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CONTENTS

1. Abstract

Ovine footrot is an infectious cause of lameness in sheep, caused by the bacterium *Dichelobacter nodosus*. *Fusobacterium necrophorum* is a secondary bacterial pathogen that increases the severity of footrot. Footrot has a major impact on the health, productivity and welfare of sheep worldwide. The estimated cost to the UK sheep industry is between £24 and £80 million per annum.

F. necrophorum is an opportunistic pathogen, meaning that it has reservoirs in healthy individuals or their environment. In footrot these were believed to be the soil of sheep pasture and sheep faeces, however, no studies had been conducted to investigate reservoirs of *F. necrophorum* in sheep. The aim of this project was to conduct two longitudinal studies to investigate reservoir sites of *F. necrophorum* in ovine footrot. A second aim was to develop a strain typing method for *F. necrophorum*, and to use this to compare communities of *F. necrophorum* at different sites in sheep and their environment.

A key finding from this study was that, contrary to prior belief, the environment was not a significant reservoir of *F. necrophorum* in footrot. Instead, *F. necrophorum* was primarily detected in sheep. Certain strains of *F. necrophorum* were found on the feet of sheep, and these were found in higher numbers and for longer time periods on feet with footrot. Footrot may therefore be important for maintaining a population of *F. necrophorum* within a sheep flock, and feet with footrot may be important for transmission of *F. necrophorum* to other sheep within the flock. The strains of *F. necrophorum* found on feet were transiently present in the mouths and faeces of sheep, and these sites may be significant in the absence of footrot. The mouths and faeces of healthy sheep may also facilitate transmission of *F. necrophorum* between flocks through movement of sheep.

2. Introduction

2.1. Ovine footrot

Footrot is an infectious dermatitis of the interdigital skin of sheep that causes lameness. This leads to poor welfare (Ley *et al.*, 1995; Goddard *et al.*, 2006), poor health and reduced productivity (Marshall *et al.*, 1991; Nieuwhof *et al.*, 2008; Wassink *et al.*, 2010), with resulting economic losses for sheep farmers. Footrot is one of the top three economically significant diseases for the UK sheep industry, with an estimated cost of between £24 and £80 million per annum (Nieuwhof & Bishop, 2005; Wassink *et al.*, 2010).

There are two clinical presentations of footrot: interdigital dermatitis (ID), characterised by inflammation of the interdigital skin, and severe footrot (SFR) where hoof horn separates from the underlying sensitive tissue (Figure 1). The causal agent of footrot is the Gram-negative bacterium *Dichelobacter nodosus* (Beveridge, 1941; Kennan *et al.*, 2011; Witcomb *et al.*, 2014).

Figure 2 Clinical presentations of footrot.
A: Healthy foot; B: Interdigital dermatitis (ID) with inflammation visible in the interdigital skin; C: Severe footrot (SFR) with separation of the sole horn and necrotic tissue.

2.2. Characteristics of *Fusobacterium necrophorum*

Fusobacterium necrophorum is a Gram-negative anaerobe (Lechtenberg *et al.*, 1988), and is a pathogen of both humans and animals (Nagaraja *et al.*, 2005). There are two subspecies of *F. necrophorum: F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* (Shinjo *et al.*, 1991). *F. necrophorum* subsp. *necrophorum* is more pathogenic (Nagaraja *et al.*, 2005) and is more commonly found in animal disease whereas *F. necrophorum* subsp. *funduliforme* is more frequently reported in human disease (Hall *et al.*, 1997).

F. necrophorum is an opportunistic pathogen with reservoirs (sites in living organisms or the environment where bacteria live and usually multiply) in healthy individuals. It causes diseases characterised by necrotic lesions and abscesses, termed necrobacilloses (Langworth, 1977; Tan *et al.*, 1996). Hepatic abscesses in cattle and pharyngotonsillitis in humans are the most well studied

examples (Tan *et al.*, 1996; Narayanan *et al.*, 1997; Aliyu *et al.*, 2004; Jensen *et al.*, 2015). *F. necrophorum* is also associated with other diseases including periodontal disease in wallabies, calf diphtheria, digital necrobacillosis in ungulates and endometritis in cattle (Ruder *et al.*, 1981; Monrad *et al.*, 1983; Panciera *et al.*, 1989; Edwards *et al.*, 2001; Antiabong *et al.*, 2013; Aghamiri *et al.*, 2014).

2.3. The role of *Fusobacterium necrophorum* in ovine footrot

Fusobacterium necrophorum has been known to play a role in ovine footrot for over a century. In the early 20th Century, *F. necrophorum* was believed to be the causal agent of ovine footrot (Mohler & Washburn, 1904). In 1941, Beveridge identified *Dichelobacter nodosus* as the causal agent. When sheep feet were inoculated with *D. nodosus,* footrot developed, however, when sheep feet were inoculated with pure cultures of *F. necrophorum*, the resulting lesions did not resemble footrot. Beveridge concluded that *F. necrophorum* was likely to be a secondary invader in footrot, and that it could increase lesion severity.

