Assessment of the Hemotek® system for the in vitro feeding of field-collected *Culicoides imicola* (Diptera: Ceratopogonidae) in South Africa

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Abstract

The optimising and standardisation of in vitro blood feeding protocols for field-collected *Culicoides* species (Diptera: Ceratopogonidae) will be of essence for the comparison of the vector competencies of various populations of viruses of veterinary importance and the establishment of laboratory colonies of putative vector species. A custom-made feeding chamber to accommodate the small size of *Culicoides imicola* Kieffer was designed for the commercially available Hemotek® system and compared to existing membrane and cotton pledge feeding methods. High feeding rates coupled to higher mean blood meal volume than that of the existing OVI device indicated that the Hemotek system will be suitable for the feeding of field-collected *Culicoides*. The Hemotek system was subsequently used to identify factors that may affect feeding success in the laboratory. Evaluated factors were the source (host) and temperature of the blood meal, time of the day of feeding, the position of the blood reservoir in relation to the midges and exposure time to the blood. While only feeding orientation and the temperature of the blood source seems to significantly affect the feeding rate, all the factors did influence the volume of blood consumed.

Keywords: Culicoides imicola, blood feeding, blood meal volume.

Introduction

The unanticipated northwards expansion and subsequent re-emergence of bluetongue virus (BTV) (Reoviridae) in northern Europe, previously considered disease free, since 2006, accentuated the need for a better understanding of the epidemiology of *Culicoides* (Diptera: Ceratopogonidae) borne diseases (Rodríguez-Sánchez *et al*., 2008; Conraths *et al*., 2009; Pascall *et al*., 2019). This need was emphasised by the detection of a novel *Culicoides*-borne arboviruses such as Schmallenberg virus (SBV) (Bunyaviridae), in Europe in 2011 (Hoffmann *et al*., 2012; Veronesi *et al*., 2013). Concurrently epizootic haemorrhagic disease virus (EHDV) (Reoviridae), also transmitted by *Culicoides*, spread northwards in North

America and has recently been detected in wild white-tailed deer, *Odocoileus virginianus*, as far north as east-central Canada (Allen *et al*., 2019).

The northwards expansion in Europe was initially ascribed to climate change, which may have led to a potential increase in the geographical occurrence range of competent *Culicoides* vector species such as, *Culicoides imicola* Kieffer (Nolan *et al*., 2008). It was, however shown that native palearctic species, previously considered to be of less importance, e.g. *Culicoides obsoletus* (Meigen) and/or *Culicoides chiopterus* (Meigen) may have played a decisive role in spreading this virus (Caracappa *et al*., 2003; Mehlhorn *et al*., 2007; Meiswinkel *et al*., 2007). These observations were confirmed by infection studies of pools potentially containing *C. obsoletus*, *Culicoides scoticus* Downes and Kettle, *Culicoides dewulfi* Goetghebuer and *C. chiopterus* demonstrating a low level of vector competence when fed on a viraemic sheep infected with BTV and field isolations from these species (Meiswinkel *et al*., 2007; Dijkstra *et al*., 2008; Carpenter *et al*., 2015).

Up to date, a relatively small proportion, 14 of 1357 described species of *Culicoides* (Borkent, 2017), is considered as confirmed vectors of viruses of veterinary or medical importance (Purse *et al*., 2015). During the last decade, several *Culicoides* species from Africa, Europe and Northern America were shown to be susceptible to laboratory infection with exotic arbovirus strains. This indicates that competence to orbiviral infection is widespread in the genus *Culicoides* (Carpenter *et al*., 2008; Venter *et al*., 2011; Del Rio López *et al*., 2012; Ruder *et al*., 2012).

Determination and comparison of the oral susceptibility, as an indication of vector competence, of field populations of *Culicoides* will infer the role of these populations in the transmission of viruses. It will also indicate to what extent populations beyond the current range of an outbreak can support the potential expansion thereof. The in vitro feeding of field-collected *Culicoides* will be indispensable for the determination and comparison of the oral susceptibility and vector competence of various *Culicoides* species and geographical populations. The optimising of artificial feeding methods and the identification of factors affecting the efficacy thereof will be essential for the establishment of sustainable laboratory colonies of putative vector species.

Due to their small size, nocturnal activity and unidentified factors that may influence the blood feeding response, the in vitro feeding of field-collected *Culicoides* remains challenging. Several diverse methods, each with its own advantages and disadvantages, for the artificial feeding of *Culicoides* midges, have been developed. Although the feeding on viraemic hosts can be considered the most reliable method to determine oral susceptibility, it necessitates large numbers of field-collected *Culicoides* coinciding with the time the infected host displays high viremia. The use of susceptible and disease-free host animals for infection and transmission studies and colony maintenance is expensive, time-consuming, requires large laboratory space and insect-proof stables. It can also be considered as unethical if reliable alternative methods are available. Alternative methods to determine oral susceptibility and subsequent transmission such as the use of embryonated chicken eggs (Jones & Foster, 1966; Boorman *et al*., 1975; Van der Saag *et al*., 2015, 2017) may be restricted to viruses that will replicate in chicken eggs.

