 across South Africa.

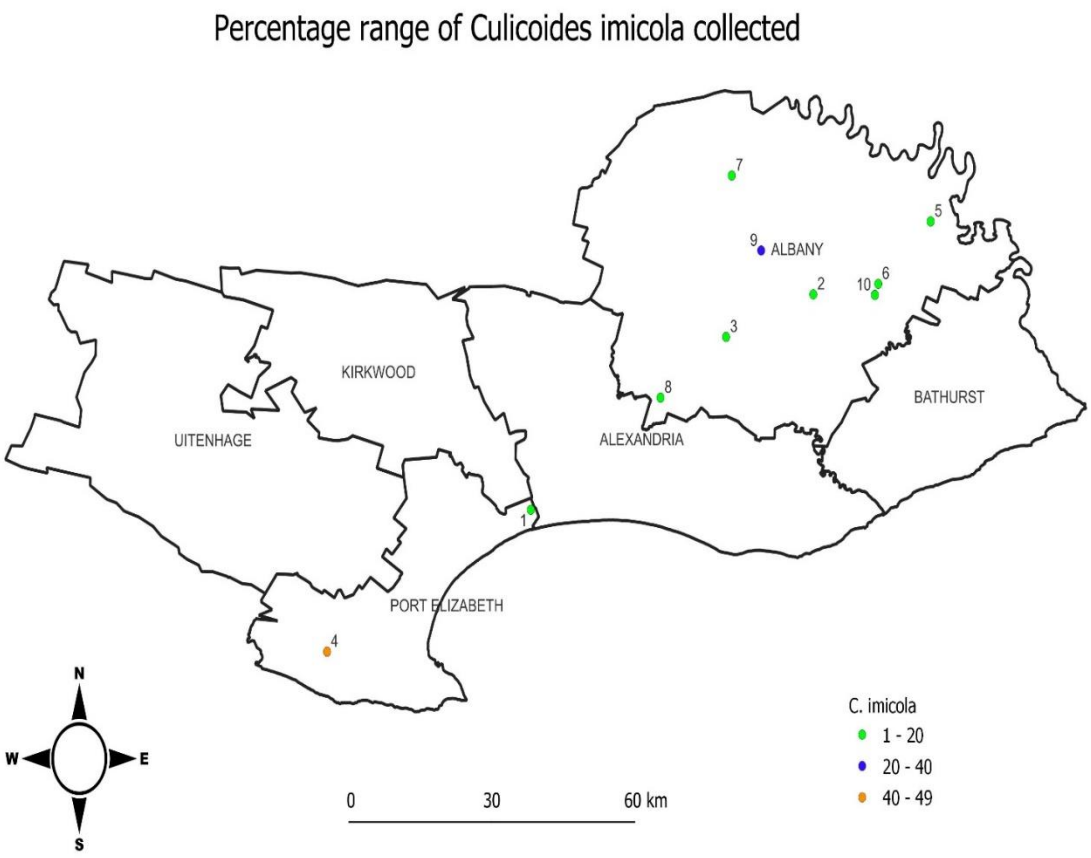


Figure 4 4: Map with percentage ranges of *Culicoides imicola* collected at the collection sites (Labuschagne 2023)

Culicoides imicola has been collected across most countries in Africa, southern Europe, and eastwards up to India. This species has also been recorded in all nine the Biomes of South Africa. *Culicoides imicola* has been shown to be able to transmit at least 11 different viruses including African horse sickness, Bluetongue, equine encephalosis, Akabane, Bovine ephemeral fever, Letsitele, Nyabira, Sabo, Shamonda, Simbu and several unidentified viruses. This species breeds in moist organically enriched soils and have been reared from mud collected from the edges of water bodies, rivers, dams, drainage and sewage channels and moist areas caused by dripping or leaking pipes around water troughs (Braverman, 1974; Braverman & Boorman, 1978; Lubega & Khamala, 1976; Meiswinkel, 1989, 1995, 1996; Labuschagne *et al.*, 2007). Some authors incorrectly assign the breeding site for *C. imicola* as dung (Braverman & Boorman, 1978; Lubega & Khamala, 1976; Nevill, 1968). The species most likely reared by these authors was *C. bolitinos*.

Precipitin tests indicated host preferences to be cattle, sheep, horses and poultry (Braverman *et al.*, 1971; Clastrier & Wirth, 1961; Dipeolu, 1976; Nevill & Anderson, 1972; Walker & Boreham, 1976; Walker & Davies, 1971).

4.1.4 *Culicoides zuluensis*

Culicoides zuluensis is a widespread and common species, often collected in light traps. *Culicoides zuluensis* can become locally abundant in favourable conditions and was collected at 9 of the collection sites but it was never the dominant species collected (Figure 4.5). At Site 2 (22.3%) and Site 10 (18.5%) the highest numbers of this species were collected. At the other 8 sites less than 20% of the total *Culicoides* specimens collected at these sites was of this species. Nevill *et al.* (1992) isolated Letsitele from this species during a six-year survey conducted from 1979-1985 across South Africa.

Culicoides zuluensis has been collected in Kenya, South Africa and Zimbabwe and were collected in 8 of the 9 biomes in South Africa, it was only absent from the Desert biome. This species breeds in mud from river, stream and drainage canal edges (Braverman & Boorman, 1978; Lubega & Khamala, 1976) and have been recorded as feeding on birds, goats, sheep and horses (Walker & Boreham, 1976).

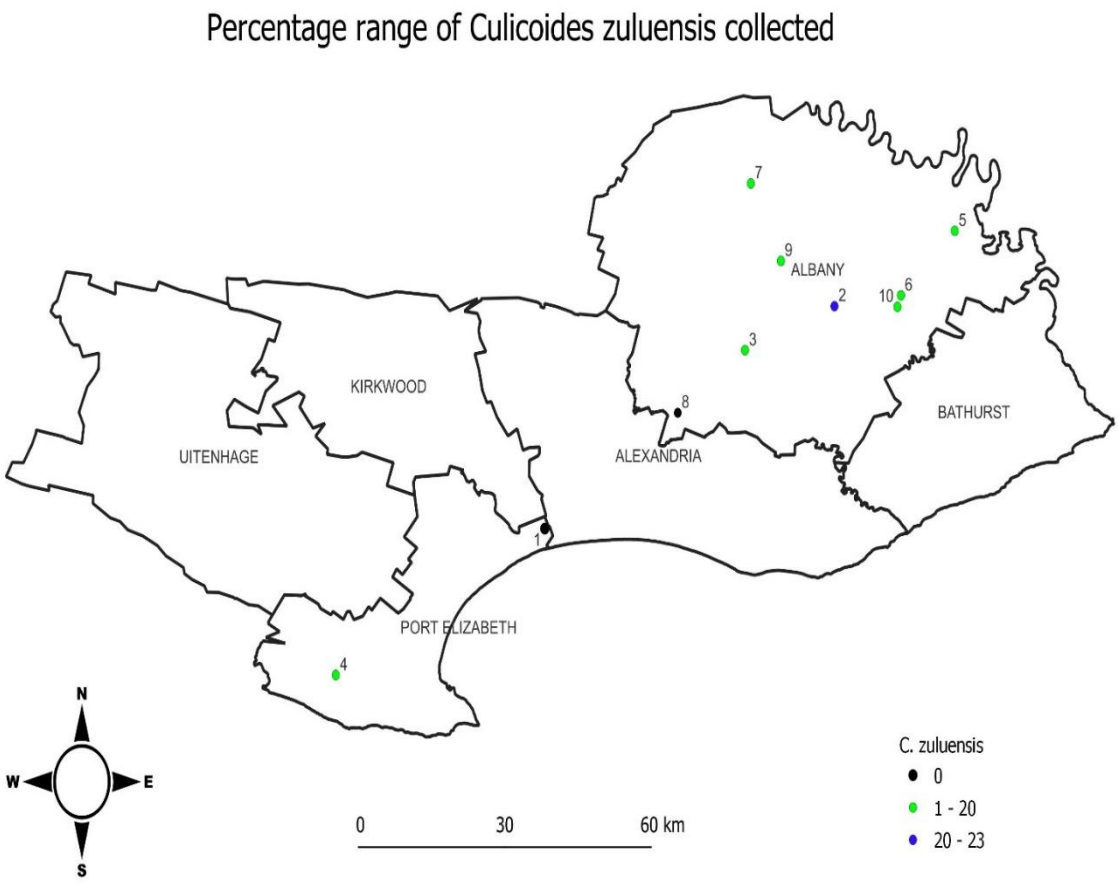


Figure 4. 5: Map with percentage ranges of *Culicoides zuluensis* collected at the collection sites (Labuschagne 2023)

4.1.5 *Culicoides sp. #33*

Culicoides sp. #33 is one of the species that still need to be described and named. Although this species is not commonly collected it was present at two of the collection sites (Figure 4.6). At Site 1 it was the second most common species collected and was present in nearly every collection made. This species has only been collected from the Western Cape, Eastern Cape and Northern Cape provinces and was collected in the following biomes: Fynbos, Succulent Karoo, Desert, Nama-Karoo, Grassland and Albany Thicket. The vector status, breeding site and host species are unknown.

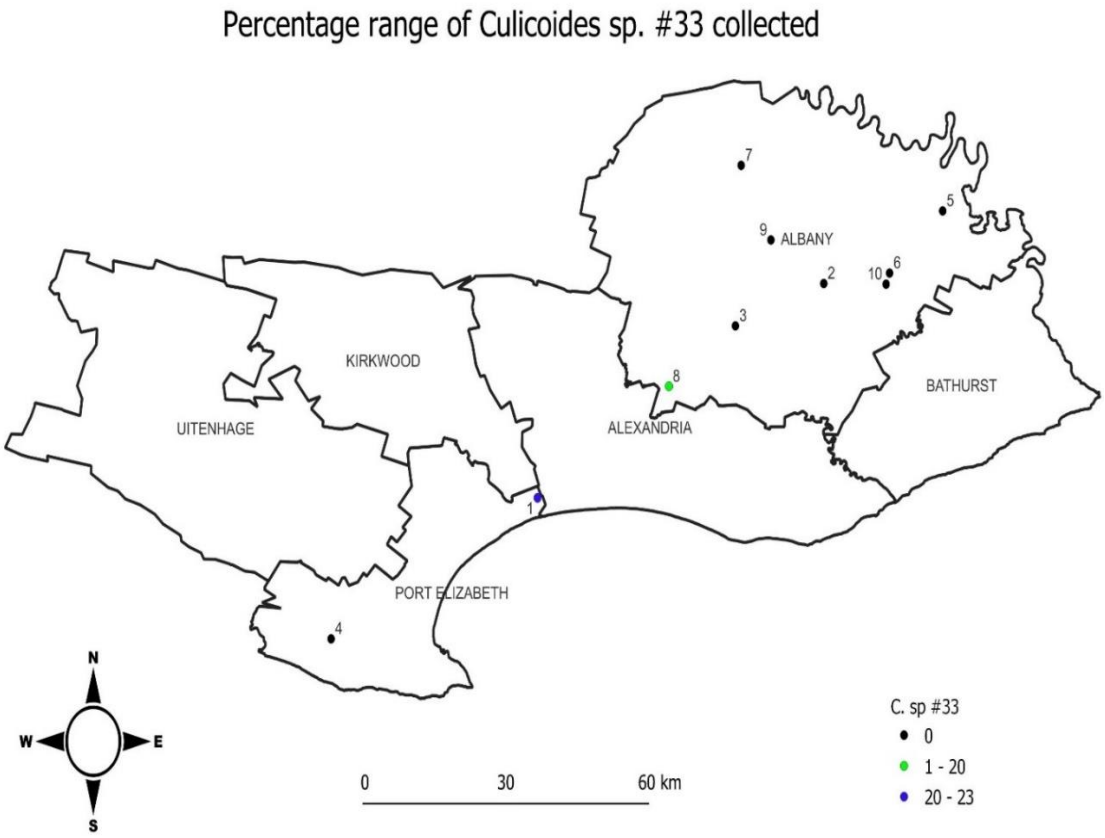


Figure 4. 6: Map with percentage ranges of *Culicoides* spp. #33 collected at the collection sites (Labuschagne 2023)

4.1.6 *Culicoides tuttifrutti*

Culicoides tuttifrutti Meiswinkel, Cornet and Dyce is a widespread species that is rarely collected in large numbers but can become locally abundant. In 2004 nearly 12,300 specimens were identified from 15,834 *Culicoides* collected at a farm in the Grahamstown area in the Eastern Cape Province (Labuschagne, 2016). This species breeds in rotting fruit (Meiswinkel & Linton 2003; Nevill *et al.*, 2007; Sebastiani *et al.*, 2001) that include fruits from *Kigelia africana* (sausage tree), *Sclerocarya birrea* subsp. *caffra* (maroela) and other indigenous fruits as well as *Ananas comosus* (pineapple).

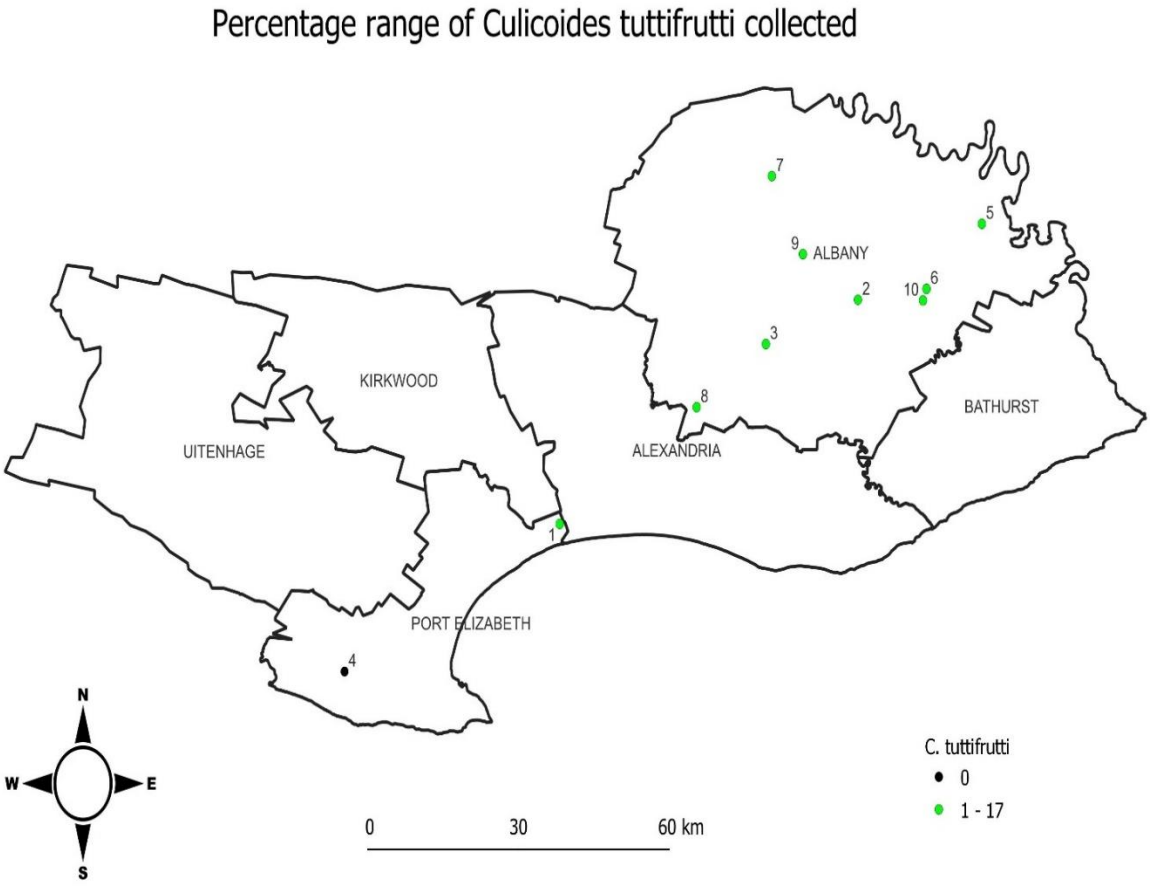


Figure 4. 7: Map with percentage ranges of *Culicoides tuttifrutti* collected at the collection sites (Labuschagne 2023)

During this survey this species was collected at 9 of the collection sites (Figure 4.7). Although it was never the dominant species during the survey it was the second most common species at Site 8, and the third most common species at Site 3. This species has only been collected from Ivory Coast, Malawi, South Africa and Zimbabwe and were collected from the following biomes in South Africa: Fynbos, Nama-Karoo, Grassland, Savanna, Albany Thicket, Indian Ocean Coastal Belt and Forests. Meiswinkel recorded this species as feeding on mammals and birds (Meiswinkel 1995). The vector status of this species is unknown.

4.1.7 *Culicoides leucostictus*

During this survey *C. leucostictus* was collected at 8 of the collection sites (Figure 4.8). Although *C. leucostictus* is a widespread and common species, it was never the dominant species collected during the survey. However, *C. leucostictus*, it was the third most common species at Sites 6 and 10.