In 1969, Roberts and Egerton studied the aetiology and pathogenesis of footrot. They suggested that *F. necrophorum* initiates invasion of the interdigital skin in interdigital dermatitis, facilitating colonisation by *D. nodosus*, and that it increases the severity of footrot lesions. This was based on observation of artificially induced footrot infections, and in fact during natural infection they observed that *D. nodosus* predominated in early lesions but that *F. necrophorum* predominated in later lesions (Egerton *et al.*, 1969).

Recent evidence supports the theory that *D. nodosus* colonisation occurs prior to colonisation with *F. necrophorum*. In a study examining load of *D. nodosus* and *F. necrophorum* over time during natural infection, Witcomb *et al.* (2014) found an increase in load of *D. nodosus* before and during an episode of ID and prior to occurrence of SFR. In contrast, the load of *F. necrophorum* only increased once SFR had occurred. The authors concluded that *D. nodosus* initiates disease and *F. necrophorum* is an opportunist once disease has occurred. Subsequent cross sectional studies have also demonstrated highest prevalence and load of *D. nodosus* on feet with ID, and highest prevalence and load of *F. necrophorum* on feet with SFR (Witcomb *et al.*, 2015; Maboni *et al.*, 2016).

The suggested role for *F. necrophorum* as a secondary opportunist in ovine footrot is consistent with its opportunistic nature in other diseases (Section 2.2 above). In many conditions, *F. necrophorum* is thought to act synergistically with other bacterial pathogens to enhance the disease severity, for example in calf diphtheria, ovine foot abscesses and bovine hepatic abscesses (Roberts, 1967; Takeuchi *et al.*, 1983; Panciera *et al.*, 1989).

2.4. Potential reservoirs of *Fusobacterium necrophorum* in sheep and their environment

It is believed that sheep faeces are the main source of *F. necrophorum* in footrot (Tan *et al.*, 1996). Roberts and Egerton (1969) observed colonisation of the interdigital skin by *F. necrophorum* when sheep were kept in pens heavily contaminated by faeces, however they did not actually test faecal samples from sheep for the presence of *F. necrophorum*. In a recent study using molecular methods, Witcomb (2012) failed to detect *F. necrophorum* in faeces collected directly from sheep, however faecal shedding of *F. necrophorum* in sheep faeces maybe intermittent, and therefore may not have been detected by Witcomb (2012) because of the small number of animals studied (n=20).

F. necrophorum has been shown to survive in soil microcosms in the laboratory (Garcia *et al.*, 1971). This paper led to an assumption that *F. necrophorum* is ubiquitous in the environment of sheep and other ungulates, and this has been widely reported in reviews, the introductions of primary research papers and veterinary textbooks e.g. (Langworth, 1977; Winter, 2004; Yeruham & Elad, 2004; Green & George, 2008; Dubreuil & Anderson, 2009; Handeland *et al.*, 2010; Underwood *et al.*, 2015). The only study so far to test soil from sheep pasture for presence of *F. necrophorum* was a pilot study by Witcomb (2012). The 20 soil samples collected from one sheep pasture in this study were negative for *F. necrophorum* DNA. In other ungulates there are reports of outbreaks of necrobacillosis associated with animals congregating at feeding or watering areas, in periods of increased rainfall (Edwards *et al.*, 2001; Handeland *et al.*, 2010) and heavily faecally contaminated pens (Monrad *et al.*, 1983). It is therefore possible that high stocking density of animals and suitable climatic conditions lead to transiently increased presence and survival of *F. necrophorum* in localised areas of pasture, however, these areas will always be contaminated with faeces. Other than Witcomb (2012) pilot data there are no soil samples analysed for the presence of *F. necrophorum* and grass has never been tested for presence of *F. necrophorum*.

F. necrophorum has been detected in the mouths of sheep (McCourtie *et al.*, 1990; Bennett *et al.*, 2009; Witcomb, 2012). Witcomb (2012) reported that 74% (26/35) of mouth swabs were positive for *F. necrophorum*, and also provided evidence from fluorescence in-situ hybridisation (FISH) studies that intact, physiologically active *F. necrophorum* were present in the oral cavity of sheep.

F. necrophorum is detected on healthy feet of sheep, and on feet with ID, but with lower frequency of detection and at lower loads than feet with SFR (Witcomb *et al.*, 2014; Frosth *et al.*, 2015; Maboni *et al.*, 2016). There are no data on the persistence (duration of carriage) of *F. necrophorum* on healthy feet.

In summary, *F. necrophorum* populations could persist on the feet of sheep, in the mouth, in faeces or on pasture. Persistence of *F. necrophorum* in a flock of sheep could occur as *F. necrophorum* move between sites within and between sheep. On a larger scale, the movement of sheep between flocks could facilitate persistence.

2.5. Associations between reservoir sites of *Fusobacterium necrophorum* and footrot

It is known that feet with footrot have an increased load of *F. necrophorum* (Witcomb *et al.*, 2014; Witcomb *et al.*, 2015). One can hypothesise that increased prevalence of footrot will result in increased shedding of *F. necrophorum* into the environment and spread to other feet and sheep, however, this has not been demonstrated. This effect could also be increased when footrot lesions are of greater severity or duration, but again no studies have investigated this. Increases in *F. necrophorum* load on feet occur subsequent to increased loads of *D. nodosus* (Witcomb *et al.*, 2014), and *D. nodosus* transmission is known to occur via pasture (Whittington, 1995). It is possible that the two bacteria share transmission pathways, but associations between *D. nodosus* load and *F. necrophorum* load at any of the sites other than feet have not been investigated.