Cotton wool pledgets soaked with a blood/virus mixture has previously been used and can be seen as a relatively simple and inexpensive method to use in large-scale laboratory trails (Jennings & Mellor, 1988; Venter *et al*., 2005; Carpenter *et al*., 2008; Van der Saag *et*

al., 2015). A drawback may be that many viruses are cell-associated (Hoff & Trainer, 1974) and that the cells settle differently in a pledget and insects might be feeding only on the serum dripping from the blood-soaked pledget. Relatively high virus titres may therefore be required to infect *Culicoides* females. To overcome this drawback, a variety of membrane feeding systems have been developed and incorporated in the research on *Culicoides*. Jones & Potter (1972) used chicken skin membranes for *Culicoides sonorensis* Wirth and Jones. Owens (1981) fed three Australian species of *Culicoides* on heated bovine blood through a stretched Parafilm® membrane. Venter *et al*. (1991) published a one-day-old chicken skin membrane feeding technique to determine the infection rate of *C. imicola* for BTV. This technique has subsequently been utilised to determine the oral susceptibility of a number of Afrotropical and European livestock-associated *Culicoides* species for a variety of viruses of veterinary importance (Goffredo *et al*., 2004; Venter, 2016a, 2016b; Paslaru *et al*., 2018). Most European *Culicoides* species, however, showed an extreme reluctance to blood-feed through membranes in the laboratory (Goffredo *et al*., 2004; Carpenter *et al*., 2006; Federici *et al*., 2019).

The volume of blood ingest during feeding may be as important as the feeding rate in the evaluation and optimising of artificial feeding methods. The volume of the blood meal will determine the number of infective particles ingested and may denote the minimum level of viremia needed to infect a competent vector (Souza-Neto *et al*., 2019). Blood meal volume may furthermore define egg batch size and reproductive success. Determination of blood meal volume, and the factors influencing this, will be a prerequisite for colonisation and comparable vector competence studies. With a mean wing length of between 0.95 mm (Morag *et al*., 2013) and 1,06 mm (Meiswinkel, 1989), *C. imicola* resorts among the smallest of hematophagous vectors render determining their blood meal volume challenging.

To reliably compare the oral susceptibility of various populations, artificial feeding methods, among other factors, need to be standardised to ensure reproducibility (Wilson & Harrup, 2018). In an effort to optimise and standardise feeding protocols, a commercially available Hemotek® feeding system, routinely used in mosquito and *Culicoides* colony maintenance, was compared to a membrane feeding, method described by Venter *et al*. (1991) and cotton pledge feeding. The Hemotek system was subsequently used to pinpoint factors that may affect feeding rates, the volume of the blood meal and survival after feeding.

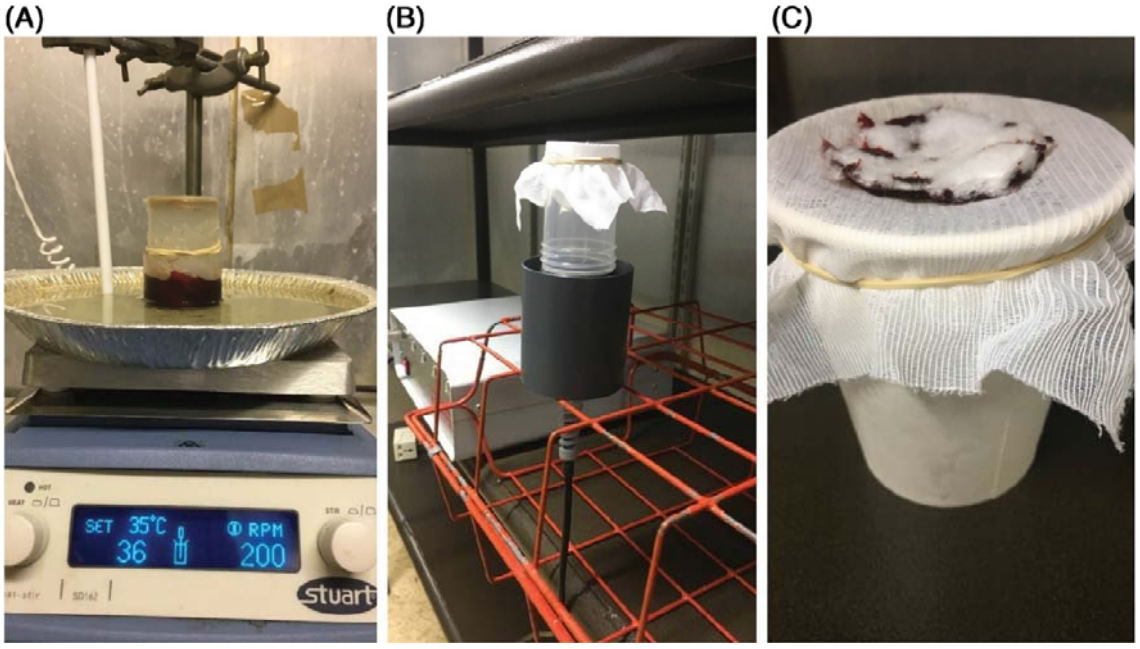

Fig. 1. Artificial feeding systems for livestock-associated *Culicoides* midges: (A) membrane feeding method as described by Venter *et al*. (1991), (B) commercially available Hemotek® system and (C) cotton pledge feeding using defibrinated cattle blood.