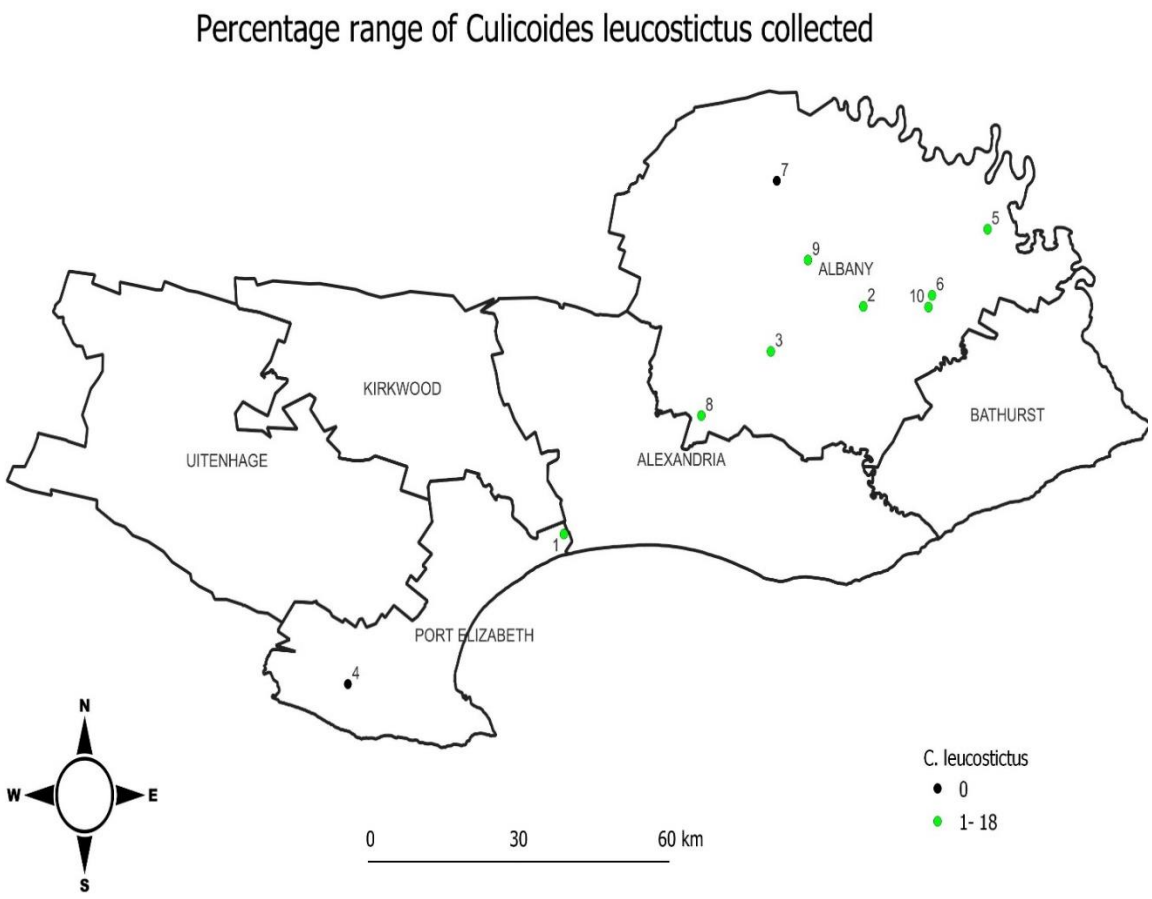


Figure 4.8: Map with percentage ranges of *Culicoides leucostictus* collected at the collection sites (Labuschagne 2023)

Culicoides leucostictus is a widespread and common species that has been collected in Cameroon, Egypt, Ethiopia, Ghana, Guinea, Kenya, Mozambique, Nigeria, Senegal, Seychelles, South Africa, Sudan, Uganda and Zimbabwe. This species has been collected in all 9 biomes of South Africa and breeds in mud from the margins of ponds, puddles, streams and in boggy areas. Decaying vegetation including banana stems, artificial, natural, crab and rot holes (Braverman, 1974; Callot *et al.,* 1967; Dipeolu, 1976; Lubega & Khamala, 1976). Precipitin tests have shown that this species mainly feed on birds, and it may become the dominant species collected near birds (Braverman & Rubina, 1976). The vector status of this species is unknown though it may become infected in the laboratory when fed on a high titre of virus (Paweska *et al.,* 2002; 2003; Venter *et al.,* 1998, 1999, 2004; Venter, *et al.,* 2006).

The current study highlighted the diversity and density of *Culicoides* species in the study area and on each participating farm.

CHAPTER 5
RESULTS AND DISCUSSION TICKS

5.1. TICK SURVEY

Livestock farming is very important in sub-Saharan African as it contributes to about 25% of the gross domestic product of the region (Van Den Bossche & Coetzer, 2008). This study identified the different tick species found on different hosts within the study area, which may adversely affect the health of livestock through disease transmission and blood feeding. Ixodid ticks are classified as 1, 2 or 3-host species. Most tick species of public health importance are three-host ticks.

Table 5.1 depicts whether t the 10 species collected during the survey, were a 1, 2 or 3 host species, host and area on host tick was collected from, other potencial hosts and attachement sites on those hosts, as well as the pathogens that can be transmitted by each of these tick species.

From the tick survey 10 species, from four genera were identified (Figure 5.1 and Table 5.2). Figures 5.2 to 5.11 depict the distribution of each species with the number range of that species collected at each of the collection sites. All 10 species have previously been recorded from the Eastern Cape Province. Six species within the genus *Rhipicephalus*, were collected and two in the genus *Amblyomma* and one species each in the genera *Haemaphysalis* and *Hyalomma. Rhipicephalus decoloratus, Rhipicephalus evertsi evertsi* and *Amblyomma hebraeum* were the 3 dominant species collected. During the study the following one-host ticks were collected: *Rhipicephalus decoloratus* and *Rhipicephalus microplus.* Two-host ticks collected were: *Hyalomma rufipes* and *Rhipicephalus evertsi evertsi.* Three-host ticks collected were: *Amblyomma hebraeum, Amblyomma marmoreum, Haemaphysalis elliptica, Rhipicephalus follis, Rhipicephalus gertrudae* and *Rhipicephalus simus.*

Table 5.1 Tick species, 1, 2 or 3 host species, hosts ticks collected from in this survey, areas attachment, hosts review studies, areas of attachment and pathogens transmitted.

Tick species	1, 2 or 3-host species	Hosts collected from in this survey	Area where ticks were attached during this survey	Hosts review studies	Areas of attachment	Pathogens transmitted
Amblyomma hebraeum	Three-host	cattle, goats, horses	Genital area, scrotum, udder	Cattle, sheep, goats, giraffe, African buffalo, elands, warthogs, rhinoceroses, Birds immature stages same hosts as adults also birds, tortoise	Adults prefer under tail, lower perineal area, udder, testes, prepuce, axilla of cattle, around feet sheep and goats, Larvae found on feet, legs, groin sternum, Head, neck, wings	*Anaplasma* sp. *Ehrlichia ruminantium* *Theileria* sp *Rickettsia africae*
Amblyomma marmoreum	Three-host	Goats	Anal area	Tortoise, Birds immature stages	Base of the legs and tail Head, neck, wings	Unknown
Haemaphysalis elliptica	Three-host	Cattle	All over the body	Dogs, cats, large wild felids	Head, neck, shoulders and severe infestations all over the body	*Babesia rossi* *Anaplasma* sp *Babesia* sp *Hepatozoon* sp *Theileria* sp

Hyalomma rufipes	Two-host	Cattle	Under the tail	Cattle, sheep, goats, large wild herbivores, rhinoceroses, guineafowl	Bare areas around anus, genitalia, hooves of sheep, immature on heads and neck of birds	*Babesia occultans* *Theileria* sp *Theileria buffeli* *Theileria taurotragi* *Ehrlichia* sp
Rhipicephalus decoloratus	One-host	cattle, horses, sheep, goats, dog	All over the body	Cattle, horses, donkeys, sheep, goats and wild animals	Back, upper legs, neck, shoulders, dewlap and belly	*Anaplasma marginale* *Babesia bigemina* *Babesia caballi* *Theileria* sp *Theileria ovis* *Theileria buffeli* *Theileria annulata* *Ehrlichia ovina* *Ehrlichia* sp
Rhipicephalus evertsi evertsi	Two-host	horses, cattle, goats	Anal area, groin, udder, scrotum	Cattle, horses, Zebra and Elands, sheep, goats, impala, blue wildebeest, African buffalo and greater kudu	Peri-anal area, inner thighs, groin, immature stages attach to external ear canal	*Babesia caballi* *Theileria* sp *Theileria bicornis* *Theileria equi* *Theileria separata*

						Theileria buffeli *Theileria taurotragi* *Theileria annulate* *Anaplasma bovis* *Anaplasma marginale* *Borrelia theileri* *Rickettsia conori* *Ehrlichia* sp
Rhipicephalus follis	Three-host	Horses	Neck and under the tail	Cattle, eland, horses, mountain zebra, immature stages prefer 4 stripped mouse	Under tail, genitalia, neck	Unknown
Rhipicephalus gertrudae	Three-host	Horses and cattle	Above eyes, Neck, shoulder	Cattle, sheep, Dogs, large wildlife, young baboons, incidents of human feeding	Ears, muzzle, hands, feet,	Unknown
Rhipicephalus microplus	One-host	Cattle	All over the body	Cattle, goats, In presence of cattle they can attach to other domestic animals and wildlife	Belly, dewlap, shoulders and flanks	*Babesia bigemina* *Babesia bovis* *Anaplasma marginale*

Rhipicephalus simus	Three-host	Cattle and horses	Inner thighs, On the tai of horses	Monogastric animals, wild carnivores, equids, Suids, Cattle and African buffalo and to a lesser extent sheep, Dogs	Tail brush and around the feet of cattle, on the tail of horses and zebra, head and shoulders of dogs and warthogs, sheep around feet, immature on murid rodents	Unknown

Previous studies have implicated *Rhipicephalus sanguineus* and *Hyalomma dromedarii* as the vectors of AHSV (Salama *et al.*, 1981; Awad *et al.*, 1981). These two tick species were not among those that were identified in the study area (Figure 5.1). *Hyalomma dromedarii* is distributed and adapted to the dry areas, this species occur mainly north of the equator though it was introduced to Namibia with the importation of camels. *Rhipicephalus sanguineus* has previously been recorded from South Africa and from the Easetrn Cape Province, though it was not collected during this current study (Horak *et al.*, 2009).

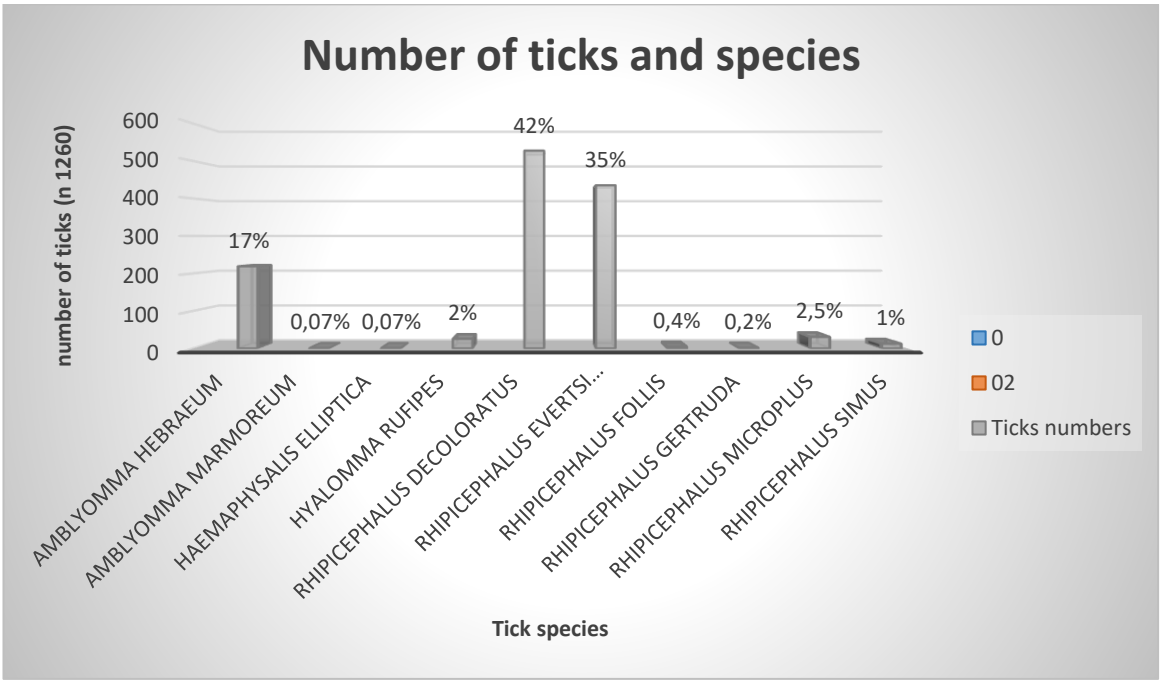


Figure 5 1: Chart showing the number of ticks and tick species

Table 5. 2 Tick species, number collected per site and total per species

species	Site 3	Site 6	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15	Site 16	Site 17	Site 18	Site 19	Site 28	Total per species
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Amblyomma hebraeum	10 (10.5)	2 (0.9)	47 (20.7)	0	4 (6.1)	0	14 (100)	29 (25.4)	84 (52.2)	2 (8.3)	6 (66.7)	11 (5.6)	10 (90.9)	219 (17.4)
Amblyomma marmoreum	0	0	1 (0.4)	0	0	0	0	0	0	0	0	0	0	1 (0.1)
Haemaphysalis elliptica	0	0	0	0	1 (1.5)	0	0	0	0	0	0	0	0	1 (0.1)
Hyalomma rufipes	0	11 (4.9)	0	0	4 (6.1)	0	0	0	11 (6.8)	0	0	0	0	26 (2.1)
Rhipicephalus decoloratus	0	0	91 (39.9)	56 (100	40 (60.6)	59 (100)	0	76 (66.7)	48 (29.8)	15 (62.5)	0	141 (71.2)	1 (9.1)	527 (41.8)
Rhipicephalus evertsi evertsi	84 (88.4)	204 (90.7)	88 (38.6)	0	17 (25.8)	0	0	6 (5.3)	15 (9.3)	0	3 (33.3)	18 (9.1)	0	435 (34.5)
Rhipicephalus follis	0	5 (2.2)	0	0	0	0	0	0	0	0	0	0	0	5 (0.4)
Rhipicephalus gertrudae	0	1 (0.4)	1 (0.4)	0	0	0	0	0	0	0	0	0	0	2 (0.2)

Rhipicephalus microplus	0	0	0	0	0	0	0	3 (2.6)	0	0	0	28 (14.1)	0	31 (2.5)
Rhipicephalus simus	1 (1.1)	2 (0.9)	0	0	0	0	0	0	3 (1.9)	7 (29.2)	0	0	0	13 (1.0)
Total	95	225	228	56	66	59	14	114	161	24	9	198	11	1,260

5.1.1 *Amblyomma hebraeum*

Amblyomma hebraeum is known as the South African bont tick which prefers cattle, sheep, goals and large wildlife, particular giraffes (*Giraffa camelopardalis*), African buffalo (*Syncerus caffer*), eland (*Taurotragus oryx*) (Madder *et al.*, 2014). A study by Horak (1991) showed that the immature stages of eight ixodid tick species were abundant on birds and *A. hebraeum* was the second most abundant species collected from birds and this species was also collected from guineafowls. While these ticks are off their preferred host and on the ground, they will attach to humansand then they can transmit *Rickettsia africae* which causes tick-bite fever in humans (Horak *et al.*, 2002).

During the current study this species was collected from cattle, goats and horses at Sites 3, 10, 14, 15, 16, 17, 18 and 19 (Figure 5.2). This is a three-host tick which is commonly found in, bushy wooded areas, of the Eastern Cape province and can also survive in open grasslands. This is a big tick with long mouth parts, is brightly coloured with a stricking pattern on the scutum of both males and female, has smooth eyes, and brown and white banded legs (Walker *et al.*, 2000).

The larvae of this species were recorded to be abundant on vegetation in late summer to early winter and less abundant in in spring to early summer in the Eastern Cape Province. *Amblyomma hebraeum* is often collected on cattle during spring and summer months in South Africa. During this study collection of the tick species were done during winter months (May to July).

This species prefers bare areas on cattle such as axillae, groin, belly, sternum and upper lower perineum. This tick species is a vector to *Ehrlichia ruminantium* which is a causal agent to heartwater. Heartwater is one of the diseases that cause major losses in ruminants (Raoult & Roux, 1997).