2.6. Methods for studying the epidemiology and ecology of *Fusobacterium necrophorum*

Longitudinal studies involve measuring the outcome of interest repeatedly over time. They are valuable in epidemiology as they can provide evidence for causal associations, and they also facilitate investigation of persistence through detection of the same organism at the same site on repeated occasions. A longitudinal study is necessary to determine reservoirs of *F. necrophorum* in sheep and their environment, and their relevance to development, severity and chronicity of footrot.

The study by Witcomb *et al.* (2014) was the first to examine the load of *F. necrophorum* on feet. As highlighted by those authors, load, measured by quantitative PCR (qPCR), can provide a more detailed indication of changes in bacterial populations than simple presence/absence data provided by standard PCR, and when measured over time load is an extremely useful epidemiological tool.

The use of species level data is not sufficiently robust to understand persistence and transmission of bacterial pathogens, and therefore strain typing is essential in these types of epidemiological studies. Strain typing has been widely used to understand the epidemiology and ecology of bacterial pathogens in livestock, using a range of methods including ribotyping, pulsed field gel electrophoresis (PFGE), multiple locus variable number tandem repeat analysis (MLVA) and multilocus sequence typing (MLST) (Shere *et al.*, 1998; Zadoks *et al.*, 2005; Vranckx *et al.*, 2011; Davies *et al.*, 2016). Ribotyping and determination of sequence variation of the leukotoxin gene have

been used to differentiate strains of *F. necrophorum* (Narayanan *et al.*, 1997; Zhou *et al.*, 2009), but understanding of the variability of *F. necrophorum* strains beyond the subspecies level is minimal.

2.7. Summary and conclusions from current knowledge

F. necrophorum is a secondary pathogen in ovine footrot that may increase severity of footrot. It has been widely stated that *F. necrophorum* is ubiquitous on pasture, but there is very little evidence to substantiate this. *F. necrophorum* has been detected in mouths, healthy feet, and feet with footrot, and is suggested to be shed in sheep faeces, however, it is unknown which of these sites are reservoirs for *F. necrophorum*. It is also unknown whether changes in load of *F. necrophorum* at these sites are associated with changes in prevalence, severity or chronicity of footrot, or changes in load of *D. nodosus*.

The study objectives were to test the following hypotheses:

1. *F. necrophorum* is present in the environment of sheep (feet, mouth, faeces, soil, grass) and persists at these sites.
2. *F. necrophorum* load at these sites increases following increased incidence and prevalence of footrot in feet and sheep.
3. *F. necrophorum* load at these sites is higher with increasing severity and chronicity of footrot in feet and sheep.
4. *F. necrophorum* load at these sites increases subsequent to increased load of *D. nodosus*.

In addition, the following objectives were developed:

1. To develop and validate a multiple locus variable number tandem repeat analysis (MLVA) typing scheme for *F. necrophorum*.
2. To use this scheme to analyse communities of *F. necrophorum* in samples from longitudinal studies of sheep and their environment.

3. Materials and methods

3.1. Study design and sampling procedures for two longitudinal studies

Two longitudinal studies (Studies A and B) were carried out. There were 10 sheep in Study A and 40 sheep in Study B. The data from these studies were also used to study *Dichelobacter nodosus;* load of *D. nodosus* is included in the statistical analyses of *F. necrophorum* data.

3.1.1. Approvals and consent

The studies were approved by the University of Warwick's local ethics committee (AWERB.33/13-14). Faecal samples from sheep were collected under Home Office Licence (PPL 70/8392). Informed consent was obtained from the farmers before each study started and farmers were compensated for inconvenience at the end of the studies. The farmers were notified of any footrot lesion score > 1, and treatment was advised. All treatments were recorded.

3.1.2. Study design and sheep sampling procedure

Study A

The study population was a flock of approximately 150 North Country Mule breeding ewes on a lowland farm in Warwickshire, England where footrot was endemic. The flock was first visited on 28-May-2014. Four lame sheep (two ewes and two lambs) were convenience-selected and six non-lame sheep were randomly selected. These 10 sheep were sampled fortnightly for four occasions. They remained as part of the larger flock throughout the study period and grazed only one pasture. At the final visit five ewes and three lambs were sampled, because two lambs had been sold. At each sampling each foot of each sheep was examined and scored for lesions of interdigital dermatitis (ID) and severe footrot (SFR) as described by Moore et al. 2005 (Table 1).