Material and methods

Midge collection

Midges were collected alive next to cattle stables at four sites of the ARC-Onderstepoort Veterinary Research (ARC-OVR) (258290S, 288110E, 1219 m above sea level), Pretoria, South Africa. Collections were done overnight using down-draught, 220 V OVI light-traps equipped with 8 W UV-light tubes, in summer, from 26 January to 1 May 2018. Midge survival was facilitated by excluding large insects from the collections using mosquito netting, mesh size 2 mm, placed around the entrance portals of the trap. Crumpled paper towel was placed into the 500 mL collection beaker-sheltered the trapped midges against the downdraught of the fan. *Culicoides imicola*, were sorted from the collected midges using identification keys and species descriptions (Meiswinkel, 1989; Labuschagne, 2016).

Handling of collected *Culicoides* before feeding was conducted as described previously (Venter *et al*., 1998). The collected midges were maintained in 250 mL unwaxed paper cups in the dark at 23.90 ± 0.82 °C, 69.02 ± 5.50% RH and fed *ad libitum* on a 10% (w/v) sucrose solution on cotton pledges. The midges were kept in the dark to minimise their phototaxis behaviour and to increase survival.

Comparison of feeding methods

A membrane feeding method, as described by Venter *et al*. (1991), was compared to a commercially available Hemotek system and cotton pledge feeding using defibrinated bovine blood. Blood was collected from the jugular vein using a trocar and cannula into a 1 L sterilised glass flask containing about 150 mL 6 mm diameter of sterilised glass beads. After collection, the flask was closed and shaken until defibrination of the blood was completed.

The fibrin was discarded, and the remaining blood decanted into sterile 20 mL screw-top glass bottles and stored at 4 °C. Between 26 January and 1 May 2018, 94 pools of field-collected *Culicoides* were fed using different feeding methods.

To prevent midges from feeding on the plasma instead of the blood cells, the observable visible plasma layer was removed with a 5 mL disposable syringe, immediately before it was used for feeding. To minimise the potential influence of the physiological composition and age structure of the midges, the feeding systems were evaluated in parallel.

OVI device

The feeding chamber of the OVI device (Fig. 1A) consists of a plastic container, 40 mm in diameter and 50 mm high, of which the bottom was replaced by a 1-day-old chicken skin membrane (Venter *et al*., 1991). The lid of the container was replaced with fine gauze. The midges were immobilised at −20 °C before they were transferred in pools of between 1000 and 1500 individuals into the feeding chamber. Pools sizes were approximated to eliminate sorting of the midges on a refrigerated chill table before feeding to decrease handling and accompanying mortality. This feeding chamber fitted into a second slightly wider, plastic container (45 mm in diameter and 40 mm high) containing 10 mL of blood and 5 mm long magnetic stirrer bar. The two containers were placed in a makeshift water bath, consisting of a disposable aluminium pie dish, on a magnetic heater stirrer to maintain the blood temperature at 36.5 °C (Fig. 1A). The blood was stirred slowly at 200 rpm during feeding. The collected midges were acclimatised for at least 24 h at 23.90 ± 0.82 °C, 69.02 ± 5.50% RH in the dark and fed within 48 h post collection. A feeding time of 35 min was allowed. After feeding, the midges were transferred to 80% ethanol and the number of *C. imicola* and number of engorged females determined.

Hemotek®

A commercially available Hemotek feeding system with a PS6 Power Unit was evaluated in parallel with the OVI system (Fig. 1B). A Hemotek FU1 feeder, fitted with a 3 mL reservoir with a stretched Parafilm membrane, was used to feed midges on defibrinated bovine blood.

For mosquito colony maintenance (Gunathilaka *et al*., 2017; Kauffman *et al*., 2017) and the feeding of some larger *Culicoides* species, e.g. *Culicoides nubeculosus* (Meigen) (Lilian *et al*., 2015) the Hemotek feeder, with the blood reservoir, is positioned on top of a holding cage and females fed through the net of the cage. *Culicoides* species belonging to the subgenus *Avaritia* are relatively small with characteristic short mouthparts (Bellis *et al*., 2014) and *C. imicola* seems unable to access and penetrate the membrane if covered by fine netting. To overcome this impediment, a custom-made feeding chamber, similar to the OVI device, was constructed for use with the Hemotek. This chamber consisted of a plastic bottle 45 mm in diameter and 74 mm high, of which the bottom was removed. The 3 mL reservoir (45 mm in diameter) of the Hemotek feeder fitted securely into the bottom of the feeding chamber to prevent midges from escaping (Fig. 1B). The feeder temperature was set at 36.50 °C.