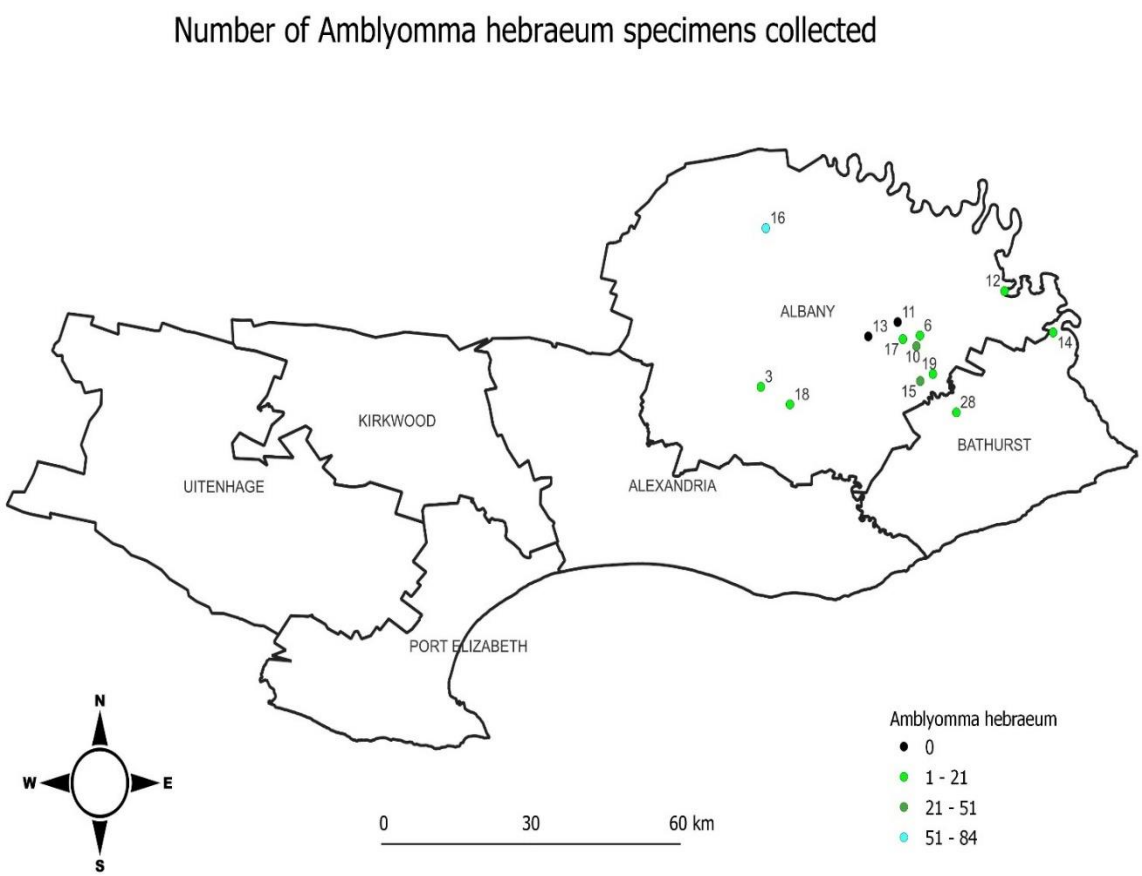


Figure 5 2: Map depicting the number range of *Amblyomma hebraeum* collected at the collection sites (Labuschagne 2023)

5.1.2 *Amblyomma marmoreum*

Amblyomma marmoreum, also called the South African tortoise tick. During this study, this three-host tick was found in Site 10 attached to cattle (Figure 5.3). In addition to tortoises these ticks are also found on cattle, sheep and goats and also on variety of wild mammals and birds. Collection of this tick indicate that it has been attached for at least 15 days on 1 host as a larval and nymphal stages each require 7 days before moulting to the next stage (Horak *et al.,* 2002).

This species completes its life cycle on tortoises, mostly on the leopard tortoise (Horak *et al.*, 1991). Adult tick rarely parasitize domestic livestock although the larvae in particular, are sometimes seen on domestic animals, wild carnivores, antelopes, helmeted guineafowls and other ground frequenting birds (Madder *et al.*, 2014).

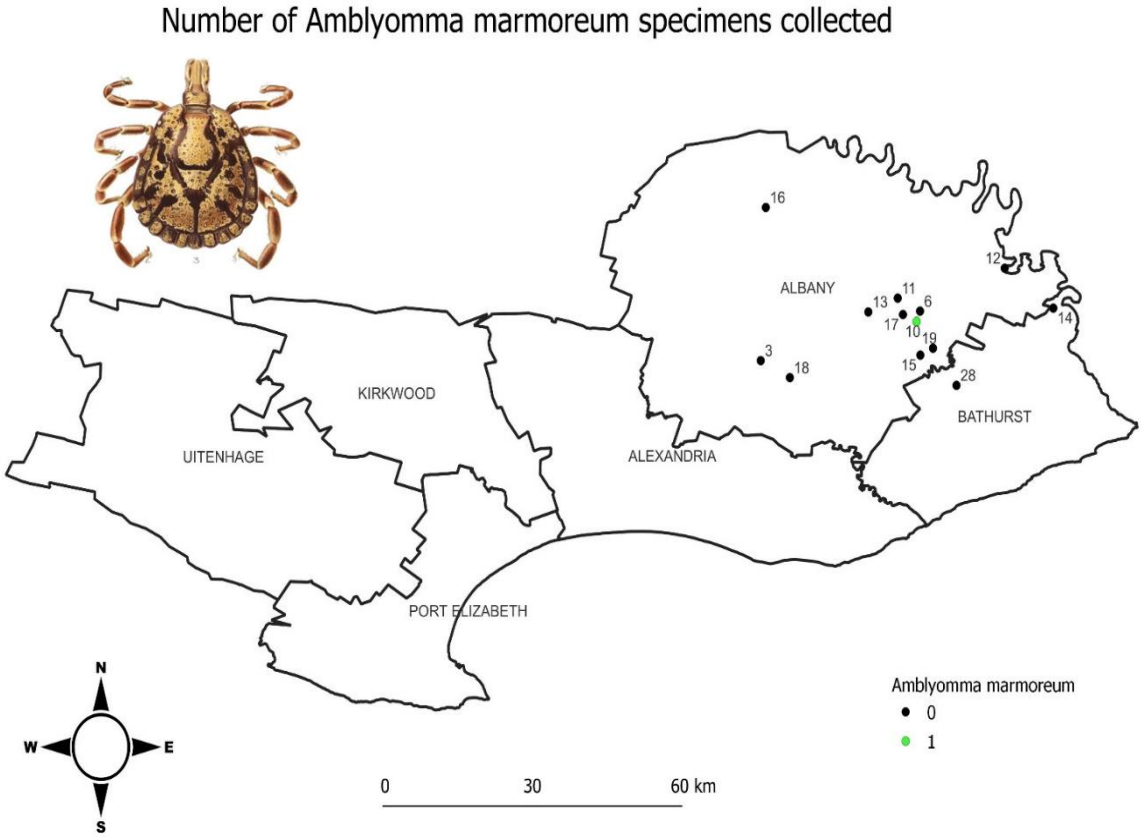


Figure 5 3: Map depicting the number range of *Amblyomma marmoreum* collected at the collection sites (Labuschagne 2023)

In colour this species is dull yellow to beige, tinged with light pink, a triangular patch posterior to the pillar and triangular posterior patches, a dark brown club shaped posteromedial strip and broad posterolateral strips may be evident in triangular posteromedial spur. Its legs are long, light to dark brown. This tick occurs in all nine provinces of South Africa (Horak et al, 2006). This tick species was predominantly collected from adult leopard tortoise, but parrot-beaked tortoise and geometric tortoise also hosts for all stages of development (Horak *et al.*, 2006).

5.1.3 *Haemaphysalis elliptica*

Haemaphysalis elliptica is know as the South African yellow dog tick. During this survey one specimen of this tick was found attached to cattle at Site 12 (Figure 5.4).

It is a long and broader tick. The marginale grove encloses one or two of its first festoons. The colour of this tick species is reddish brown. The dorsal median margin of palpal segment II gradually widening anteriorly from segment's mid-length (Apanaskevich *et*

al., 2007). It is a three-host tick and its adults also attach to larger wild carnivores (Apanaskevich *et al.,* 2007). In other surveys adult ticks are commonly found on dogs, cats and large wild carnivores and in particular the large wild felids. The larvae and nymphs mostly infest rodents. In severe infestation the adult ticks are attached all over the body but lesser infestations they attach to the head, neck and shoulders (Madder *et al.*, 2014).

Nyangiwe *et al.,* (2006) collected ticks from 200 dogs at dip tanks in the Eastern Cape Province. They found that 132 of these 200 dogs were heavily infested with ticks. Eight tick species were recorded, with *Haemaphysalis leachi* as the dominant species. The following year Apanaskevich *et al.,* (2007) redescribed the *Haemaphysalis leachi* subgroup. *Haemaphysalis leachi* do not occur in South Africa but do occur from Zimbabwe northwards up to Egypt. Thus, all previous records of *Haemaphysalis leachi* in South Africa is most likely *Haemaphysalis elliptica. Haemaphysalis elliptica* is a species belonging to the *Haemaphysalis leachi* subgroup. The main difference between these species are the size of the tick, and the shape of the lateral margin of the ventral spur on palpal segment II (Apanaskevich *et al.*, 2007).

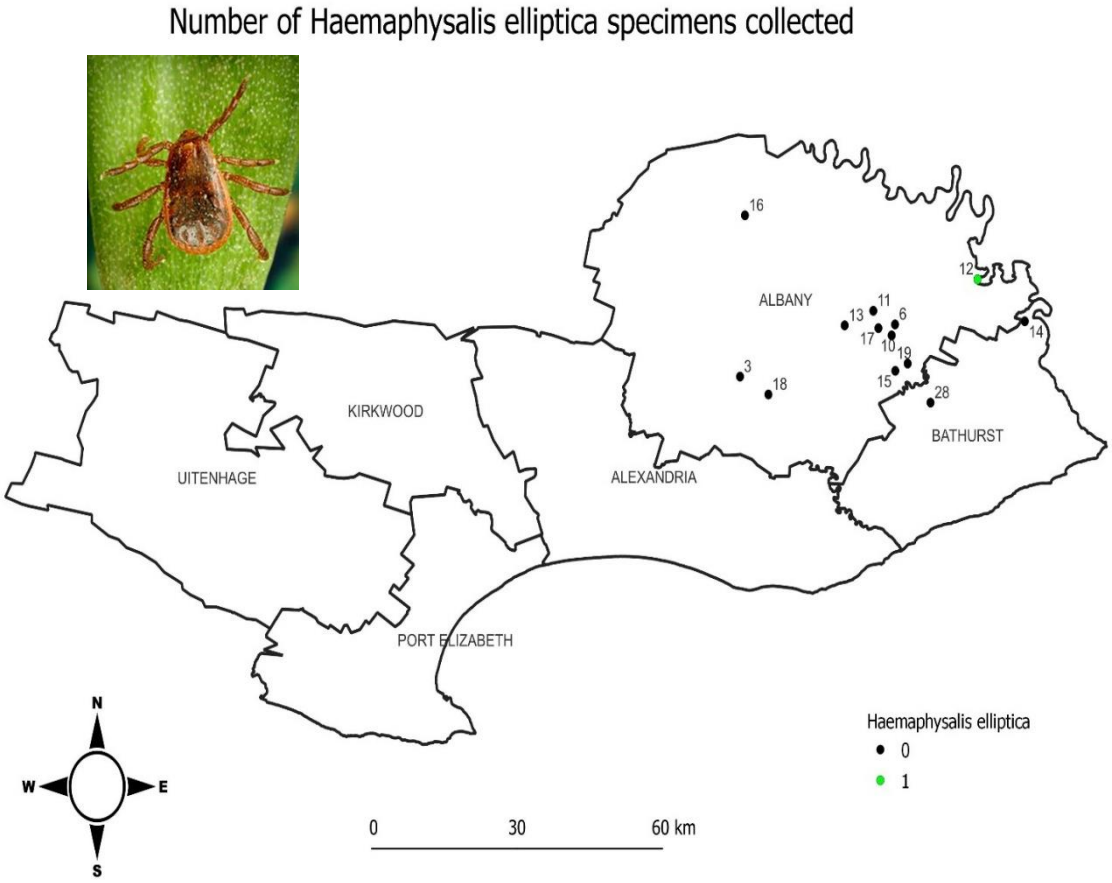


Figure 5 4: Map depicting number range of *Haemaphysalis elliptica* collected at the collection sites (Labuschagne 2023)

Haemaphysalis elliptica is present in East and Southern Africa, Democratic Republic of Congo, Kenya, Mozambique, South Africa, Tanzania, Uganda, Zambia, Zimbambwe, Ethiopia, Malawi and Rwanda (Apanaskevich *et al.,* 2007). This tick can be found throughout South Africa. In the south-east, non-seasonal rainfall area they are found in large numbers on dogs from June to September. In the northeastern summer rainfall areas, they are found mostly from January to April (Horak *et al.,* 2002).

Haemaphysalis elliptica prefers the following hosts of carnivore species, domestic dogs, domestic cats, lions, and leopard. The hosts for immature stages are the rodents and they may stay on the same host until they reach an adult stage. The adult species of *Haemaphysalis elliptica* has been found in a number of collections from a single host.

Haemaphysalis elliptica are the vectors of *Babesia canis rossi*. In South Africa *Babesia canis rossi* cause virulent Babesiosis in domestic dogs (Lewis *et al.,* 1996). This tick species has been recorded in South Africa as transmitting of *Rikettsia conori* which cause tick bite fever in humans. *Haemaphysalis elliptica* has been recorded as a common species on humans working in the fields and this might be because of its preference for carnivores (Horak *et al.,* 2002).

5.1.4 *Hyalomma rufipes*

In this survey *Hyalomma rufipes* Koch. was collected in Sites 6 and 12 from cattle (Figure 5.5). It is widely distributed across South Africa but is rarely collected in the winter rainfall areas of the Western Cape. The preferred hosts for adult ticks are cattle, sheep, goats, horses, large wild herbivores, rhinoceroses (Makwarela *et al.,* 2023). Immature stages like to attach and feed on scrub hares and ground frequenting birds like guineafowl. Preferred areas of attachment on cattle are the bare areas around anus, genitalia and on the hooves of sheep (Walker, 2003). The immature stages of these ticks are commonly found on the necks of scrub hares and on head and neck of birds.

These ticks have dark brown bodies, long mouthparts, an extensively punctate scutum, beady eyes, and long red and white banded legs (Makwarela *et al.,* 2023). The adult tick prefers larger animals in both domestic animals and wildlife. In a study this tick was found attached to goats and horses. These ticks have been found on cattle in four countries that is Namibia, Botswana, South Africa and Mozambique (Biggs & Langenhoven, 1984). This tick is a two-host tick and it takes a year to complete its cycle. These ticks will prefer the upper and the lower perineum. The adult ticks are sometimes found on the ground scouting for a passing host. During this study, collections were done in winter. The season with highest infestation was recorded in summer (December to February). *Hyalomma rufipes* is the vector of *Babesia occultans*, which is responsible for bovine Babesiosis in South Africa. This tick is also a vector of *Anaplasma marginale*, which is responsible for the outbreaks of Anaplasmosis in South Africa. These ticks also cause tissue lesions in cattle that can lead to secondary bacterial infections that can result in abscesses. *Hyalomma rufipes* is the main vector of Crimean-Congo haemorrhagic fever virus. This disease causes a debilitating disease of humans with a fatality rate of about 30% without any treatment

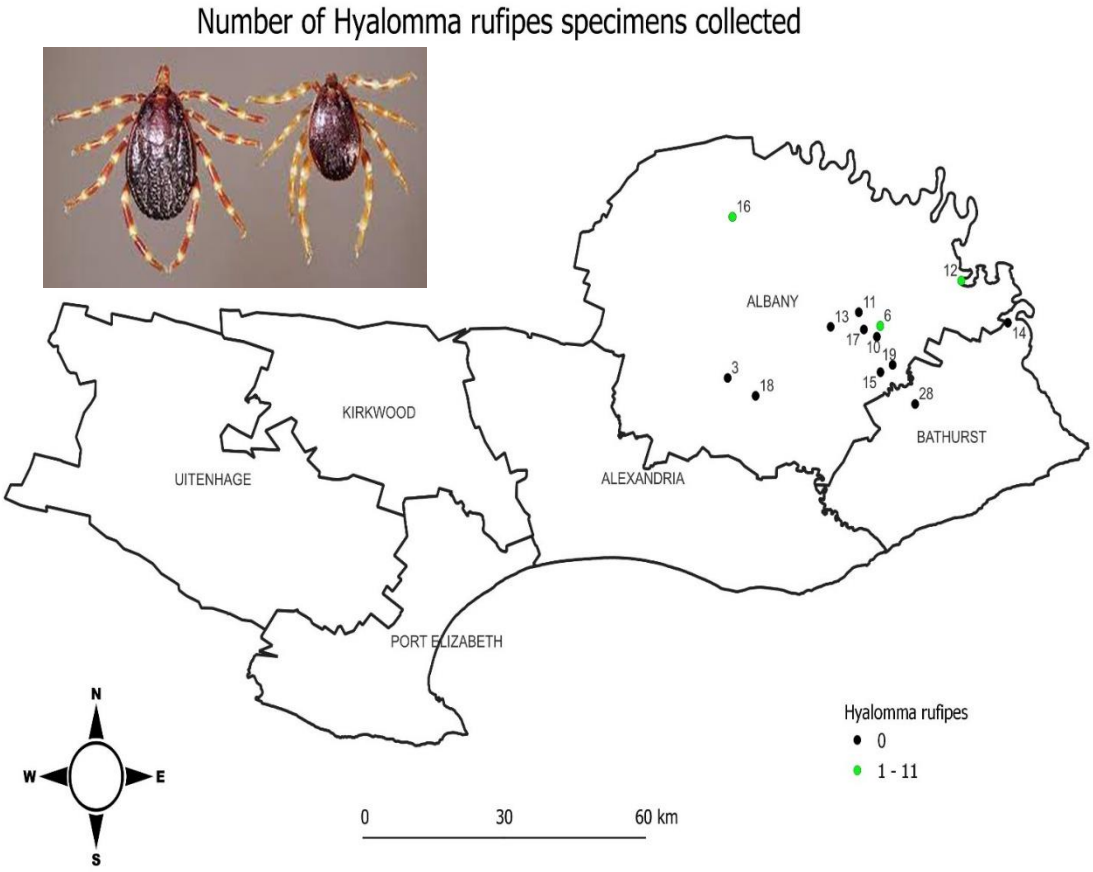


Figure 5 5: Map depicting number range of *Hyalomma rufipes* collected at the collection sites (Labuschagne 2023)

5.1.4 *Rhipicephalus decoloratus*

Rhipicephalus decoloratus was found in nine different sites namely Sites 3, 10, 11, 12, 13, 15, 16, 17 and 19. This tick was collected from cattle, horses, sheep, goats and dogs and the collections were mostly done in winter (Figure 5.6).