Table 1 Scoring system for lesions of interdigital dermatitis (ID) and severe footrot (SFR) from Moore et al. 2005

Lesion score[a]	Description
Interdigital dermatitis	
0	Clean interdigital foot with no dermatitis (scald) lesion or fetid smell
1	Slight interdigital dermatitis, irritation of the skin but dry
2	Slight interdigital dermatitis with a fetid smell <5% skin affected
3	Moderate interdigital dermatitis with a fetid smell 5-25% skin affected
4	Severe interdigital dermatitis with a fetid smell >25% skin affected
Severe footrot	
0	A clean digit with no lesion
1	An active or healing footrot lesion with a degree of separation of the sole
2	An active footrot lesion with a marked degree of separation of the sole
3	An active footrot lesion with extensive under-running of the wall hoof horn (may include under-running of the sole)
4	An active footrot lesion with complete under-running of the wall hoof horn (may include under-running of the sole)

[a] One score for each lesion was recorded per foot

The interdigital skin of each foot was sampled using a sterile cotton swab (EUROTUBO Collection swab; Delta Lab, Rubi, Spain). After the feet were sampled the gingival crevice (gum-tooth margin) of the lower incisors was sampled using a sterile cotton swab. The samples collected are summarised in Table 2.

Table 2 Summary of samples collected from sheep in the two longitudinal studies

Sample type	No. per visit		Samples collected per sampling episode
	Study A	Study B	
Foot swabs	40	160	One swab sample per foot
Mouth swabs	10	40	One swab sample from gingival crevice per sheep
Faeces	NA	40	Study B only: one rectal faecal sample per sheep

Study B

The study population was 120 Suffolk cross Wiltshire Horn ewe lambs on a farm in Warwickshire, England. The flock was chosen based on a known history of footrot.

On 07-Feb-2015 baseline samples from the study pasture were taken, and the pasture was left empty until sampled again on 17-Feb-2015.

On 18-Feb-2015, a study group of 40 individuals was selected from a group of 120 ewe lambs (1 year old females that had not been bred). All 120 sheep were observed for lameness and divided into three groups: non-lame, lame and those where lameness was uncertain. The non-lame sheep were examined and lesion scored as described for Study A. Forty healthy sheep (non-lame, no SFR lesion, ID lesion scored ≤ 1) were identified and samples collected as described for Study A, except a rectal faecal sample was also collected from each sheep using a clean, gloved finger (Table 2). If insufficient faecal material was present, a rectal swab was taken.

These 40 animals formed the study group for the longitudinal study, and were moved to the study pasture. The study sheep and pasture were sampled every week from 25-Feb-2015 to 01-Jul-2015. There were total of 20 sampling occasions including the samples taken on 18-Feb-2015.

3.1.3. Procedures for collecting environmental samples

Soil and grass samples

Soil and grass samples were collected from the pasture grazed by the study group. Two high traffic areas and one low traffic area were sampled (Table 3). High traffic areas were those where sheep congregated or visited more frequently based on observation of the sheep and information from the farmer. Low traffic areas were those where sheep did not congregate or visit frequently.

Table 3 Details of high and low traffic areas for Studies A and B

Location	Details	Samples		
		Type	Depth [a]	No. per visit
Study A				
High traffic 1	Large tree used for shelter	Soil	0-1cm	3
			4-5cm	3
		Grass	---	1[b]
High traffic 2	Open gateway to adjacent field	Soil	0-1cm	3
			4-5cm	3
		Grass	---	0[b]
Low traffic	20m × 20m area	Soil	0-1cm	5
			4-5cm	5
		Grass	---	5
Study B				
High traffic 1	Ring feeder	Soil	0-1cm	3
			4-5cm	3
		Grass	---	2-3[b]
High traffic 2	Water trough by hedge	Soil	0-1cm	3
			4-5cm	3
		Grass	---	1-3[b]
Low traffic	20m × 20m area	Soil	0-1cm	5
			4-5cm	5
		Grass	---	5

[a] Depth from which soil sample taken relative to surface of pasture
[b] Grass collected where present

In low traffic areas, samples were taken from 5 points on a 20m × 20m quadrant that were selected at random each week. In high traffic areas, a sample was taken from the centre of each area and at 1m and 2m distant (Figure 2). Sections of soil at 4-5cm and 0-1cm depth (relative to the soil surface) from each sampling point were collected using a soil corer, and transferred to sterile universal containers. Grass samples were picked and transferred to sterile universal containers.