As with the OVI system, the midges were immobilised at −20.00 °C and transferred, in pools, into the feeding chamber after which the top of the chamber was covered with fine net fixed with elastic bands (Fig. 1B). To mimic the OVI feeding device, the FU1 feeder was used in an upside-down position with the blood reservoir at the bottom of the chamber (Fig. 1B). Acclimation time and feeding preparation, i.e. 35 min at 23.90 ± 0.82 °C, 69.02 ± 5.50% RH

in the dark, as described for the OVI device. As for the OVI device, the midges were transferred to 80% ethanol after feeding and the number of *C. imicola* and engorged females determined.

Cotton pledget feeding

In parallel to membrane feeding, midges were fed on 4 cm diameter cotton wool pledgets, saturated in 5 mL defibrinated bovine blood, placed on top of the netting of the holding cups (Fig. 1C). This eliminated the need to immobilise and transfer of the midges before feeding. However, before blood feeding, collected midges were fed on a 10% sucrose solution post collection and maintained at 23.90 ± 0.82 °C, 69.02 ± 5.50% RH in the dark for at least 24 h before offered a blood meal. As for membrane feeding, the sucrose solution was removed 12 h before blood feeding. The midges were allowed to feed for 2 h in the dark after which they were immobilised at −20.00 °C and transferred to 80% ethanol.

In a second series of pledget feeding evaluations, the midges were blood fed immediately after field collection. The collected midges were not offered a sucrose meal post collection and were fed immediately after exiting the collection boxes for 2 h. Feeding conditions and environmental factors were the same as previously described.

The evaluated feeding success indicators were the feeding rate, the volume of the blood meal and survival after feeding. To determine the feeding rate, *Culicoides* females were immobilised after feeding at −20 °C and transferred to 80% ethanol, after which the blood-engorged females and total number of female *C. imicola* were counted. The number of replicates conducted with each feeding device is indicated in Table 1.

To determine the volume of blood ingested engorged *C. imicola* females, they were immediately after feeding, randomly sorted in pools of 10 on a refrigerated chill table and stored at −20.00 °C. Twenty replications of each treatment were done. These females were homogenised in 120 µL distilled water with two stainless steel beads in an Eppendorf® using a TissueLyser (Qiagen®, South Africa). After homogenisation for 5 min, samples were vortexed and 100 µL transferred to an ELISA plate and absorbance read at 410 nm range using a SpectraMax spectrophotometer. Three readings were taken per sample. The spectrophotometer readings were converted to blood meal volume with a calibration curve. Calibration curves were obtained by the dilution of known volumes of the blood, in distilled water, used for feeding. These curves were generated on the day of feeding as the absorption of the defibrinated blood can change over time (de Beer *et al*., n.d.).

To compare survival, females were immobilised immediately after feeding at −20.00 °C for 1 min and the blood-engorged *C. imicola* females separated on a refrigerated chill table. These females were randomly pooled in groups of 50 and transferred to 250 mL paper cups and maintained on a 10% sucrose solution on cotton pledges at 23.90 ± 0.82 °C, 69.02 ± 5.50% RH in the dark. Survival was determined 24 h post feeding. Six groups of 50 engorged females were evaluated for each feeding system/regime.

Factors that can potentially influence feeding success of an artificial feeding system was evaluated in a second series of experiments. Based on the relatively high feeding rates obtained, the Hemotek system as described above, was used for these evaluations. The feeding protocol and feeding success indicators, as described for the comparisons of the feeding systems, was used to evaluate the following factors.

Table 1. Feeding rate, blood meal size and survival rate as obtained for the artificial feeding of field-collected female *Culicoides imicola.*