It is known as blue tick because of the colour of the engorged females. It is the most common and widespread tick species in Africa and are vectors of various species of *Babesia*, *Theileria*, and Ehrlichia that cause African Babesiosis and Anaplasmosis. The most preferred host for this tick is cattle. This tick also feed on sheep, goats, donkeys, horses and wild animals. The preferred feeding sites on cattle for all stages of this tick is the back, upper legs, neck, shoulders, dewlap and belly (Walker, 2003).

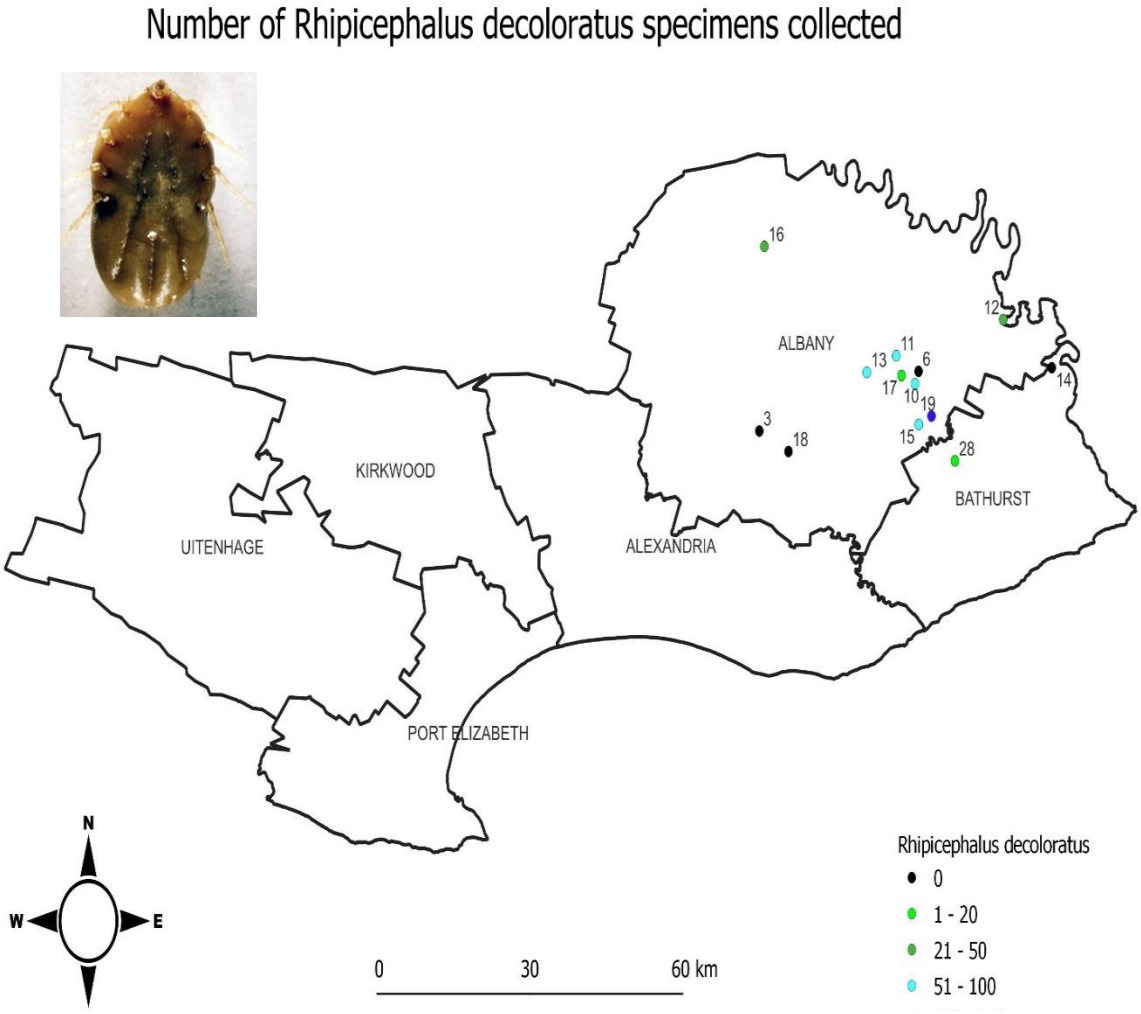


Figure 5 6: Map depicting the number range of *Rhipicephalus decoloratus* collected at the collection sites (Labuschagne 2023)

These are small inconspicuous ticks with thin legs and short mouthparts (Makwarela *et al.,* 2023) it is a one-host tick as its larva falls from vegetation and attach to available host and complete its developmental stages on that host. The colour of males is brownish yellow, and their gut is darker and can be seen through the moderate sclerotized scutum of the females (Makwarela *et al.,* 2023). These ticks are indigenous to Africa and they pose a risk to domestic animals as they are vectors of tick-borne diseases. In South Africa, this tick has been found in savannah, fynbos biomes and grasslands. It is found in all nine provinces of South Africa. Cattle are the preferred host of *R. decoloratus* adult ticks. They can be found all over the body of cattle (Makwarela *et al.,* 2023).

At Dohne Research station two breeds of cattle namely Bonsmara and Nguni were both exposed to *R. decoloratus* and *Rhipicephalus microplus* to investigate the tick resistance of these two breeds and to study the dynamics of the two tick species in the Eastern Cape. *Rhipicephalus microplus* was found to be the most abundant species at pastures grazed by bonsmara cattle, followed by *R. decoloratus*. There were lower number of both tick species on pastures grazed by Nguni cattle (Nyangiwe *et al.*, 2011).

5.1.6 *Rhipicephalus evertsi evertsi*

Rhipicephalus evertsi evertsi was distributed in 8 sites namely Sites 3, 6, 10, 12, 15, 16, 18 and 19 and was collected from horses, cattle and goats (Figure 5.7). It is the red legged tick distributed widely into central and southern parts Africa. It is a 2-host tick with the larvae and nymphs completing its developing stages on one host and the dropping off into the vegetation where it moults into an adult and then attach other another host. These are medium sized ticks with dark brown highly punctate scuta, beady eyes, and legs with an orange to red colour (Makwarela *et al.*, 2023).

Rhipicephalus evertsi evertsi has a preference for horses in all stages of development, it is also a common parasite of cattle, goats and sheep, this species was available on both goats and cattle at all 72 dip tanks, a total of 334 goats and 316 cattle were infested (Nyangiwe & Horak, 2007). It is the commonest species in donkeys in Yeman. Sheep are only good hosts to the adult species of *R. evertsi evertsi*. Although this species is widely distributed in southern and eastern Africa and is hosted by a variety of species, but adult ticks are seldom abundant (Walker *et al.*, 2000)

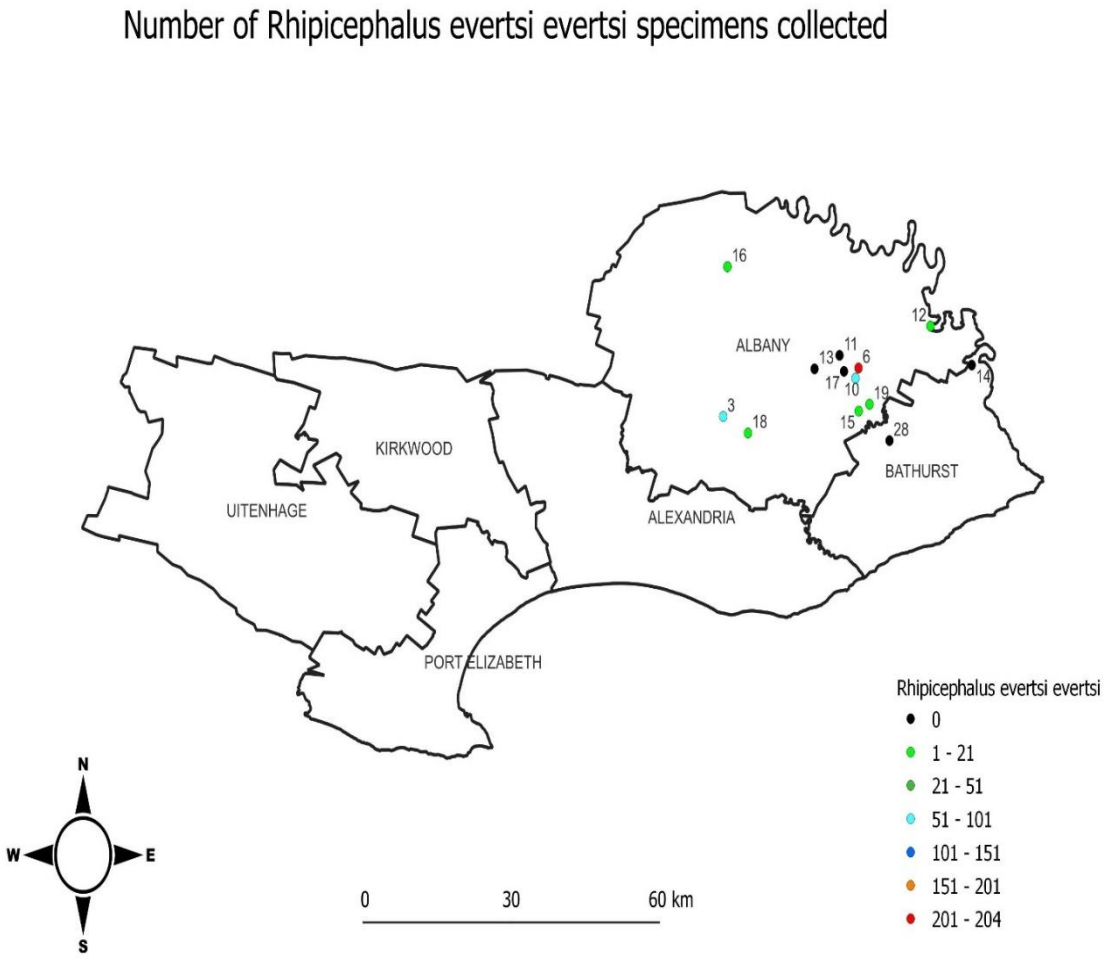


Figure 5 7: Map depicting the number range of *Rhipicephalus evertsi evertsi* collected at the collection sites (Labuschagne 2023)

5.1.7 *Rhipicephalus follis*

Rhipicephalus follis Dönitz was found attach to horses at Site 6 (Figure 5.8). This tick is commonly distributed on the mountainous areas on the east of South Africa. Previous studies indicate that the preferred hosts for this species are large ruminants such as cattle, eland and also horses and Cape Mountain zebra. In a study by Theiler and Robinson in 1953 they collected these ticks from undetermined hosts (Walker, 1990). The immature stage would prefer the four stripped grass mouse. In the Cradock area in the Eastern Cape Province infestations by this tick were found throughout the year on cattle (Walker *et al.*, 2000).

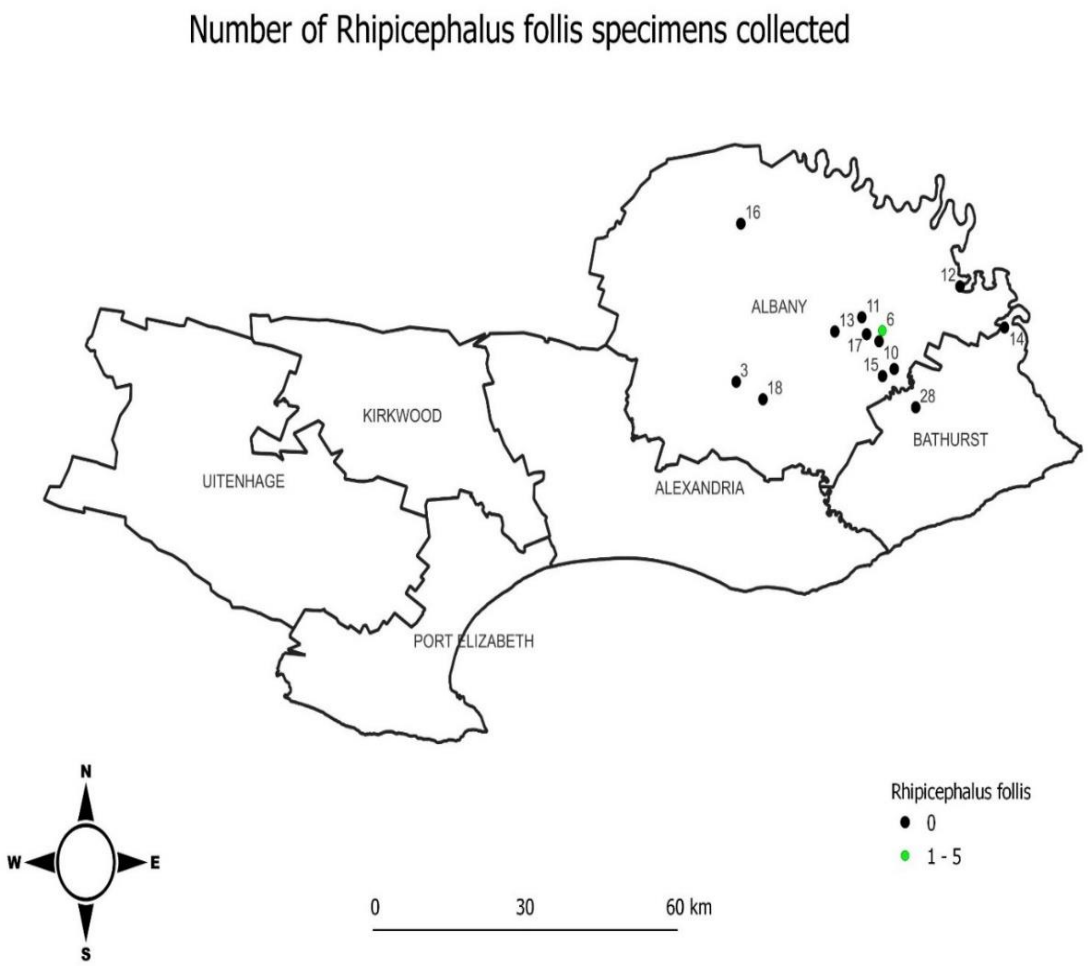


Figure 5 8: Map depicting the number range of *Rhipicephalus follis* collected at the collection sites (Labuschagne 2023)

5.1.8 *Rhipicephalus gertrudae*

Rhipicephalus gertrudae Feldman-Muhsam was collected at Sites 6 and 10 from horses and cattle (Figure 5.9). It is a three-host tick which the adults prefer to feed on cattle, sheep and dogs as well as several large wildlife species and will also feed on primates. There were records of mortalities of young baboons in Namibia resulted from heavy infestation by this tick (Horak *et al.,* 2002). It was found attached to cattle and horses. There were about twenty-one incidences of these ticks feeding on humans (Horak *et al.,* 2002). This tick is numerous on sheep during May to October in the southern and western coastal areas of South Africa (Walker *et al.,* 2000). In South Africa, collections of this tick were done in the semi arid and winter rainfall areas with dry summers (Horak *et al.,* 2017). This tick species has replaced *Rhipicephalus simus* as the most common species

on dogs in the southwestern Cape and in the central Free State Province (Nyangiwe *et al.*, 2006).