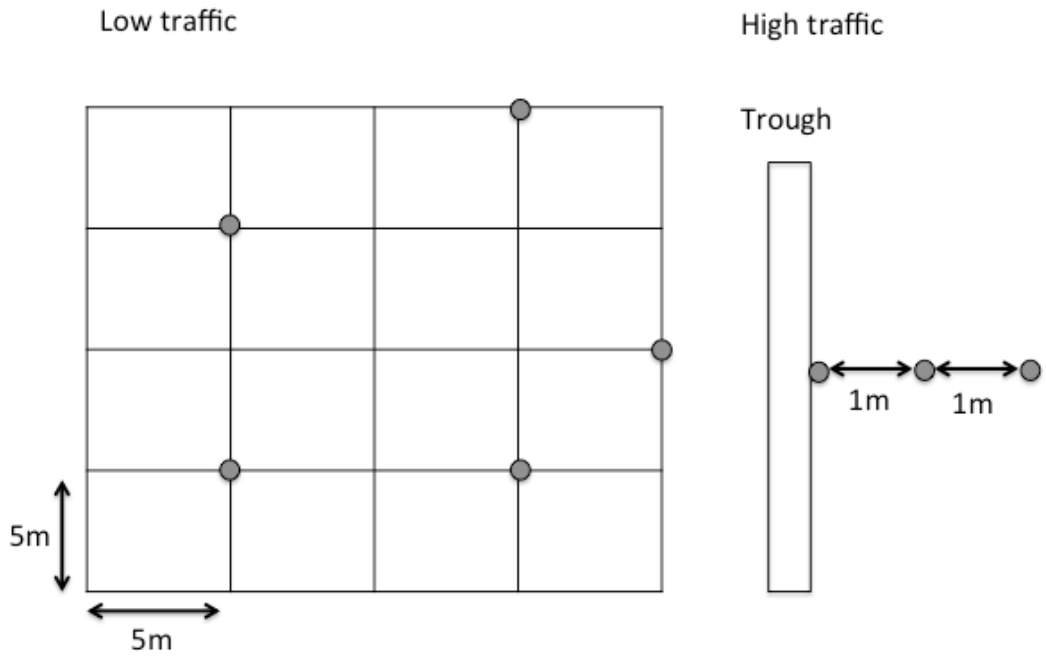

Figure 2 Sampling strategy for low and high traffic areas of pasture
Sampling points are shown using grey circles. For the low traffic quadrant, nodes are numbered from 1 – 25 and five were selected using a random number generator. For the high traffic area, a sample was taken at the site and at 1m and 2m distant.

Faecal samples (Study A only)

In Study A, faecal samples were collected from pasture. Five fresh faecal samples were collected from five sites on the pasture. External and internal (i.e. no direct contact with the environment) sections of each faecal sample were collected, giving ten samples per visit.

3.1.4. Collection of climate data

Collection of weather data

Daily data on total rainfall (mm) and minimum, maximum and mean temperatures (°C) were accessed from the Warwick weather station (http://warwick-weather.com/; last accessed Aug 2015, no longer available). Data were recorded from ten days before the start of each study until the end of the study.

3.2. Laboratory methods for the detection and quantification of *Fusobacterium necrophorum*

3.2.1. Sample selection for laboratory analysis

All samples from Study A were analysed. All environmental samples (soil and grass) from Study B were analysed plus a subset of sheep samples. This included samples from 19 diseased sheep from 2 weeks before a period of footrot (or start of the study) to 2 weeks after the period of footrot (or end of the study). Samples from every fourth week from 2 sheep that scored ID0 and SFR0 for the duration of the study were analysed. Samples from weeks 1-3 were analysed for these 21 sheep, plus a further randomly selected 9 sheep.

3.2.2. DNA extraction

DNA extraction from all sample types (swabs, soil, faeces and grass) was performed using the method described by Purdy (2005). The centrifugation times for the ethanol precipitation stage were increased from 5 to 30 minutes. Samples were processed in batches of 16 including an extraction blank (500µl sterile phosphate buffered saline (PBS)) processed in each batch as a negative control. The extracted DNA was stored at -20°C.

For soil, grass and faeces samples the weight of each sample was recorded prior to extraction. There was approximately 0.5g of soil and grass and 0.1g of faeces per sample. Swabs were thawed before processing, and then transferred to a sterile 2ml screw-cap microcentrifuge tube using sterile tweezers and any PBS remaining in the cryotube was added to the tube.

Polyethylene glycol (PEG) precipitation

The DNA extracted from the environmental samples (soil, grass and faeces) was further purified by PEG precipitation using a method adapted from Selenska and Klingmuller (1991). An aliquot of 25µl of DNA was transferred to a sterile 1.5ml microcentrifuge tube and 5µl 5M sodium chloride and 25µl 30% PEG 6000 (Sigma-Aldrich Ltd., Gillingham, UK) were added, the sample was mixed and precipitated overnight at 4°C. The sample was then centrifuged at 13,000 ×*g* for 20 minutes and the supernatant removed. The pellet was re-suspended in 1ml of 70% ice-cold ethanol and centrifuged at 13,000 ×*g* for 5 minutes and the supernatant removed. The ethanol wash step was repeated and then the resulting pellet was air-dried for at least 30 minutes. The pellet was then re-suspended in 50µl 10mM Tris pH 7.5 and stored at -20°C.

3.2.3. Quantitative PCR (qPCR) for *Fusobacterium necrophorum*

A TaqMan® qPCR targeting the *rpoB* gene (RNA polymerase beta subunit) of *F. necrophorum* (Witcomb *et al.*, 2014) was used to analyse all samples (Table 4). The probe was labelled at the 5'-

end with the fluorescent dye FAM (6-carboxyl-fluorescein) and at the 3'-end with the non-fluorescent quencher BBQ (Black Berry Quencher).

Table 4 qPCR primers and TaqMan® probe for quantification of *F. necrophorum* (*rpoB*)

	Primer sequence (5' to 3')
Forward primer	AACCTCCGGCAGAAGAAAAATT
Reverse primer	CGTGAGGCATACGTAGAGAACTGT
TaqMan® probe[a]	6FAM-TCGAACATCTCTCGCTTTTTCCCCGA-BBQ

[a] 6FAM = 6 carboxy-fluoroscein, BBQ = Black Berry Quencher

All qPCR assays were performed using an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Warrington, UK). Cycling conditions and amplification reactions were based on Witcomb *et al*. (2014). Amplification reactions are detailed in Table 5. Cycling conditions consisted of an initial denaturation at 95°C for 20s followed by 40 cycles of 95°C for 30s, 61°C for 30s. Each sample was run in technical triplicate for quantification purposes; and only samples that were positive for all three technical replicates were used in further analysis.