	Feeding rate (%)		Blood meal volume (µL)		Survival rate at 24 h (%)	
	Replicates	**Mean ± STD**	**Replicates**	**Mean ± STD**	**Replicates**	**Mean ± STD**
Feeding System						
OVI	29	15.33 ± 15.95**a**	20	0.04 ± 0.01**a**	6	36.89 ± 14.22**a**
Hemotek®	29	55.32 ± 17.61**b**	20	0.06 ± 0.03**b**	6	57.33 ± 13.31**b**
Pledget: 24 h post collection	20	26.81 ± 11.82**a**	20	0.03 ± 0.02**ac**	6	45.33 ± 12.63**ab**
Pledget: immediately post collection	16	78.61 ± 8.70**c**	20	0.02 ± 0.01**c**	6	93.00 ± 6.16**c**
Blood meal Source						
Bovine	13	33.35 ± 12.69**a**	20	0.02 ± 0.01**a**	6	31.33 ± 7.34**a**
Ovine	13	23.74 ± 9.90**a**	20	0.04 ± 0.01**b**	6	28.67 ± 12.82**a**
Equine	13	23.70 ± 14.56**a**	20	0.02 ± 0.01**a**	6	42.00 ± 31.87**a**
Time of day of feeding						
Mid-day	14	35.44 ± 13.51**a**	20	0.01 ± 0.01**a**	6	48.00 ± 13.56**a**
Late afternoon	14	34.87 ± 13.49**a**	20	0.01 ± 0.01**a**	6	58.33 ± 7.53**a**
After sunset	13	41.47 ± 12.73**a**	20	0.01 ± 0.01**a**	5	58.00 ± 12.65**a**
Exposure time (min)						
5	12	25.24 ± 14.03**a**	20	0.02 ± 0.01**a**	6	69.00 ± 6.78**a**
20	12	22.96 ± 10.97**a**	20	0.03 ± 0.01**a**	6	59.33 ± 10.78**a**
35	12	31.21 ± 15.64**a**	20	0.05 ± 0.02**b**	5	60.00 ± 23.28**a**
50	12	32.49 ± 18.81**a**	20	0.05 ± 0.03**b**	6	56.33 ± 15.7**a**
Blood temperature (°C)						
32.50	13	51.86 ± 13.28**a**	20	0.03 ± 0.01**a**	6	80.00 ± 20.47**a**
34.50	13	59.50 ± 11.95**a**	20	0.01 ± 0.01**b**	6	74.67 ± 16.81**a**
36.50	13	61.31 ± 12.14**a**	20	0.04 ± 0.02**a**	6	78.67 ± 20.03**a**
38.50	13	56.55 ± 9.57**a**	20	0.06 ± 0.03**c**	6	82.92 ± 19.68**a**
40.50	12	39.91 ± 11.96**b**	20	0.03 ± 0.01**a**	6	57.00 ± 17.33**a**
Feeding orientation						
Down	14	23.54 ± 13.36**a**	25	0.01 ± 0.01**a**	3	27.57 ± 19.46**a**
Up	14	9.02 ± 4.92**b**	17	0.01 ± 0.01**a**	3	51.00 ± 23.52**a**

ANOVA with Tukey's test was applied to differentiate between the treatment effects on the proportion of engorged females, blood meal volume and midge survival rate. Numbers for each treatment in the same column followed by different alphabetic letters are significantly different at the 5% level.

STD, standard deviation.

Blood meal source

Culicoides imicola preferably feed on bigger mammals (Meiswinkel, 1989). To determine potential host preferences, 13 pools of field-collected midges were fed on either defibrinated bovine, equine or ovine blood, between 2 and 17 February 2018.

Time of day of feeding

Previous studies indicated that *C. imicola*, as characteristically to most *Culicoides* species, display host seeking flight activity peaks that coincide with sunset (Venter *et al*., 2019). Between 16 February and 2 March 2018, 14 pools of field-collected midges were fed either before midday, late midday or immediately after sunset. To determine if the natural host seeking activity of the females may impact on the blood feeding response in the laboratory.

Exposure time

Midges were exposed on either blood-soaked cotton pledgets or the Hemotek system for up to 12 h as described by Van der Saag *et al*. (2015). Between 20 and 26 March 2018, 12 replicates were conducted for feeding periods of 5, 20, 35 and 50 min, using a Hemotek system.

Blood temperature

The normal mean body temperature for domesticated mammals range between 38.00 °C (horses) and 39.00 °C (pigs). However, most infectious diseases will induce a febrile state (for cattle mean rectal temperature above 39.40 °C). To determine the potential influence of the blood temperature on the feeding response, 13 pools of field-collected midges were fed on blood maintained at 32.50, 34.50, 36.50, 38.50 and 40.50 °C, respectively between 3 and 13 April 2018.

Feeding orientation

In mosquito feeding and colony maintenance, the Hemotek feeder, with the blood reservoir, is placed on the top of the holding cage and the insects must fly upwards to reach the blood source. In contrast, in the OVI device the blood reservoir is positioned at the bottom of the feeding chamber (Fig. 1A). FU1 feeder was evaluated in both the upwards and bottom position to determine the potential influence of feeding orientation on feeding success (Fig. 2). Between 23 February and 3 March 2018, 14 replicates were conducted for each position.

Fig. 2. Hemotek® system feeder in the upwards and downwards feeding positions used for the feeding of livestock-associated *Culicoides* midges.

Statistical analyses

The proportion of females that fed was calculated based on the presence/absence of visible blood in the abdomen. Pairwise analysis of variance (ANOVA) was used to differentiate between the treatment effects on the proportion of engorged females, blood meal volume and midge survival rate. As data were normally distributed standard (parametric) methods were used, Tukey's test was applied.

Results

All given results are presented in the Table 1.