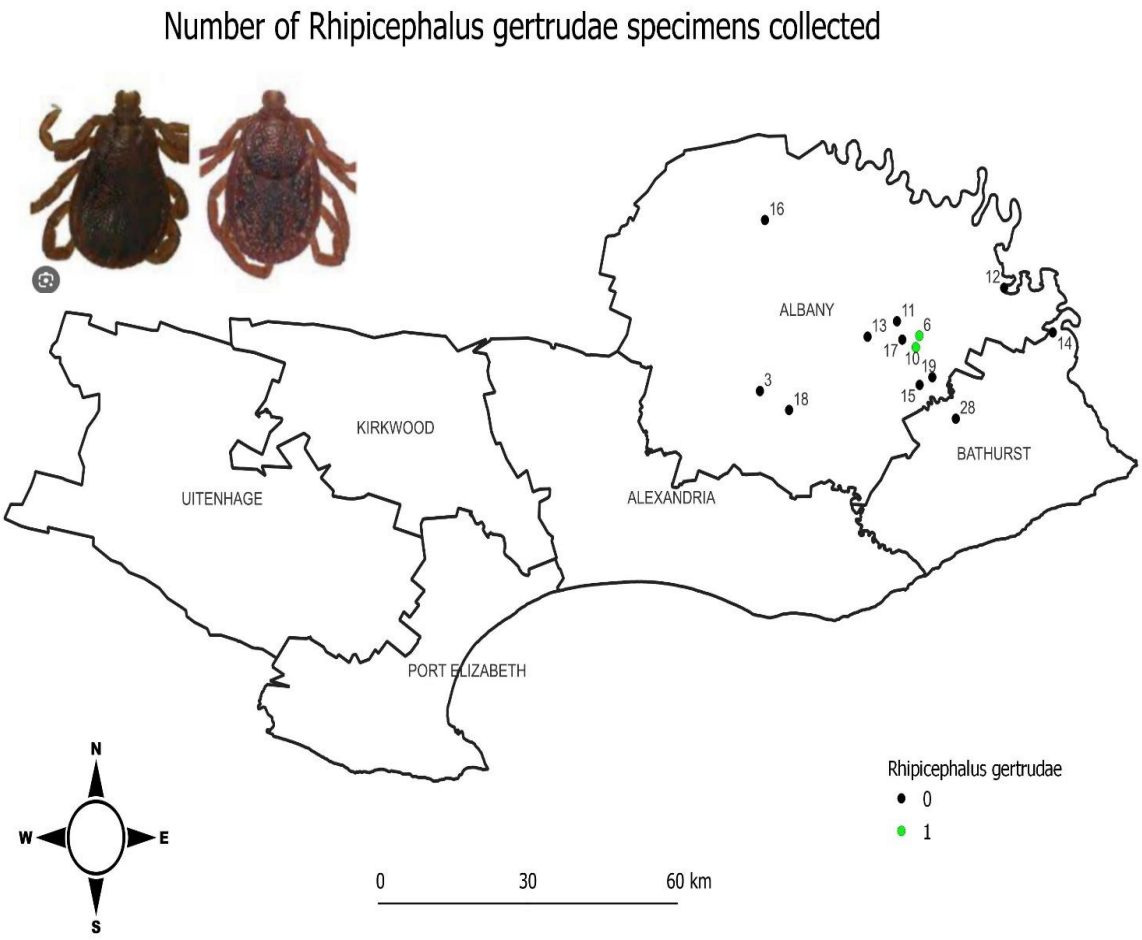


Figure 5 9: Map depicting number range of *Rhipicephalus gertrudae* collected at the collection sites (Labuschagne 2023)

5.1.9 *Rhipicephalus microplus*

Rhipicephalus microplus Canestrini is also known as the Asian blue tick and was collected from cattle at Sites 15 and 19 during this study (Figure 5.10). This tick is known to prefer cattle but a study by Nyangiwe (2007) indicated that goats were also good hosts for this tick that normally infest cattle. This tick prefers to attach to the belly, dewlap, shoulders and flanks. *Rhipicephalus microplus* is considered as the most important parasite of livestock in the world (Estrada-Peña *et al.*, 2006).

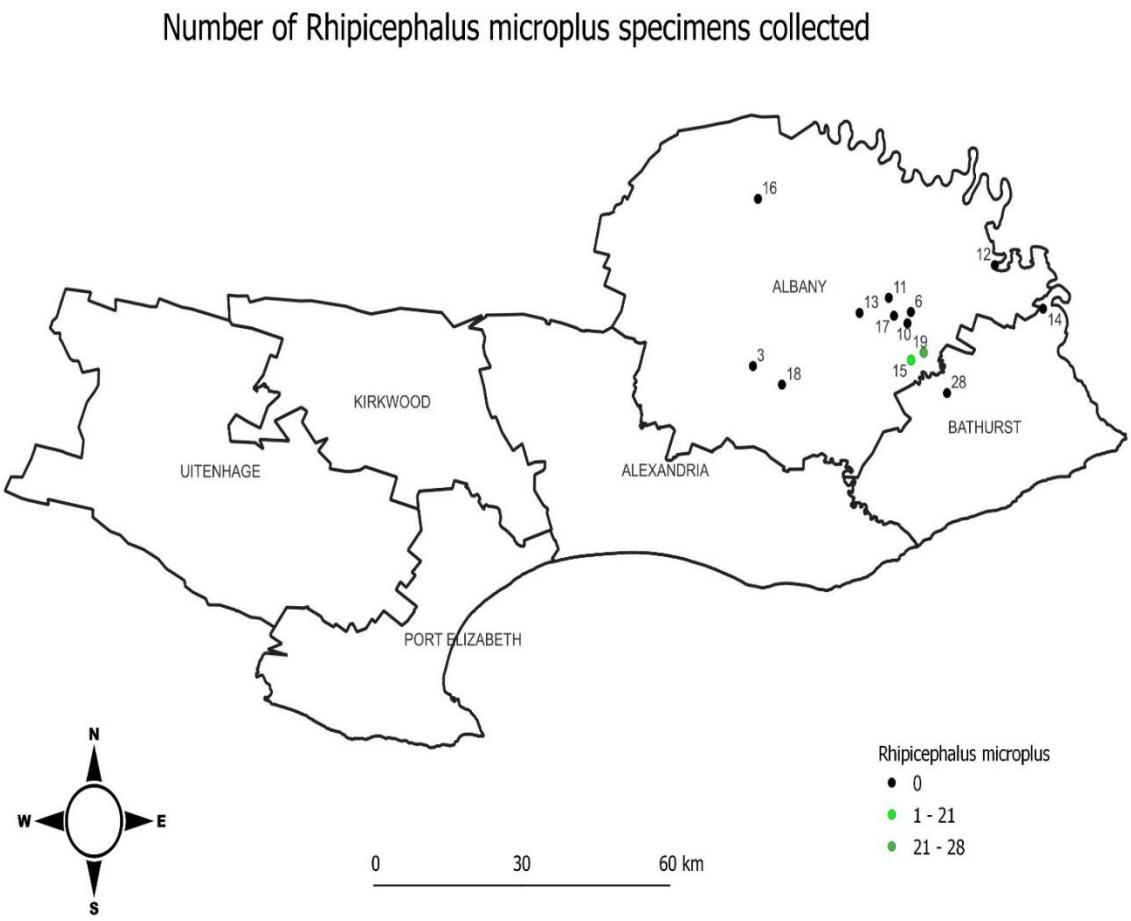


Figure 5 10: Map depicting number range of *Rhipicephalus microplus* collected at the collection sites (Labuschagne 2023)

In appearance *R. microplus* and *R. decoloratus* are similar and this can lead to misidentifications. But generally adult *R. microplus* are bigger and slightly redder in colour, when compared to *R. decoloratus*. This tick originated from Asia and poses a risk to cattle worldwide including Africa. *Rhipicephalus microplus* spends about three weeks on a host to complete the three stages of development. This tick species transmits both *Babesia bovis* and *Babesia bigemina,* which is cause of Babesiosis (redwater) in cattle (Walker, 2003). *Rhipicephalus microplus* also transmit bacterial pathogens including *Anaplasma marginale,* which is cause of Anaplasmosis or gallsickness in cattle and *Borrelia theileri* causing spirochaetosis in cattle. Heavy infestations may lead to hide damage and formation of scar tissue at the feeding sites (Walker, 2003).

This tick species has been recorded in South Africa in all nine provinces with high abundances in Northwest, Limpopo, KwaZulu Natal, Mpumalanga, Gauteng and coastal areas of Western and Eastern Cape Province (Makwarela *et al.*, 2023).

5.1.10 *Rhipicephalus simus*

Rhipicephalus simus Koch is also called as the glossy brown tick. This tick species was collected from horses and cattle during this survey at Sites 3, 6, 16, and 17 (Figure 5.11). This tick species has been identified on dogs from southern Mozambique in the northeast to at least as far south as Grahamstown, Eastern Cape Province in the southeast (Nyangiwe *et al.,* 2006). The preferred hosts of adult tick are monogastric animals like domestic and wild carnivores, equids and suids, cattle and African buffalo and to a lesser extent sheep are also hosts to this tick. Incidents of human bites has also been reported. For humans this tick is a vector of *Rickettsia conori*, the causative agent of Mediterranean spotted fever in humans (Horak *et al.*, 2002)

It is a conspicuous large tick with a large, dark, shiny scutum and has a denser distribution of small interstitial punctuations (Madder *et al.,* 2014; Nyangiwe *et al.,* 2013). It is a three-host tick and is mostly found in the regions with savanna climate in the country (Yawa *et al.,* 2018). *Rhipicephalus simus* is one of the three tick species commonly found on domestic dogs in South Africa along with *Heamaphysalis elliptica* and *Rhipicephalus sanguineus* (Horak *et al.,* 1987, 2009).

The main host of an adult *R. simus* is cattle but they can also be found on other domestic animals like sheep, goats and horses. They also infest large wildlife carnivores, zebra, warthogs and rhinoceros. These ticks prefer to attach to tail brush and feet of cattle. These ticks are found on the tail of horses and zebra and on head and shoulders of dogs and warthogs and on sheep attach around feet. Adults are commonly found on large host in summer, larvae in autumn and winter and nymphs during winter and spring (Walker, 2003).

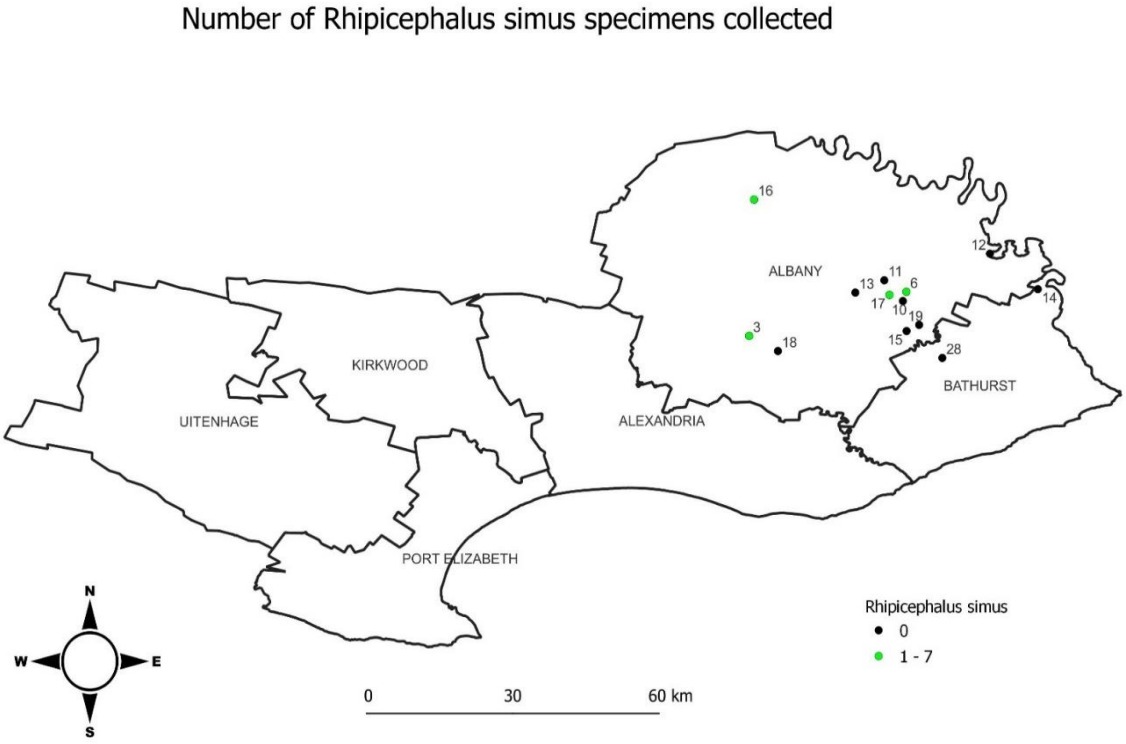


Figure 5. 11: Map depicting number range of *Rhipicephalus simus* collected at the collection sites (Labuschagne 2023)

Rhipicephalus simus is capable of transmitting pathogens to susceptible hosts. This tick species can transmit the bacterium *Anaplasma marginale* to cattle which is cause Anaplasmosis or Gallsickness in cattle (Madder *et al.*, 2014). It is also a vector of the bacterium *Rickettsia africae* causing African tick typhus to humans and while feeding on calves and lambs this tick also produces toxins that cause paralysis in calves and lambs (Walker, 2003).

CHAPTER 6

RESULTS AND DISCUSSION: PCR AND ELISA

6.1 *CULICOIDES* SPP. PCR RESULTS

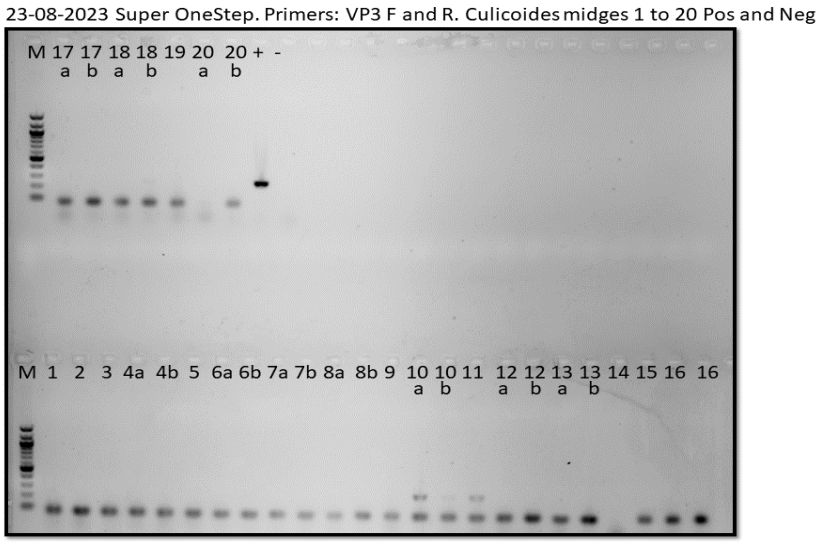
Due to ease of use and the rapid nature of performing PCR, the use of PCR in the detection of viruses in both the host and the vectors are replacing the conventional virus isolation. A benefit of PCR is that it can be used on older dried out vector samples or samples that have been stored in ethanol. Virus isolation is a longer and more time-consuming process of testing for virus, but the benefit of this type of testing is that live virus will be picked up. There is a risk that with the standard PCR test, without the use of specific primers targeting live virus, both vaccine and live virus may be picked up (Bréard *et al.,* 2003; Zientara *et al.,* 2004).

Culicoides and tick samples were tested with an inhouse PCR for the presence of virus. All samples that tested positive with the inhouse PCR was sent to be tested with the WOAH accredited and validated test. Only samples that test positive with the WOAH accredited and validated test can be considered positive. With the inhouse PCR 5 samples of *Culicoides* spp. tested positive (Table 6.1 and Figure 6.1) and were sent for further testing with the WOAH accredited and validated PCR test to verify the results. Three of the pools that tested positive were from the autumn season (*Culicoides bolitinos, C.sp. #54* and *C. tuttifrutti*) and two of the pools were from winter season (*C. imicola and C. bolitinos*). All four of these species are in the same subgenus (*Avaritia*) and *C. imicola, C. bolitinos* and *C. tuttifrutti* are part of the imicola group within this subgenus. The positive pools were from Sites 1, 3, 5, 7 and 8. Two pools of *Culicoides bolitinos* tested positive, one from Site 1 (Port Elizabeth) and the other from Site 3 (Grahamstown). On further testing with the WOAH accredited and validated PCR test, 2 pools of *Culicoides* species tested positive for the virus. These two was a pool from Site 1 consisting of two specimens of *C. bolitinos* and another pool from Site 7 consisting of 6 specimens of *C. tuttifrutti* tested positive for the presence of AHSV, both the positive samples were from the autumn season (Figure 6.1).