Table 5 Details of master mix components used in qPCR amplifications

Master mix component	Working concentration	Final (reaction) concentration	Volume per reaction (µl)
Primer forward	10µM	900nM	2.25
Primer reverse	10µM	900nM	2.25
TaqMan® probe	10µM	250nM	0.625
TaqMan® Universal Mastermix	2 ×	1 ×	12.5
BSA	10mg ml^{-1}	1mg ml^{-1}	2.5
Nuclease free H_20	-	-	3.875
DNA	Various	Various	1.0
Total	-	-	25.0

3.3. Statistical analysis of qPCR data from longitudinal studies

3.3.1. Classification of footrot lesions

All statistical analyses were carried out using the R (v3.3.2) statistical environment (R Development Core Team, 2008) with the R studio user interface (v1.0.136). (Bacterial load data + 1) were log_{10} transformed for statistical analyses. For the purpose of statistical analysis, footrot status was defined as presented in Table 6. Feet could be classed as healthy or having footrot, and footrot could be

further categorised as ID or SFR. The footrot status of a sheep was determined in the same way based on the most severe lesion recorded on her feet at that time.

Table 6 Classification of footrot status for statistical analysis

Term	Lesion score
Healthy	ID ≤ 1, SFR 0
Footrot (includes ID and SFR)	ID > 1 and/or SFR > 0
ID	ID > 1 and SFR 0
SFR	SFR > 0

3.3.2. Chi-squared test for goodness of fit

Expected frequencies for number of positive samples by sample type were calculated by multiplying the overall detection rate by the total number of samples of each type collected. A Chi-squared test was then used to determine goodness of fit. P-values of ≤ 0.05 were considered significant.

3.3.3. Associations between footrot status and load of *Fusobacterium necrophorum*

Due to the presence of repeated measures in the data, two level binomial mixed effects models were used to determine associations between footrot status and load of *F. necrophorum* on positive foot swabs, mouth swabs, and faecal samples (Study B only). The outcome variable was the presence/absence of footrot, and data were grouped by foot (foot swabs) or sheep (mouth swabs and faecal samples) to account for repeated observations. The $\log_{10}$ transformed load of *F. necrophorum* was used as the explanatory variable. Associations between load and footrot status were considered significant when 95% confidence intervals of the coefficient for load did not include 0.

3.3.4. Survival analysis

Non-parametric maximum likelihood estimation (Kaplan-Meier estimate) of survival of *F. necrophorum* positive samples was carried out. The event was a sample becoming negative for *F. necrophorum*. The Wilcoxon two sample permutation was used to test for differences in survival probablilities between groups.

3.3.5. Binomial mixed effects model construction

Data from foot swabs from Study B were analysed using binomial logistic mixed effects models where presence/absence *F. necrophorum* on feet was the outcome. A three level model was used to account for repeated observations over time of feet, and feet clustered within sheep. A mean-

centred polynomial term for time (week + week^2 + week^3 + week^4) was included in all analyses. Explanatory variables were lagged to the previous week. Explanatory variables were initially tested individually in univariable models and then a multivariable model was developed using a manual forward selection process. The Aikaike information criterion (AIC) was used to compare the relative fit of models. Variables were retained in the model when 95% confidence intervals of the coefficient did not include 0, and when the AIC value for the model was lower.

3.3.6. Linear mixed effects model construction

A linear mixed effects model was constructed with $\log_{10}$ *F. necrophorum* load data from positive foot swabs from Study B as the outcome variable. A three level model was used as for the binomial model for foot swabs above. Lagged explanatory variables were tested in univariable and multivariable models as described above.

3.3.7. Associations and correlations between explanatory variables

Associations between continuous and ordinal explanatory variables were examined using Spearman's correlation tests. Associations between binary categorical variables were examined using a Chi-squared test, and associations between a binary categorical variable and a continuous or ordinal variable were examined using a Mann Whitney U test.

3.4. Multiple locus variable number tandem repeat analysis (MLVA) typing of *Fusobacterium necrophorum*

3.4.1. Identification of tandem repeat regions in the *Fusobacterium necrophorum* genome

Seventy-three tandem repeat regions were identified from the whole genome shotgun sequence of *F. necrophorum* ATCC 51357 (GenBank Accession number AJSY00000000.1) using the Tandem Repeats Finder software v.4.08 (Benson, 1999). Three loci (Fn13, Fn42 and Fn69) showed good amplification and sufficient polymorphism for use in MLVA typing when tested with eight *F. necrophorum* subsp. *necrophorum* isolates.

3.4.2. MLVA PCR reactions and cycling conditions

PCR primers used to amplify the three selected MLVA targets and their tandem repeat sizes are given in Table 7. PCR reactions were carried out in a final volume of 25µl and are detailed in Table 8. Cycling conditions were 95°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 55°C (Fn13 and Fn69) or 62°C (Fn42) for 30 sec, 72°C for 30 sec, followed by final extension at 72°C for 10 min. All PCR reactions were carried out on an Eppendorf Mastercycler ep gradient machine (Eppendorf, Hamburg, Germany) with DNA extracted from *F. necrophorum* subsp. *necrophorum* DSM 21784 as

the positive control and nuclease free H_2O as the reagent blank. PCR products were visualized after ethidium bromide-stained agarose gel electrophoresis and imaged using a Gene Flash imager (Syngene Bio Imaging, Cambridge, UK).