Evaluation of feeding systems

The highest mean feeding rate, 78.61 ± 8.70%, obtained in the 29 pools fed immediately after collection on blood-soaked pledgets was significantly different ($P < 0.001$) when compared to the others. The mean feeding rate of 55.32 ± 17.61% obtained in the 29 pools fed on the Hemotek system was significantly different ($P < 0.001$) when compared to the OVI system (mean 15.33 ± 15.95%) or for midges fed 24 h post collection on pledgets, 26.81 ± 11.82%.

Similar to the feeding rate, the mean blood meal volume obtained with the Hemotek system was significantly different ($P < 0.001$) compared to the membrane- and the pledget feeding systems. The mean blood meal volume of 0.06 ± 0.03 μL, obtained with the Hemotek, was significantly different ($P < 0.01$) compared to that of the others. The mean volume obtained with the OVI system, 0.04 ± 0.01 μL, was significantly different compared to the females fed 24 h after collection on pledgets ($P < 0.05$). The difference between mean blood meal volume of females fed immediately after collection, 0.02 ± 0.01 μL, and 24 h after collection, 0.03 ± 0.02 μL, was not statistically significant.

The highest mean survival of 93.0 ± 6.16% 24 h post feeding obtained for females fed immediately after collection on pledgets was significantly different ($P < 0.001$) compared to that for the other systems ranging from 36.9 ± 14.22% in the OVI to 57.33 ± 13.31% in the Hemotek system.

Blood meal source

The mean feeding rate, ranging from 33.35% ±12.69 for females fed on bovine blood to 23.74 ± 9.90% and 23.70 ± 14.56% for those fed on ovine and equine blood, did not differ significantly. In contrast, the mean blood meal volumes, ranging from 0.02 ± 0.01 μL to 0.04 ± 0.01 μL for females fed on equine/bovine or ovine blood, respectively, differ significantly. The highest mean blood meal volume, as obtained in females fed on ovine blood, was significantly different compared to those that fed on bovine ($P < 0.001$) or equine ($P < 0.01$) blood. The blood meal volume in females fed on bovine or equine blood did not differ significantly. The mean survival rates, ranging from 28.67 ± 12.82% in females that fed on ovine blood to 42.00 ± 31.87% for those fed on equine blood, did not differ significantly.

Time of day of feeding

The highest mean feeding rate of 41.47 ± 12.73% obtained immediately after sunset was not significantly different than that obtained before mid-day, 35.44 ± 13.51% or late afternoon,

34.87 ± 13.49%. Similar mean blood meal volumes, 0.01 ± 0.01 µL, were taken up in the midday, late afternoon and after sunset. The survival rates, 24 h after feeding, ranging from 48.00 ± 13.56% for females fed before midday to 58.33 ± 12.65% for those fed in the late afternoon were not statistically significantly different.

Exposure time

The mean feeding rate ranging from 22.96 ± 10.97% in females fed for 20 min to 32.49 ± 18.81% in females feeding for 50 min did not differ significantly. On the other hand, the mean blood meal volume, ranging from 0.02 ± 0.01 µL in females exposed for 5 min to 0.05 ± 0.03 µL in those exposed for 50 min differ significantly ($P < 0.001$). The mean blood meal volume in females exposed for 50 min was similar to those that fed for 35 min, 0.05 ± 0.02. Similarly, the mean meal volume in females that were exposed for 5 min was not significantly different than that of those exposed for 20 min, 0.03 ± 0.01 µL. The blood meal volume in females exposed for 20 min was, significantly different ($P < 0.05$) compared to those exposed for 35 min. The highest mean survival rate of 69.00 ± 6.78% for females which were exposed for 5 min was not significantly different from the lowest mean survival rate of 56.33 ± 15.77% for females which were exposed 50 min.

Blood temperature

The highest mean feeding rate, 61.31 ± 12.14%, was obtained on blood maintained at 36.50 °C. The mean feeding rate at 36.50 °C was significantly different compared to that at 40.50 °C (39.91 ± 11.96%) ($P < 0.001$). It was, however, not significantly different from that at 32.50 °C (51.86 ± 13.28%), 34.50 °C (59.50 ± 11.95%) or 38.50 °C (56.55 ± 9.57%). The highest mean volume of blood, 0.06 ± 0.03 µL, consumed on blood maintained at 38.50 °C was significantly different ($P < 0.001$) compared to that of any other temperature. No statistically significant difference was observed in female survival 24 h after feeding. The survival ranged from 57.00 ± 17.33% for females fed on blood maintained at 40.50 °C to 82.92 ± 19.68% for those maintained at 38.50 °C.

Feeding orientation

The mean feeding rate of 23.54 ± 13.36% was significantly different ($P = 0.001$) in females feeding downwards, compared to 9.02 ± 4.92% in those feeding upwards. The mean blood meal volume 0.01 ± 0.01 µL was similar in these two groups. The higher mean survival 24 h after feeding in females feeding upwards, 51.00 ± 23.52%, was not significantly different from 27.57 ± 19.46% for those feeding upwards.