Table 6. 1 Culicoides pools tested for African horse sickness virus (AHSV) by PCR

Sample Number	Midge (Species)	Site No	Date of sample	No of specimens	AHSV PCR
1	*C. pycnostictis*	5	28/06/2017	10	Neg
2	*C. nivosus*	5	28/06/2017	10	Neg
3	*C. imicola*	5	28/06/2017	20	Neg
4a	*C. leucostictis*	5	28/06/2017	3	Neg
4b	*C. leucostictis*	5	28/06/2017	7	Neg
5	*C. onderstepoortensis*	5	28/06/2017	10	Neg
6a	*#33*	1	04/03/2016	6	Neg
6b	*#33*	1	04/03/2016	4	Neg
7a	*C. bolitinos*	1	04/03/2016	2	Positive
7b	*C. bolitinos*	1	04/03/2016	18	Neg
8a	*C. bolitinos*	7	16/04/2019	9	Neg
8b	*C. bolitinos*	7	16/04/2019	7	Neg
9	*#54*	7	16/04/2019	20	Neg
10a	*C. tuttifrutti*	7	16/04/2019	6	Positive
10b	*C. tuttifrutti*	7	16/04/2019	4	Neg
11	*C. bolitinos*	3	19/06/2010	20	Neg
12a	*C. bolitinos*	3	10/06/2010	13	Neg
12b	*C. bolitinos*	3	10/06/2010	7	Neg
13a	*C. bolitinos*	1	03/03/2020	19	Neg
13b	*C. bolitinos*	1	03/03/2020	1	Neg
14	*C. tuttifrutti*	1	03/03/2020	20	Neg
15	*C. subschultzei*	5	28/07/2017	10	Neg
16a	*C. imicola*	5	28/07/2017	17	Neg
16b	*C. imicola*	5	28/07/2017	3	Neg
17a	*C. onderstepoortensis*	5	28/07/2017	7	Neg
17b	*C. onderstepoortensis*	5	28/07/2017	3	Neg

18a	*C. onderstepoortensis*	5	28/07/2017	9	Neg
18b	*C. onderstepoortensis*	5	28/07/2017	1	Neg
19	*#54*	8	23/03/2020	10	Neg
20a	*C. tuttifrutti*	8	23/03/2020	9	Neg
20b	*C. tuttifrutti*	8	23/03/2020	1	Neg



M = Quick-Load 100 bp DNA Ladder (NEB #B7025)

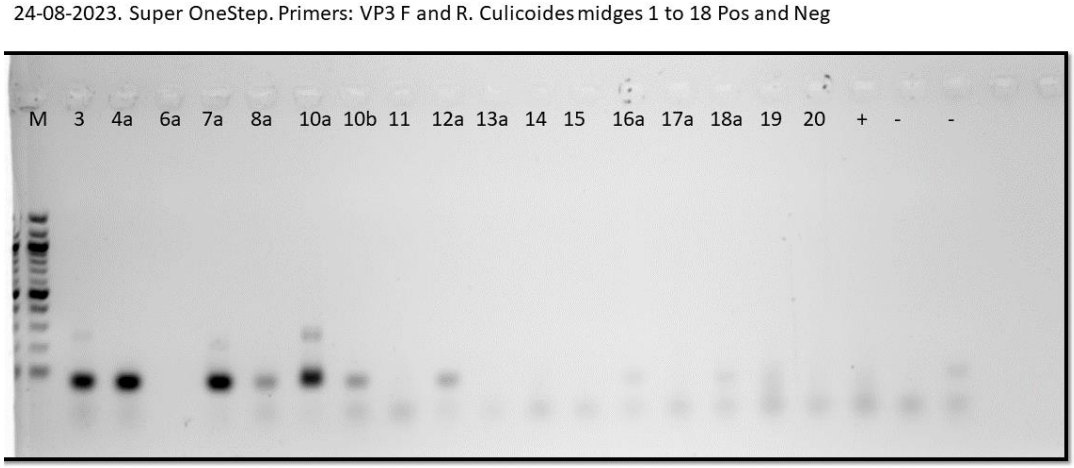


Figure 6 1: Argarose gel results of Culicoides species pools tested

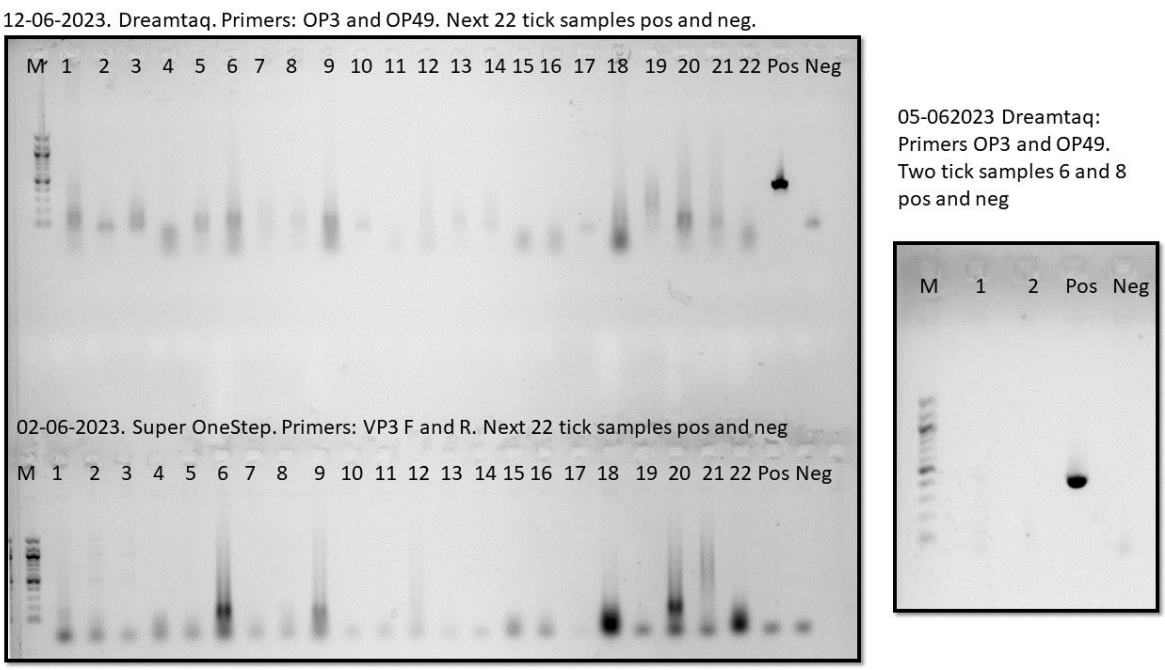
6.2. TICK SPECIES PCR RESULTS

Ticks have been implicated in the transmission of AHSV and there are studies that have been conducted to support the findings (Salama *et al.*, 1981; Awad *et al.*, 1981). The question remains whether AHSV can overwinter in tick species in the study area. Though 60 tick specimens that were sent for testing, three (n=3) tested positive for AHSV. Faint bands were observed on the gels for specimens 28, 30 and 51(Table 6.2 and Figure 6.2). These three samples were then sent for confirmation testing with the WOAH accredited and validated PCR test to confirm the results of the inhouse test, unfortunately no virus was detected. Twenty-two of the tick specimens were also tested for Lumpy skin disease virus (LSDV). On the gels no bands were observed though there were smearing effects observed.

Table 6. 2 Ticks specimens test for African horse sickness virus (AHSV) and Lumpy skin Disease (LSDV) by PCR

#	Tick (Species)	Site no	Date of sample	No of specimens	AHSV PCR	LSDV PCR
1	*R. decoloratus*	19	17/05/2019	2	2 x Neg	2 x Neg
2	*A. hebraeum*	19	17/05/2019	2	2 x Neg	2 x Neg
3	*R. decoloratus*	3	30/05/2019	2	2 x Neg	2 x Neg
4	*R. evertsi evertsi*	3	30/05/2019	2	2 x Neg	2 x Neg
5	*A. hebraeum*	3	30/05/2019	2	2 x Neg	2 x Neg
6	*R. decoloratus*	10	20/03/2020	2	2 x Neg	2 x Neg
7	*R. evertsi evertsi*	10	20/03/2020	2	2 x Neg	2 x Neg
8	*A. hebraeum*	10	20/03/2020	2	2 x Neg	2 x Neg
9	*R. decoloratus*	11	24/05/2019	2	2 x Neg	2 x Neg
10	*R. evertsi evertsi*	16	8/06/2021	2	2 x Neg	2 x Neg
11	*A. hebraeum*	16	8/06/2021	2	2 x Neg	2 x Neg
12	*R. decoloratus*	17	16/05/2018	2	2 x Neg	2 x Neg
13	*A. hebraeum*	17	16/05/2018	2	2 x Neg	2 x Neg
14	*R. evertsi evertsi*	18	30/05/2018	2	2 x Neg	2 x Neg
15	*A. hebraeum*	18	30/05/2018	2	2 x Neg	2 x Neg

16	*R. evertsi evertsi*	6	15/10/2020	2	2 x Neg	2 x Neg
17	*R. decloroatus*	13	20/03/2019	2	2 x Neg	2 x Neg
18	*R. decloroatus*	12	24/06/2019	2	2 x Neg	2 x Neg
19	*R. evertsi evertsi*	12	24/06/2019	2	2 x Neg	2 x Neg
20	*R. decoloratus*	15	17/05/2019	2	2 x Neg	2 x Neg
21	*A. hebraeum*	15	17/05/2019	2	2 x Neg	2 x Neg
22	*A. hebraeum*	14	25/03/2019	2	2 x Neg	2 x Neg
23-24	*R. microplus*	15	05/07/2023	2	2 x Neg	nd
25-28	*A. hebraeum*	15	05/07/2023	4	3 x Neg (28?)	nd
29-31	*R. evertsi evertsi*	15	05/07/2023	3	2 x Neg (30?)	nd
32-35	*R. decoloratus*	15	05/07/2023	4	4 x Neg	nd
36-38	*A. hebraeum*	16	26/07/2023	3	3 x Neg	nd
39-41	*R. decoloratus*	16	26/07/2023	3	3 x Neg	nd
42-43	*R. evertsi evertsi*	16	26/07/2023	2	2 x Neg	nd
44-45	*H. rufipes*	16	26/07/2023	2	2 x Neg	nd
46-49	*A. hebraeum*	28	04/07/2023	4	4 x Neg	nd
50-52	*R. microplus*	19	05/07/2023	3	2 x Neg (51?)	nd
53 – 55	*A. hebraeum*	19	05/07/2023	3	3 x Neg	nd
56 – 57	*R. evertsi evertsi*	19	05/07/2023	2	2 x Neg	nd
58 – 60	*R. decoloratus*	19	05/07/2023	3	3 x Neg	nd



M = Quick-Load 100 bp DNA Ladder (NEB #B7025)

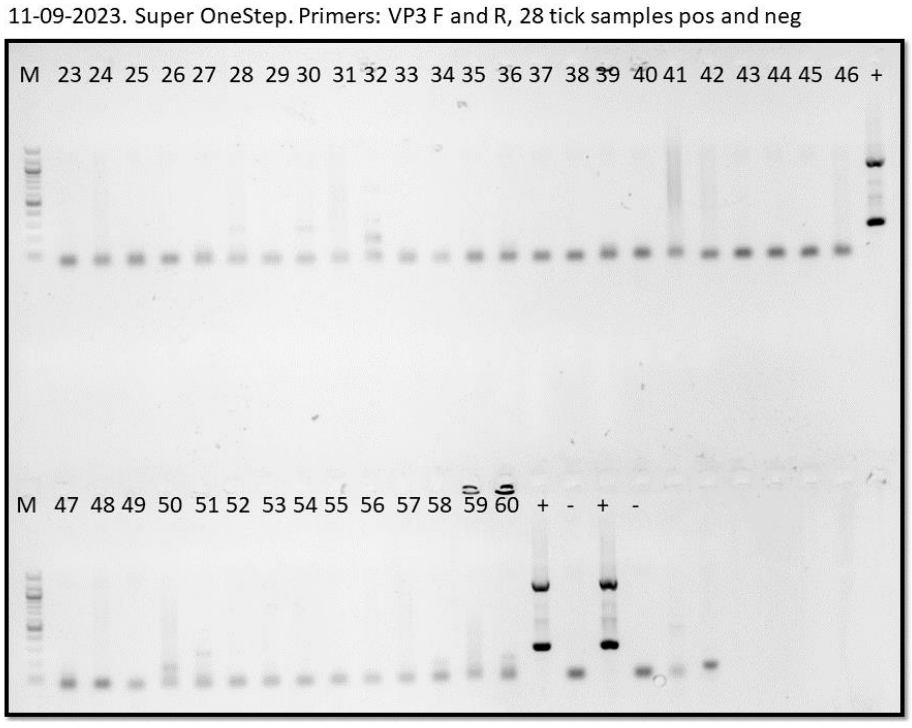


Figure 6 2: Argarose gel results of tick species pools tested

6.3. ANTIBODY TESTS RESULTS BLOOD OF DOGS

Blood samples were collected from 100 dogs from November 2022 to September 2023. Each dog was sampled four times, once every season, for the duration of the above period. Samples were sent to ARC-OVR for analysis using an AHS indirect ELISA to test for the presence IgG antibodies in the serum.

None of the blood samples tested positive for the presence of antibodies against AHSV with the indirect ELISA, though each animal were tested four times. A wide range of breeds, ages and size were tested. The breeds included to name but a few: Jack Russel, Border Collie, Boerboel, Laborador, Great Dane and cross breeds (Table 6.3). The size of the dogs ranged from extra large (Great Dane) to small (Jack Russel), the ages of the animals ranged from 9 months to 13 years. The majority of the older dogs had been living on that property since they were puppies. Thus, one would expect that if any animal were to have had any antibodies against AHSV, it would have been these older animals. Based on it could be concluded that their animals had not been exposed to the virus through the bites of infected *Culicoides* midges.

Table 6. 3 Antibody test results form the dogs

Site number	Dogs	breed	sex	Age	size	No times bled	Blood results
Site 11	1	Jack Russell	M	2 yrs	small	4	negative
	2	Border collie	M	16 yrs	medium	4	negative
	3	Boerboel	M	2 yrs	large	4	negative
	4	Jack Russell	M	1 yr	small	4	negative
	5	Boerboel	F	2 yrs	large	4	negative
Site 9	6	E Springer spaniel	M	12 yrs	medium	4	negative
	7	E Springer spaniel	M	9 yrs	medium	4	negative
	8	Great dane	M	18 months	Huge	4	negative
	9	Labrador	M	3 yrs	large	4	negative
	10	Border collie	M	20 months	medium	4	negative
	11	Border collie	M	20 months	medium	4	negative

Site 8	12	Border collie	F	10 yrs	medium	4	negative
	13	Jack Russell	F	13 yrs	small	4	negative
	14	Dachshund X	F	9 yrs	medium	4	negative
	15	x breed	F	3 yrs	large	4	negative
	16	x breed	M	5 yrs	large	4	negative
Site 20	17	Border collie	M	2 yrs	medium	4	negative
	18	Rotweiler	F	4 yrs	large	4	negative
	19	Rotweiler	M	4 yrs	large	4	negative
	20	Pitbull	M	9 months	medium	4	negative
	21	Pitbull	F	9 months	medium	4	negative
	22	x breed	M	6 yrs	large	4	negative
	23	x breed	M	9 yrs	medium	4	negative
	24	x breed	F	11 yrs	large	4	negative
	25	x breed	M	7 yrs	large	4	negative
	26	Boerboel	M	11 months	large	4	negative
	27	x breed	F	13 yrs	medium	4	negative
	28	x breed	M	3yrs	small	4	negative
Site 21	29	Greyhound	M	3 yrs	large	4	negative
	30	Greyhound	M	1 yr	large	4	negative
	31	Greyhound	F	3 yrs	large	4	negative
	32	Boerboel	M	1 yr	large	4	negative
	33	Boerboel	M	3 yrs	large	4	negative
Site 22	34	Border collie	F	6 yrs	medium	4	negative
	35	Labrador	F	3 yrs	large	4	negative
	36	Border collie	F	18 months	medium	4	negative
	37	Border collie	F	4 yrs	medium	4	negative
Site 6	38	Daschund X	M	15 yrs	medium	4	negative
	39	Jack Russell	F	12 yrs	small	4	negative
Site 23	40	Border collie	M	2 yrs	medium	4	negative
	41	Border collie	M	1 yr	medium	4	negative
	42	Border collie	M	7 yrs	medium	4	negative
	43	Border collie	M	3yrs	medium	4	negative
	44	Boerboel	M	3 yrs	large	4	negative
	45	Border collie	M	5 yrs	medium	4	negative
	46	Jack Russell	M	4 yrs	small	4	negative
	47	Border collie	M	5 yrs	medium	4	negative

Site 7	48	German Shepherd	M	3 yrs	large	4	negative
	49	Australian shepherd	M	5 yrs	large	4	negative
	50	x breed	M	11 yrs	large	4	negative
Site 3	51	Border collie	F	2yrs	medium	4	negative
	52	x breed	M	5 yrs	medium	4	negative
	53	x breed	F	12 yrs	medium	4	negative
	54	German short hair pointer	M	13 yrs	large	4	negative
Site 24	55	Border collie	M	13 yrs	medium	4	negative
	56	Border collie	M	18 months	medium	4	negative
Site 13	57	x breed	F	15 yrs	medium	4	negative
	58	x breed	M	8 yrs	medium	4	negative
	59	Labrador	F	4 yrs	medium	4	negative
Site 15	60	Pitbull	M	2 yrs	large	4	negative
	61	Jack Russell	M	8 yrs	small	4	negative
	62	Jack Russell	F	7 yrs	small	4	negative
	63	Border collie	F	3 yrs	medium	4	negative
	64	Pitbull	F	1 yr	large	4	negative
Site 25	65	x breed	F	2 YRS	medium	4	negative
	66	Jack Russell	F	4 yrs	small	4	negative
	67	Pitbull	M	1 yr	large	4	negative
	68	x breed	F	7 yrs	medium	4	negative
	69	x breed	M	9 yrs	medium	4	negative
	70	x breed	M	6 yrs	large	4	negative
	71	x breed	M	5 yrs	medium	4	negative
	72	x breed	M	18 months	small	4	negative
	73	x breed	F	11 yrs	medium	4	negative
Site 16	74	Border collie	M	2 yrs	medium	4	negative
	75	Border collie	M	1 yr	medium	4	negative
	76	Border collie	M	7 yrs	medium	4	negative
	77	Border collie	M	3 yrs	medium	4	negative
	78	Border collie	M	3 yrs	medium	4	negative
	79	Boerboel	M	5 yrs	large	4	negative
	80	Jack Russell	M	4 yrs	small	4	negative
	81	Border collie	M	5 yrs	medium	4	negative

Site 21	82	Greyhound	M	3 yrs	large	4	negative
	83	x breed	M	1 yr	large	4	negative
	84	x breed	M	2 yrs	medium	4	negative
	85	Jack Russell	F	3 yrs	medium	4	negative
Site 26	86	Pitbull	F	1 yr	medium	4	negative
	87	Border collie	F	6 yrs	medium	4	negative
	88	x breed	M	5 yrs	large	4	negative
	89	x breed	F	2 yrs	large	4	negative
	90	x breed	M	3 yrs	small	4	negative
	91	x breed	M	13 yrs	medium	4	negative
	92	Jack Russell	M	11 yrs	small	4	negative
	93	Greyhound	M	9 yrs	large	4	negative
	94	x breed	F	15 yrs	medium	4	negative
	95	x breed	F	13 yrs	large	4	negative
Site 27	96	Greyhound	M	3yrs	Huge	4	negative
	97	x breed	M	4 yrs	large	4	negative
Site 19	98	x breed	M	4 yrs	medium	4	negative
	99	Jack Russell	M	2 yrs	small	4	negative
	100	Jack Russell	F	2 yrs	small	4	negative

Blood samples were collected from 100 dogs once in each of the four seasons of the year. 400 blood samples were submitted for testing. All these dogs tested negative for AHS antibodies. Different breeds, ages, and sizes of dogs were bled and all of them tested negative for AHS antibodies.