Table 7 Primers identified to develop MLVA PCR for *Fusobacterium necrophorum*

Target	Repeat size (bp)	Primers	Sequence (5' to 3')[a]
F. necrophorum ATCC 51357 contig 2	17	Fn13(F) Fn13(R)	6FAM™-AATTCAAAATGATTTCTCCCTACCT TGAGAAAGAAGATAAATGGAAAACG
F. necrophorum ATCC 51357 contig 11	11	Fn42(F) Fn42(R)	PET®-TTCCCAAAATAGCAGAAAAACATAC ACCGAAAATTCAATATCAAAATCAA
F. necrophorum ATCC 51357 contig 4	12	Fn69(F) Fn69(R)	NED™-TTGATTATCCATTTTCCTTTTTGAC CAATCCTACCTCGATTATTTCTTCA

[a] Sequence of forward primer includes fluorescent marker attached to 5' end. Fluorescently labelled forward primers were sourced from Applied Biosystems, Warrington, UK; reverse primers from Sigma-Aldrich Ltd., Gillingham, UK.

Table 8 PCR reaction mixtures for detection and characterisation of *F. necrophorum* and vector sequencing

Master mix component	Working concentration	Final (reaction) concentration	Reaction volume (µl)
Primer forward	10µM	400nM	1
Primer reverse	10µM	400nM	1
Bioline MyTaq™ Red Mastermix	2 ×	1 ×	12.5
BSA	100µg ml^{-1}	4µg ml^{-1}	1
Betaine	5M	0.2M	1
Nuclease free H_20	-	-	8.5
DNA	Various	Various	1
Total	-	-	25

Determining PCR amplicon size using fragment analysis

The size, in base pairs, of PCR products was determined using fragment analysis: samples were submitted to DNA Sequencing and Services™ (College of Life Sciences, University of Dundee, UK)

and results analysed with Peak Scanner 2 Software (Applied Biosystems, Warrington, UK). Sanger sequencing of the PCR products from each of the three assays for *F. necrophorum* DSM 21784 were used as a reference for the number of repeats to be calculated from the size in base pairs for each sample. A variation in expected size of PCR amplicon of ±2bp was tolerated.

3.4.3. MLVA typing of *Fusobacterium necrophorum* communities from swab samples

The number of MLVA variants within a locus was determined by fragment analysis. The minimum number of strains in a community was calculated as equal to the greatest number of MLVA variants at one locus. The maximum number of strains detected in a community was calculated by multiplying the number of variants at each locus together. Each unique pattern of MLVA variants within these samples was assigned a unique “community type” number.

4. Results

4.1. Results for Study A

4.1.1. Prevalence of footrot during Study A

There were 152 foot observations over the course of the study. Lesion scores observed were 0 – 4 for ID and 0 – 3 for SFR. Only one foot had footrot for more than 2 weeks. Prevalence of footrot increased during the course of the study with highest prevalence in week 5 (Figure 3).

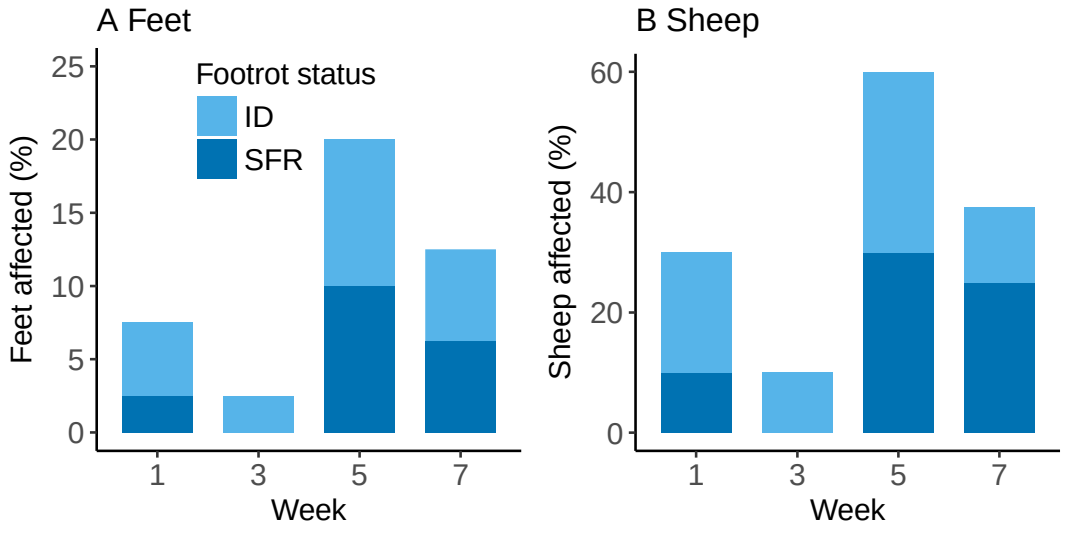

Figure 3 Footrot prevalence during Study A
A: percentage of feet affected, B: percentage of sheep affected.