Discussion

In the evaluation of the four feeding systems, the highest mean feeding (78.4%) and survival rate (93.0%) in field-collected *C. imicola* were found in females fed immediately post-collection on blood-soaked cotton wool pledgets. The mean volume of consumed blood was, however, lower compared to that for the other methods. When the field-collected midges were offered a 10% sucrose solution after collection up to 12 h before exposure to the blood-soaked pledgets feeding- and survival rates decrease significantly. The high feeding rate obtained immediately post-collection may be indicative of the dehydrated state of the midges, after spending a few hours in the trap rather than a blood feeding response. This is illustrated by the fact that, in some cases, even males will take blood from the pledgets. This may partly

explain the lower volumes of blood consumed by females fed immediately post collection. In vector competence studies, the lower blood meal volume may relate directly to lower infection, or a refractory status, in *Culicoides* as reported for pledget feeding (Jennings & Mellor, 1988; Venter *et al*., 2005; van der Saag *et al*., 2015; Barber *et al*., 2018; Federici *et al*., 2019). The higher survival rate in this group may be resulting from the fact that they were not immobilised at −20.00 °C and transferred to a feeding chamber before feeding.

The high feeding rate obtained with the Hemotek system coupled to a mean blood meal volume, higher than that of the OVI device, signified that the Hemotek can be used for the comparative blood feeding of field collected South African *Culicoides* species. An advantage of the Hemotek, compared to that of the OVI system, is that lower volumes, 3 mL instead of 10 mL, of blood can be used in infection experiments. The blood temperature can be regulated more accurately in the Hemotek and the blood is in closed system which will reduce potential bacterial contamination and prevent non-target insects from feeding. A disadvantage may be that the blood is not agitated during feeding to ensure a homogenous distribution of blood cells and pathogens. It was speculated that the slight vibration of membrane by the action of a stirring bar may increase the feeding response (Davis *et al*., 1983; Lehane, 2005).

As reflected by the sex ratio and physiological composition of the collected midges, light traps near mammal hosts, mainly collect *Culicoides* females actively in search of a blood meal (Venter *et al*., 2019). This phenomenon, however, does not inevitably lead to a blood feeding response in the laboratory as reflected by the reluctance of especially European species to take a blood meal under artificial conditions (Jennings & Mellor, 1988; Goffredo *et al*., 2004; Carpenter *et al*., 2006; Federici *et al*., 2019). This phenomenon may have contributed the large variation in feeding rate within and between replicates of the same feeding regime in the current study. In addition to the physiological condition of the female, factors that may initiate the probing response include vibration, surface texture, skin, hair and feather thickness, carbon dioxide and other odour levels, visual stimuli, contact-chemical stimuli and heat and moisture levels (Friend & Smith, 1977; Davis *et al*., 1983; Lehane, 2005). Some of which may be intricate to mimic in the laboratory.

Of the factors evaluated, only the temperature of the blood and feeding orientation seem to have a significant effect on the feeding rate. The highest proportional feeding response was obtained on blood maintained between 34.50 °C and 38.50 °C. This temperature corresponds with the mean body temperature of the host animal and agrees with previous studies that indicate heat as an important probing stimulant for several blood feeding insects (Friend & Smith, 1977; Lehane, 2005). The temperature of the blood has previously been shown to play a significant role in the feeding response of some *Culicoides* species. e.g. a 30% reduction in the feeding rate of *Culicoides impunctatus* Goetghebuer was found if the temperature of blood was decreased from 40.00 to 36.00 °C (Blackwell *et al*., 1994). Davis *et al*. (1983) consider the warm membrane in contact with the heated blood as a feeding stimulus for *Culicoides mississippiensis* Hoffman.

The significance of heat as a feeding stimulant was emphasised by the finding that the mean volume of blood consumed on blood maintained 38.50 °C was more than 1.7 times higher than at 36.50 °C and between 5 and 20 times higher than that at 32.50 or 34.50 °C, respectively. The lower volume of consumed blood at the lower temperature range may have contributed to the lower volumes of blood consumed on blood-soaked pledges at room temperature. The control mechanism behind this phenomenon remain unclear but the fact that

blood viscosity is inversely related to temperature may play a role (Lehane, 2005). The role that body heat may play in the location and preferential feeding location on host still needs to be determined. It will be of interest to determine to what extent this phenomenon will be influenced by environmental temperatures.

The higher proportions of females that fed if the blood reservoir was at the bottom, instead of the top, of the feeding chamber may once again signify the detrimental physical condition of the midges after light trap collection. Some of the females which fed in the downwards position may have lacked the energy to fly towards the blood source. This is also reflected by the somewhat higher survival in females which fed upwards.

Although longer exposure to the blood did not increase the proportions of females that fed, longer exposure of, up to 35 min, did increase the volume of blood imbibe. The minimum time needed for a *Culicoides* female to fully imbibe is not known. For most periodic ectoparasites, like *Culicoides*, obtaining blood from a host can be considered as perilous and evolutionary pressures may have minimised host contact time (Lehane, 2005).