On the 16/05/2023 blood was collected from three horses at site no 8 and two of them came back positive of AHS. At this same site on the 05/06/23 blood samples were collected from 3 dogs and these tested negative for AHS antibodies. On the 3 and 7 May 2023 *Culicoides* samples were collected from site 8. Unfortunately, ticks were not collected during this month. The supposed scenario here is, that if horses tested positive for AHSV and the dogs tested negative, then that dogs do not play a role in the transmission of AHSV in this area.

In a study that was conducted in Egypt in 1981 by Salama *et al.,* AHSV was isolated and identified from naturally infected dogs in Upper Egypt. One hundred and eleven dog blood samples were collected from the border of Egypt and Sudan in the Aswan Province where AHS epizootics occur. Blood samples were in OCG diluent (Salama *et al.,* 1981).

In another study, a dog died of AHS at the Malelane Research Unit in Mpumalanga without ingesting any horse meat. There was no known history of this dog eating infected horse meat as it was in an enclosure being fed commercial dry food (O'Dell, 2017; Van Sittert *et al.,* 2013). If there was no other source of infection for this dog, other than vector borne transmission, it would mean that in the commercial dry food there were equine products which would be contradictory to what they wrote on their labels of an ISO-certified manufacturer. The question also arises to whether the virus will be able to withstand the manufacturing process (O'Dell, 2017; Van Sittert *et al.,* 2013).

Looking at Site 3 of this study 34 different *Culicoides* species have been collected from this site. All most every year, there are outbreaks of AHSV that are reported to the local state veterinary office. Dogs in Site 3 have been bled four times, once in each season but none of them tested positive for AHSV antibodies. With the number of *Culicoides* species collected from this site, and that no dog tested positive for AHSV antibodies, it can be postulated that *Culicoides* don't prefer to feed on dogs. Looking at breeds of dogs that were bled in this site, and other sites *Culicoides* species may prefer to feed on particular breeds of dogs. Without further study and blood meal analysis of blood fed *Culicoides* midges it would be impossible to say conclusively that *Culicoides* species do not feed on dogs.

A study that was conducted by Braverman and Chizov-Ginzburg (1995) to establish the role of dogs as the hosts to AHSV. They state that studies that have been conducted in South Africa, Kenya and Egypt have shown that the dogs can be infected with AHS and can act as the hosts (Braverman & Chizov-Ginzburg, 1996). It is therefore possible to transmit the virus from dog to dog and from dog to horse. In similar studies in Kenya and Egypt it was found that dogs acquire infection by ingesting infected horse meat and blood. *Culicoides bolitinos, C. pycnostictus* and *C. imicola* were present in all 10 sites, and *C. bolitinos* and *C. imicola* are the principal vectors of AHSV. If similar studies that were

conducted in South Africa and dogs were found to be positive of AHS there might be other factors that might have played a role in those studies. In all the sites where AHS occur annually dog blood samples were collected all the seasons of the year. Looking at the results of the study by Braverman and Chizov-Ginzburg (1995), the biting midges that were caught in the dog kennel were 10 times smaller than at other animal houses. A precipitin test that was conducted of blood-fed *Culicoides* species that were enclosed on a farm in Israel and Zimbabwe of which all these farms housed dogs and were all negative of the dog blood (Braverman & Chizov-Ginzburg, 1996). In a total of 401 blood meals from seven *Culicoides* species were positive of other animal but all negative for the dogs. Out of 1 123 blood meals from 11 *Culicoides* species surveyed near Harare none were positive of canine blood. This study of Braverman and Chizov-Ginzburg further implies that in a study in Israel and Zimbabwe 194 and 241 specimens of *Culicoides imicola* were precipitin tested and there was no canine blood in all these specimens (Braverman & Chizov-Ginzburg, 1996).

In previous studies dogs are known to be only non equine species that contract the pulmonary form of AHS after infection with AHSV. All documented natural cases of dogs have resulted from ingestion of infected horse meat. The role of dogs in the epidemiology of AHSV if any is unknown (Coetzer & Guthrie, 2004).

In another study by Hanekom et al., (2023), dog bloods from the biobank at a veterinary teaching hospital in Tshwane were used for testing. The high population density in big city might have played a role in the positive outcome of results because of lack of preferred host which means only dogs were available for *Culicoides* species to bite. According to Hanekom midges circumvent canine blood meals in low density farm areas but might feed on dogs in near suburban areas due to lack of alternative host from dogs (Hanekom *et al.,* 2023).

The current study sites were all mainly farms and that might be the reason why there were no positive dog results. Site 26 is situated in the urban area, but there are other animal species in that vicinity, on which the *Culicoides* species could have fed.

CHAPTER 7
CONCLUSIONS AND FUTURE WORK

7.1. CONCLUSIONS

African horse sickness virus was isolated from 2 pools of *Culicoides* collected from the field. These were 1 pool of *C. bolitinos* and another pool of *C. tuttifrutti.* Both these species are placed in the subgenus *Avaritia,* with *C. imicola* they form part of the Imicola group. *Culicoides bolitinos* was the most abundant species collected, with *C. imicola* the third most abundant and *C. tuttifrutti* the sixth abundant species. Both *C. bolitinos* and *C. imicola* were present at all 10 collection sites while *C. tuttifrutti* were present at 9 of the collection sites.

The presence of both the vectors of AHSV and BTV, namely *C. bolitinos* and *C. imicola*, increases the risks of disease outbreaks if the virus enters the area. *Culicoides bolitinos* breeds in cattle, African buffalo and the blue wildebeest dung (Paweska, 2003). In cattle farms with horses, it is recommended that horses be stabled at night to minimise their contact with *Culicoides* species. *Culicoides imicola* breeds in moist, organic rich soils and can become super abundant under ideal conditions and more than a million specimens of this species can be collected at night in a single light trap, this increases the risk of an infected midge biting an animal.

From the results of this study, dogs don't play a major role in the transmission of AHSV in the study area, thus it is unlikely that they are silent reservoir hosts of AHSV, as all of the dogs tested negative for AHSV antibodies. Though any sick dog, in especially in AHS endemic areas needs to be examined and samples collected for AHSV screening.

Climate play a huge role in the occurrence of vector borne diseases in South Africa and worldwide. Epidemics of these vector borne diseases may become more frequent in certain parts of North Africa as *Culicoides* spp. extends its range, and this may lead to a serious threat to the distribution of infection to Middle East and Mediterranean (Van Den Bossche & Coetzer, 2008).

The identification of 49 *Culicoides* species in the study area, which is about 50% of the total species found in South Africa, raises concerns as both the main vectors and all the laboratory vectors were collected during this study. With the ever-increasing number of game reserves being established in the area, there are a danger of AHSV, and other viruses being brought in with animals being translocated to stock these farms. As was shown by the Spain and Portugal outbreak in 1987, competent vectors can pickup and spread a virus from subclincally infected animals to healthy hosts.

The persistence of AHS serotype 2 outbreaks in the study area, indicates that the virus overwinters in the study area in the adult *Culicoides* populations. This was corroborated by the isolation of the AHSV from the pools of *Culicoides* submitted for testing. Different breeding habitats of *Culicoides* species, temperatures or humidity variations and cold tolerance varies among *Culicoides* species and this may play a role in the persistence of AHSV in an area (Paweska *et al.*, 2002).

Vaccination of all horses against AHS with the Onderstepoort Biological Products (OBP) live polyvalent, attenuated vaccine is the best control measure, which horse owners should undertake.

The eradication of different tick species is very important although it might not be economical feasible. It is also important to consult your state/private veterinarian or animal health technician to determine the most suitable tick treatment for your herd. Dogs based on their age, size and any existing health conditions must also be treated for ticks.

The convectional method for the control of tick infestations is through dipping or spraying with acaricides. Pour on formulations and ascaricide impregnated eartags and bands have been developed to fight tick infestations. Try and avoid tick resistance ask for advice from your state/private veterinarian or animal health technician. For soft tick and Rhipicephalus sanguineus control, spray inside the animal houses with acaricide with a long residual effect (Jongejan & Uilenberg, 1994).

Host resistance to ticks is one of the biological methods that can be used for tick control. Although this control system has a problem as cattle can build resistance to only one tick species.

In Australia the development of Anti tick vaccine is the major new system against *Rhipicephalus microplus*. This approach has already been shown to work in principle by Allan and Humphreys (Jongejan & Uilenberg, 1994). Farmers in general, horse owners, dog owners and individuals, veterinary personnel will find the results of this study very fruitful.

Due to climate change a lot has happened in the occurrence and distribution of the vector borne diseases, highlighting the importance of an integrated research approach. It is important to study the vectors, the pathogens, the interactions between host, vector and pathogen as well as role the environmental factors in disease distribution and maintenance.

7.2. FUTURE WORK

This study has shown that the *Culicoides bolitinos* was the dominant species followed by *Culicoides pycnostictus*. Although *Culicoides imicola* has been incriminated as the most important vector in the transmission of AHSV, there are also growing concern that other *Culicoides* species may also act as competent field vectors of AHSV in South Africa (Paweska *et al.,* 2003). There is little that is known of the vector status of *C. pycnostictus*, and the previous studies do not really incriminate this species as the vector to AHSV. Further research is needed especially in the endemic areas, to investigate the role of this species, in the transmission of AHSV.

Research on AHS, *Culicoides* and tick species distribution in the Eastern Cape Province needs to be extended to cover a larger area, compared to this study. With 49 different *Culicoides* species identified in the Sarah Baartman district municipality, the species diversity could increase if a larger area, including the former Transkei were to be sampled.

Various researchers have expressed views regarding the vectors of AHS as mosquitoes, ticks and other biting flies. In Sudan during an AHS outbreak, it was postulated that horn flies (*Lyperosia minuta*) were involved as they were observed around the animals and there was a remarkable absence of other blood sucking arthropods (Du Toit, 1944). A study is needed to focus on these flies and other blood feeding flies to establish their role in the transmission of AHSV.

These outbreaks of BTV8 did not stop even during the cold temperatures in north–west Europe but continued. It was then suspected that ticks might be playing a significant role in transmission of BTV8 in that region. A study was initiated to establish if ticks are involved in these transmissions of BTV8 in North-West Europe, both hard (*Ixodidae*) and soft (*Argasidae*) ticks were tested. Bluetongue virus serotype 8 was picked up by PCR in 20 out of 24 female ticks infected by capillary feeding and it was found that BTV8 could survive in hard ticks for at least 21 days and up to 26 days in soft ticks (Bouwknegt *et al.*, 2010). Further research is needed so that we can remove ticks as vectors of AHSV as it is indicated in this study.

Future research areas that need to be investigated is the role of *C. tuttifrutti* in the transmission of AHSV and other orbiviruses. A study on blood meal analysis of blood fed *Culicoides* midges are also needed. Without further study and blood meal analysis of blood fed *Culicoides* midges it would be impossible to say conclusively that *Culicoides* species do not feed on dogs

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ANNEXURE 1: CONSENT FORM

PARTICIPANT INFORMATION SHEET

Ethics clearance reference number: 2019/CAES-HREC/122

Research permission reference number:

12/11/1/1/23

Title: African Horse Sickness Epidemiology: *Culicoides* spp. diversity, vectors and overwintering of the virus in the Eastern Cape province of South Africa.

Dear Prospective Participant

My name is Ayanda Patrick Mtyapi, I am doing research under the supervision of Prof James Oguttu, a professor in the Department of Agriculture & Animal Health. My study will lead to the award of MSc (Agricultural Science) Degree from the University of South Africa. We are inviting you to participate in a study titled "African Horse Sickness Epidemiology: *Culicoides* diversity, vectors and overwintering of the virus in the Eastern Cape province of South Africa".

WHAT IS THE PURPOSE OF THE STUDY?

I am conducting this research to determine whether the African horse sickness virus can overwinter in adult *Culicoides* populations in the Eastern Cape province of South Africa. To investigate the diversity of Culicoides spp in the study area. It is also to determine whether ticks do carry African horse sickness virus. To determine the tick species from various host in this study that are infected with the AHSV . To also investigate the presence of African horse sickness antibodies in dogs.

WHY AM I BEING INVITED TO PARTICIPATE?

As a horse owner in the Sarah Baartman municipality, we ask you to participate in the study to help researcher with the collection of *Culicoides*, collection of tick

species from various host and collection of blood sample from dogs. The total number of farms to participate for this study will be all horse farms in the Sarah Baartman district municipality.

WHAT IS THE NATURE OF MY PARTICIPATION IN THIS STUDY?

The study involves the following areas:

- Collection of *Culicoides spp*
- Collection of tick species from various hosts
- Collection of blood samples from dogs

  Your participation in this study will be to allow and assist researcher in the collection of the above samples from your animals.

The expected duration of participation in this study will be 12 months for collection of *Culicoides spp.* Tick species will be collected once a month for the duration of the study. Blood samples will be collected from dogs for four repetitions for the duration of the study.

CAN I WITHDRAW FROM THIS STUDY EVEN AFTER HAVING AGREED TO PARTICIPATE?

Participating in this study is voluntary and you are under no obligation to consent to participation. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a written consent form. You are free to withdraw at any time and without giving a reason.

WHAT ARE THE POTENTIAL BENEFITS OF TAKING PART IN THIS STUDY?

If you participate in this study the following questions about AHS can be answered

1. Does AHSV overwinter in adult *Culicoides* populations in the Eastern Cape province of SA?
2. Is there more than one *Culicoides spp* in the study area in which AHSV overwinters?
3. Are ticks in the study area infected with AHSV?
4. Which ticks species found on the various hosts in the study area carry the AHSV?

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5. Are the dogs in the study area sero-positive for AHSV?

ARE THERE ANY NEGATIVE CONSEQUENCES FOR ME IF I PARTICIPATE IN THE RESEARCH PROJECT?

There are no foreseeable risks of harm or side effects to you or your dogs and horses by participating in this study. The inconveniences to you will be the use of your slight electricity needed for electric insect trap that will be running the whole night on the day of collection as well as the valuable time that you will sacrifice to bring your animals for ticks collection or your dogs for collection of blood samples.

WILL THE INFORMATION THAT I CONVEY TO THE RESEARCHER AND MY IDENTITY BE KEPT CONFIDENTIAL?

Yes, your name will not be recorded anywhere and no one will be able to connect you to any information you will give researcher. All information will be given a code number or a pseudonym and you will be referred to in this way in the data, any publications, or other research reporting methods such as conference proceedings.

HOW WILL THE RESEARCHER(S) PROTECT THE SECURITY OF DATA?

Hard copies of your information will be stored by the researcher for a period of five years in a locked cupboard/filing cabinet at the University of South Africa, for future research or academic purposes; electronic information will be stored on a password protected computer. Future use of the stored data will be subject to further Research Ethics Review and approval if applicable.

Anonymous data collected may be used for other purposes, such as a research report, journal articles and/or conference proceedings. However names of participants will not be included in such publications. It will not be possible to link individuals in publications and people in the study area.

WILL I RECEIVE PAYMENT OR ANY INCENTIVES FOR PARTICIPATING IN THIS STUDY?

Participation in this study is voluntary and participants are not entitled to any payment.

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HAS THE STUDY RECEIVED ETHICS APPROVAL

This study has received written approval from the Research Ethics Review Committee of the College of Agriculture and Environmental Sciences, Unisa. A copy of the approval letter can be obtained from the researcher if you so wish.

HOW WILL I BE INFORMED OF THE FINDINGS/RESULTS OF THE RESEARCH?

If you would like to be informed of the final research findings, please contact Mr Ayanda Patrick Mtyapi on +27 46 622 7112 / +27 795001162 or ayandamtyapi@ymail.com

Should you require any further information or want to contact the researcher about any aspect of this study, please contact Mr Ayanda Patrick Mtyapi on +27 466227112 / +27 795001162 or ayandamtyapi@ymail.com

Should you have concerns about the way in which the research has been conducted, you may contact Prof James Oguttu, e-mail:. Contact the research ethics chairperson of the CAES General Ethics Review Committee, Prof EL Kempen on 011-471-2241 or kempeel@unisa.ac.za if you have any ethical concerns.