It was shown that *C. imicola* females can consume up to 75% of their body weight in a single blood meal under artificial conditions (Venter *et al*., 2005). In *Culicoides*, the alimentary canal, in addition to the midgut, consists of a cuticle-lined diverticulum known as the crop. Sugar meals are normally sent to the crop to be regurgitated into the midgut as required. This may be of importance for water conservation as no absorption takes place from the crop. It is not known to that extent the dehydrated state of the female after collection may have prevented blood from reaching the mid-gut, where it is usually held. This may adversely affect infection studies since the mid-gut of the females may not be exposed to the full dose of virus immediately after ingestion thereof (Lehane, 2005).

In contradiction to feeding rate which was only significantly influenced by two of the factors evaluated, all five factors play a more decisive role in the volume of blood consumed. Although it was shown that the source (host) of the blood may influence the volume of blood ingested, it remains to be determined to what extent the diet and physical condition of the donor animal will influence the quality and acceptability of the blood. It also remains to be determined to what extent the use of anticoagulants (de Beer *et al*., 2016), feeding stimulants such as ATP and/or membrane type (Blackwell *et al*., 1994) will influence not only the feeding rate but also blood meal size and survival.

Corresponding with the flight activity of the females (Venter *et al*., 2019), it seems that larger volumes of blood may be consumed later in the day compared to that earlier in the day.

In addition to the factors evaluated, the feeding response can be affected by a range of factors including ambient temperature, insect age, mating status, stage of the gonotrophic cycle as well as previous feeding history (Lehane, 2005). To minimise the potential influence of some of these in the current study, individual factors were evaluated in parallel utilising midges from a single population collected in a single night under identical environmental conditions in the current study.

In relation to blood meal volume and infection prevalence, it can be noted that the minimum level of viraemia required to infect competent *Culicoides* vectors is not known. Considering the relatively small volume of blood taken up during artificial or natural feeding, variation in the blood meal volume may have a direct effect on the number of infective particles imbibed

and as such the potential infection in a competent vector. Based on the blood meal volume of *C. imicola*, ranging from 0.010 to 0.060 µL, a viraemia of $10^5 \log_{10}$ID virus/ml will theoretically expose the vector to approximately 1 $TCID_{50}$ of virus (Venter *et al*., 2005). In Australia, Muller *et al*. (1982) calculated the volume of the blood meal taken by a single *Culicoides brevitarsis* Kieffer, which is of similar size to the *C. imicola*, about 0.03 µL and obtain an infection prevalence of 0.2% in midges fed on cattle with a BTV viraemia of 10^2 $TCID_{50}$/ml. It can, however, be mentioned that Bonneau *et al*. (2002) succeeded in infecting *C. sonorensis* when feeding on a BTV-infected sheep with no detectable viraemia. This implies that any titre of viraemia may be sufficient to establish an infection in at least a proportion of biting vectors and that the number infected will depend upon the biting- and competence rate of the species involved. The proportion of competent vectors that eventually become infected will, however, depend on the titre of the virus in relation to the volume of blood imbibe. Variations in the blood meal volume may as such have a substantial effect on the laboratory determination and reliable comparisons of the infection rate between species and populations and emphasise the need for standardised feeding protocols.

Female *Culicoides* need a blood meal to complete their gonotrophic cycle. The relationship between blood meal volume and fecundity in *Culicoides* is not clear. It is accepted that one batch of eggs is matured after each blood-meal taken (Kettle, 1962; Walker & Boreham, 1976). Veronesi *et al*. (2009) reported a mean egg batch size of 6.8 eggs / *C. imicola* female after blood feeding using the OVI system. This batch size was considerably lower than that of 69 eggs / female reported by Nevill (1967) after feeding on the shaven ear of rabbit or that of 53–65 (Braverman & Linley, 1994), 263 (Walker & Boreham, 1976) or 69 eggs / female (Barceló & Miranda, 2018) in field-collected gravid females. Although the quality of the blood and incubation temperatures could play a role, it seems that lower volumes of blood are taken up during artificial feeding compared to that on a host under natural conditions. Infection prevalence determinations based on artificial feeding may therefore underestimate the real situation.

Although the current study indicates that the method used for artificial blood feeding may have a limited effect on survival, 24 h after exposure, potential long-term effects on survival and fecundity still needs to be determined.

Conclusions

Comparison of feeding systems indicated that feeding rate as well as the volume of blood consumed were higher in the Hemotek than that of the OVI system. The Hemotek system can be used for the blood feeding of field-collected livestock-associated South African *Culicoides* species. The study highlighted some of the factors that may affect the feeding success under artificial conditions. The volume of the blood imbibes is more prone to be influenced by external factors compared to number of females that feed. The temperature of the blood source especially plays a significant role in influencing blood feeding response as well as the volume of blood imbibe. Although artificial feeding will identify susceptible *Culicoides* species, standardised protocols will be essential to obtain comparable and reproducible results between species and populations.

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The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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