Thank you for taking time to read this information sheet and for participating in this study.

Thank you.

Mr Ayanda Patrick Mtyapi
Tel : 0466227112
Cell: 0795001162 /E-mail: ayandamtyapi@ymail.com

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INFORMED CONSENT TO PARTICIPATE IN THIS STUDY

I, _____ (participant name), confirm that the person asking my consent to take part in this research has told me about the nature, procedure, potential benefits and anticipated inconvenience of participation.

I have read (or had explained to me) and understood the study as explained in the information sheet.

I have had sufficient opportunity to ask questions and am prepared to participate in the study.

I understand that my participation is voluntary and that I am free to withdraw at any time.

I am aware that the findings of this study will be processed into a research report, journal publications and/or conference proceedings, and my participation will be kept confidential unless a consent is granted.

I have received a signed copy of the informed consent agreement.

Participant
Signature……..Date……

Researcher's Name & Surname……(please print)

Researcher's
signature……..Date……

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ANNEXURE 2: SECTION 20 APPROVAL

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

Enquiries: Ms Mama Laing • Tel: +27 12 319 7442 • Fax: +27 12 319 7470 • E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1/23 (2659SS)

Mr Ayanda Patrick Mtyapi
Department of Rural Development and Agrarian Reform
Upper George Street
Grahamstown
6139

Email: ayandamtyapi@ymail.com; Ayanda.Mtyapi@drdar.gov.za

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Dear Mr Mtyapi,

Your Section 20 amendment request as submitted on 30 August 2022, requesting an amendment to the Section 20 permissions granted on 2019-11-27 and 2020-06-01 under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following amended research/study, with the following conditions:

Conditions:

1. All conditions stipulated in the approvals on 2019-11-27 and 2020-06-01 (attached) must still be complied with;
2. The only allowable deviations from the conditions stipulated in the approvals on 2019-11-27 and 2020-06-01 (attached) are:
   2.1 The expiry date may be extended to 30 December 2024;
3. This amendment is only valid for samples that are to be collected from date of signature of this amendment;

4. Only blood samples from dogs may be collected under this extension in compliance with the following conditions:
   - **4.1** Samples may only be collected from the Grahamstown State Veterinary area for which a letter of support dated 31 August 2022 was provided;
   - **4.2** 5 days prior to the handling of dogs and collection of blood samples, written confirmation must be obtained from the responsible state veterinarian that the revlevant area(s) is/are still not under any restriction for disease control purposes. Records must be stored for a period of 5 years for auditing purposes;
   - **4.3** Any suspect outbreak of a controlled or notifiable animal disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984) must be immediately reported to the responsible state veterinarian.

Title of research/study: African horse sickness epidemiology: Culicoides diversity, vectors and overwintering of the virus in the Eastern Cape Province, South Africa
Researcher (s): Mr Ayanda Patrick Mtyapi
Institution: UNISA Florida Science Campus
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/23 (2659SS)

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2022 -09- 02

agriculture,
forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Name, Department of Agriculture, Forestry and Fisheries

7. All samples to be transported must be packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996);
8. All samples must be sent to the ARC-OVR for analyses;
9. This Section 20 approval expires on 30 May 2020.

Title of research/study: African horse sickness epidemiology: Culicoides diversity, vectors and overwintering of the virus in the Eastern Cape Province, South Africa
Researcher (s): Mr Ayanda Patrick Mtyapi
Institution: UNISA Florida Science Campus
Your Ref./ Project Number:
Our ref Number: 12/11/1/1/23

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -11- 27

SUBJECT: **RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)**

UNISA university of south africa

UNISA-CAES ANIMAL RESEARCH ETHICS COMMITTEE

Date: 03/12/2019

NHREC Registration # : N/A
REC Reference # : 2019/CAES_HREC/122
Name : Mr AP Mtyapi
Student #: 37070002

Dear Mr Mtyapi

Decision: Ethics Approval from 03/12/2019 to completion

Researcher(s): Mr AP Mtyapi
ayandamtyapi@ymail.com

Supervisor (s): Prof JW Oguttu
joguttu@unisa.ac.za; 011-471-3353

Dr K Labuschagne
labuschagnek@arc.agric.za; 012-529-9177

Working title of research:

African horse sickness epidemiology: *Culicoides* diversity, vectors and overwintering of the virus in Eastern Cape Province, South Africa

Qualification: MSc Agriculture

Thank you for the application for research ethics clearance by the UNISA-CAES Animal Research Ethics Committee for the above mentioned research. Ethics approval is granted until the completion of the project, **subject to submission of yearly progress reports. Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

Due date for progress report: 30 November 2020

The committee makes the following recommendations for the researcher to consider:

1. That ticks be pooled according to animals they were collected from, to allow further analysis of the samples for future follow-up studies. This will ensure that the data collected will be maximised.
2. That a maximum of 120 dogs be targeted to ensure that the statistically significant minimum of 100 is obtained even after drop-outs.

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ANNEXURE 3: UNISA ANIMAL ETHICS APPROVAL

3. That a photograph be taken of each dog to ensure that there is proof that the same animals were used during each visit. This will strengthen the academic integrity of the research.

*The **low risk application** was **reviewed** by the UNISA-CAES Animal Research Ethics Committee on 02 December 2019 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
2. Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study should be communicated in writing to the Committee.
3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
4. Any changes that can affect the study-related risks for the research participants, particularly in terms of assurances made with regards to the protection of participants' privacy and the confidentiality of the data, should be reported to the Committee in writing, accompanied by a progress report.
5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
*The reference number **2019/CAES_HREC/122** should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

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Yours sincerely,

Mrs A Wilson
Deputy Chair of UNISA-CAES Animal REC
E-mail: cheata@unisa.ac.za
Tel: (011) 471-2321

Prof MJ Linington
Executive Dean : CAES
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ANNEXURE 4: VACCINATION PROTOCOL

agriculture,
forestry & fisheries
Department:
Agriculture, forestry & fisheries
REPUBLIC OF SOUTH AFRICA

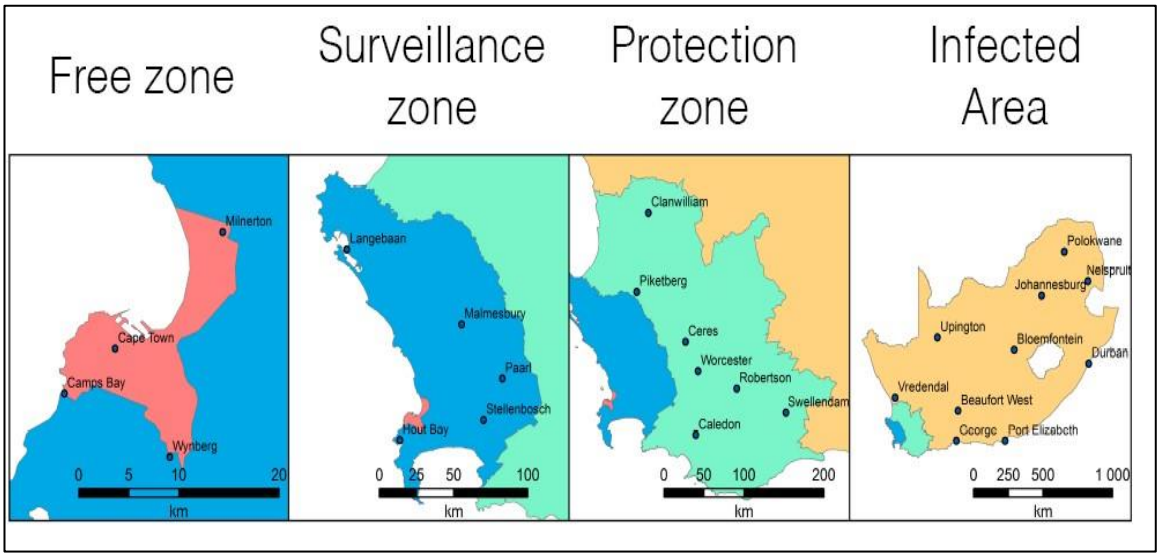
IMPORTANT INFORMATION REGARDING VACCINATION AGAINST AFRICAN HORSE SICKNESS (AHS) IN SOUTH AFRICA

AHS is a controlled animal disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84) and certain measures have been prescribed for AHS control. Please take note of the following:

Zoning

Certain parts of the Western Cape Province have been legislated as the "AHS controlled area" in terms of the Animal Diseases Act 1984 (Act no 35 of 84). The AHS controlled area is considered low risk for AHS and is made up of three zones: the AHS protection zone, the AHS surveillance zone and the AHS free zone. The rest of South Africa, outside the AHS controlled area, is considered endemic for AHS and is known as the AHS infected zone. Please contact your local Animal Health Technician (AHT) or State Veterinarian (SV) to find out what AHS zone your animals are in.

The map below is kindly supplied by the Equine Health Fund and shows a detailed view of each control zone:



(Red= AHS free zone, blue=AHS surveillance zone, turquoise= AHS protection zone, orange=AHS infected zone)

Vaccination

Always ensure that your animals are vaccinated by the correct person, at the correct time of year, with a vaccine registered in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47) and according to the AHS zone they are in. Always handle, store and administer vaccine carefully according to the manufacturer's instructions and ensure that it is kept cold until it is given to the animal.

Regulation 1 of the Animal Diseases Act 1984 (Act no 35 of 84)

States that an "efficient remedy means a remedy, approved by the director under section 1(6) of the Act". Section 1 of the Animal Diseases Act 1984 (Act no 35 of 84) states that a 'remedy' means any stock remedy which has been registered under the Fertilizers, Farm Feed, Agricultural Remedies and Stock Remedies Act, 1947 (Act 36 of 1947), including any medicine or veterinary medicine as defined in section 1 of the Medicines and Related Substances Control Act, 1965 (Act 101 of 1965.";

The only vaccine currently registered in terms of Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47) for AHS and therefore the only "efficient/effective remedy" as described above is the live virus vaccine produced by Onderstepoort Biological Products (OBP).

Please see the below except from Table 2 of the Regulations to the Animal Diseases Act 1984 (Act no 35 of 84) regarding vaccination against AHS.

Excerpt from Table 2 of the Regulations to the Act regarding vaccination for AHS

Animal Disease	***Controlled Veterinary act to be performed in respect of-***	
	Susceptible animals	***Contact animals***
1	***4***	***5***

African Horse sickness	1. *All equines in the Republic except equines in the African Horse sickness free zone and the African Horse sickness surveillance zone as described in Table 1, shall between the ages of 6 and 12 months, then between the ages of 12 and 18 months and then again once every* 2. *year thereafter be immunized with an effective remedy by the responsible person; Provided that the director in a particular case may determine that such immunization must be carried out by an officer or veterinarian.* *Equines in the African Horse sickness free zone and surveillance zone as described in Table 1 shall only be immunized with the written permission of the director.*	1. *Contact animals in a controlled area shall be isolated and immunised as determined by the director.* 2. *Contact animals outside the controlled area shall not be moved into a controlled area without the permission of the director.*

On 26 March 2015, the period of vaccination was reduced to the low midge activity period of the year in order to decrease the risk of midge transmission of vaccine virus. Ensure that all administrations of vaccine are given within the correct period as described in the table below:

Table 2 showing AHS vaccination times per AHS zone each year

AHS Zone	**Vaccination Period**
AHS free zone	Permission for vaccination will only be given from 1 June 31 October each year
AHS surveillance zone	Permission for vaccination will only be given from 1 June to 31 October each year

AHS protection zone	All equines in this area must be vaccinated within the period of 1 June to 31 October each year
AHS infected zone	Strong recommendation is made to vaccinate during the period from 1 June to 31 October each year

The following bullet points describe the control measures for vaccination against AHS:

- In the AHS infected zone all equines must be vaccinated every year against AHS with a vaccine registered in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47) such as the OBP AHS vaccine. It is strongly recommended that vaccination against AHS only take place between 1 June and 31 October each year.
- In the AHS Protection zone all equines must be vaccinated every year between 1 June and 31 October against AHS with a vaccine registered in terms of Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47) such as the OBP AHS vaccine. All administrations of vaccine must be given within this period.
- In the AHS Free and Surveillance zones equines may not be vaccinated against AHS with any remedy, whether *registered* or not, without written permission from State Veterinarian: Boland. Permission will only be given to vaccinate from 1 June to 31 October each year. All administrations of vaccine must be given within this period. If permission is granted, vaccination may only be performed by a veterinarian. To apply for permission to vaccinate or to obtain more information please see www.myhorse.org.za or contact SV: Boland (svboland@elsenburg.com) or send an email to vaccinate@myhorse.org.za.

Vaccination against AHS must be performed by a veterinarian in the following circumstances:

- For movement subject to state veterinary control (e.g. from the AHS infected zone to the AHS controlled area or from the AHS protection zone to the AHS surveillance or AHS free zone), vaccination against AHS must have been

performed with a vaccine registered in terms of Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47) by a veterinarian, not more than 24 months prior to movement and not less than 40 days prior to movement and recorded in the passport of the horse by the veterinarian.

- Where permission to vaccinate against AHS is given in the AHS surveillance or free zones.
- Where export certification requires vaccination against AHS, vaccination must be performed and recorded in the passport of the equine by a veterinarian

(*Table 2, Regulation 1 and Section 1 of the Animal Diseases Act 1984 (Act no 35 of 84))*

Movement of equids subject to AHS control

According to Regulation 20 (1) (A) (vii) of the Animal Diseases Act 1984 (Act no 35 of 84), movement of equids from the AHS infected zone to the AHS controlled area or to a zone of higher AHS control in the AHS controlled area is subject to state veterinary control and you will need a veterinary movement permit. Please see www.myhorse.org.za or contact SV: Boland (svboland@elsenburg.com) or email move@myhorse.org.za to apply for a permit or to obtain more information. (*Regulation 20 of the Animal Diseases Act 1984 (Act no 35 of 84))*

Reporting of AHS

According to Section 11 and Regulation 12 of the Animal Diseases Act 1984 (Act no 35 of 84), if you are concerned that your animals may have AHS or if an equid dies suddenly with signs that suggest AHS may be involved, you must report this to your local AHT or SV immediately. If you have had a case of AHS on your property you should also inform your neighbours and anyone who brings equids to your property. This will help ensure they can take the precautions to keep their animals safe.

(Section 11 and Regulation 12 of the Animal Diseases Act 1984 (Act no 35 of 84))

How can I help prevent my equids from getting AHS?

- Vaccinate your equines against AHS every year with a vaccine registered in terms of the Fertilizers; Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47).

- Take precautions to help prevent midges, the AHS vector, from biting your equines by doing the following:
    - Put your equines in stables or shelters from two hours before sunset to two hours after sunrise;
    - Spray your equines with a registered insect repellent and insecticide as per the manufacturer's instructions;
    - Midges gather and breed in moist or muddy areas. Eliminate midge breeding areas by removing pools or puddles of standing water, siting compost or dung heaps away from the equines and managing muddy areas e.g. around leaking taps and water troughs;
    - Do not allow equines to graze on wet, marshy land at the high risk times of the day if possible;
- Ask you neighbours and the owner or manager of any property you take your equines to if there have been any cases of AHS so you can take extra precautions to keep your animals safe;
- If you are concerned that your animals may have AHS, contact your private veterinarian or AHT or SV immediately.

ANNEXURE 5: SITES AND CO-ORDINATES

Site no	East	South
1	E 25° 50' 89"	S 33° 40' 72"
2	E 26° 29' 75"	S 33° 17' 56"
3	E 26˚ 18' 08"	S 33˚ 22' 32"
4	E 25° 22' 68"	S 33° 55' 91"
5	E 26° 46' 33"	S 33° 10' 03"
6	E 26˚ 39' 16"	S 33˚ 16' 48"
7	E 26° 18' 55"	S 33° 24' 16"
8	E 26° 09' 01"	S 33° 29' 05"

9	E 26° 23' 00"	S 33° 13' 12"
10	E 26˚ 38' 48"	S 33˚ 18' 00"
11	E 26˚ 36' 18"	S 33˚ 15' 18"
12	E 26° 50' 30"	S 33° 11' 50"
13	E 26˚ 32' 24"	S 33˚ 16' 54"
14	E 26° 56' 57"	S 33° 16' 27"
15	E 26° 39' 18"	S 33° 21' 54"
16	E 26˚ 18' 48"	S 33˚ 04' 48"
17	E 26˚ 37' 00"	S 33˚ 17' 12"
18	E 26° 22' 00"	S 33° 24' 30"
19	E 26° 40' 59"	S 33° 21' 06"
20	E 26˚ 37' 06"	S 33˚ 08' 00"
21	E 26° 35' 00"	S 33° 15' 18"
22	E 26° 23' 30"	S 33° 07' 23"
23	E 26° 22' 24"	S 33° 05' 06"
24	E 26° 38' 12"	S 33° 02' 18"
25	E 26° 08' 48"	S 33° 11' 48"
26	E 26° 33' 30"	S 33° 18' 24"
27	E 26° 25' 54"	S 33° 13' 16"
28	E 26° 44' 06"	S 33° 25' 24"