4.1.2. Climate data for Study A

Mean temperature ranged from 12.2°C to 17.6°C. Rainfall was high from 10 days before the first visit to the second visit and it then became drier, with one two-week period with less than 1mm rainfall between the second and third visits.

4.1.3. Detection and quantification of *Fusobacterium necrophorum* for Study A

Detection of Fusobacterium necrophorum by sample type

F. necrophorum was detected in 34% of all samples (Table 9). There were more positive foot and mouth samples, and fewer positive environmental and faecal samples than expected by chance ($p < 0.01$).

Table 9 Frequency of detection and load of *F. necrophorum* by sample type

Sample type	Frequency of detection		*rpoB* copies in positive samples	
	No.	%	Minimum	Maximum
Foot swabs	76/152	50.0	1.42×10^2 swab^{-1}	8.37×10^7 swab^{-1}
Mouth swabs	30/38	78.9	1.16×10^2 swab^{-1}	1.08×10^6 swab^{-1}
Faeces	1/40	2.5	7.27×10^6 g^{-1}	7.27×10^6 g^{-1}
Soil	7/88	8.0	3.24×10^3 g^{-1}	1.02×10^5 g^{-1}
Grass	1/24	4.2	1.71×10^4 g^{-1}	1.71×10^4 g^{-1}

Variation in load of Fusobacterium necrophorum with disease

Foot swabs with higher loads of *F. necrophorum* were more likely to be from feet with ID or SFR than foot swabs with lower loads (OR 3.64, 95% CI 2.04-8.18; Figure 4). Load of *F. necrophorum* on mouth swabs was not associated with footrot status (Figure 4).

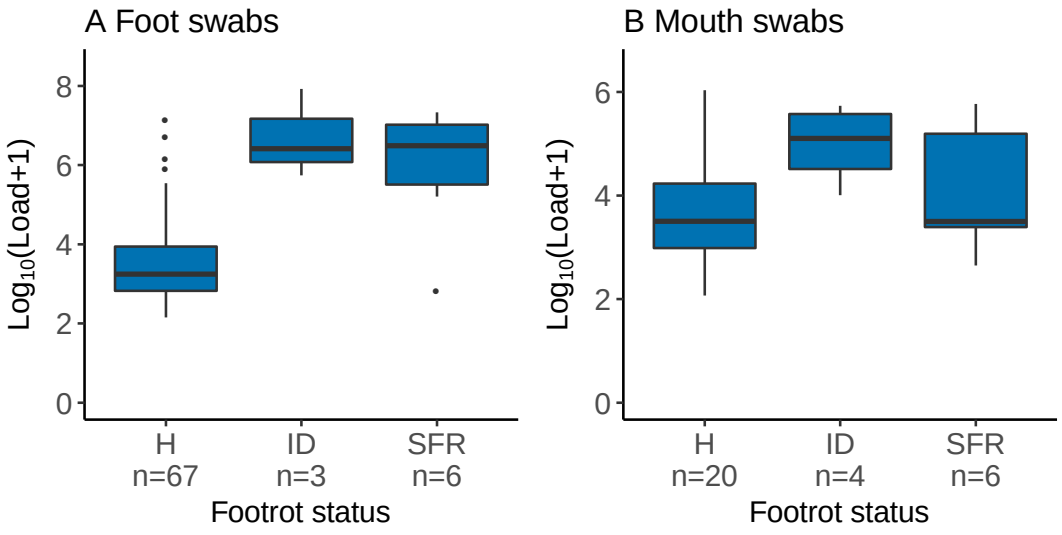

Figure 4 Load of *F. necrophorum* on foot and mouth swabs by footrot status
A: foot swabs and B: mouth swabs.

Variation in detection of Fusobacterium necrophorum over time

Detection of *F. necrophorum* on foot swabs and in environmental samples (soil and grass) decreased over the course of the study (Table 10). Only one faecal sample was positive for *F. necrophorum* (week 3). Detection in mouth samples was high throughout the study (60-90% of swabs positive per visit).

Table 10 Detection of *F. necrophorum* in all sample types by week

	Samples with detectable *F. necrophorum*									
Week	Foot swabs		Mouth swabs		Soil		Grass		Faeces	
	No.	%	No.	%	No.	%	No.	%	No.	%
1	39/40	97.5	9/10	90.0	6/22	27.3	0/6	0	0/10	0
3	17/40	42.5	9/10	90.0	1/22	4.5	1/6	16.7	1/10	10.0
5	11/40	27.5	6/10	60.0	0/22	0	0/6	0	0/10	0
7	9/32	28.1	6/8	75.0	0/22	0	0/6	0	0/10	0

Variation in detection of Fusobacterium necrophorum between environmental sampling locations

There were few positive soil samples (Table 11). The majority (6/7) of positive soil samples were from surface soil, and 6/7 were from high traffic areas. The one positive grass sample was from a low traffic area.

Table 11 Detection of *F. necrophorum* at different pasture sampling sites

Sample type and location	Positive samples	
	Number	Percentage
Soil		
High traffic	6/48	12.5
Low traffic	1/40	2.5
Surface (0-1cm)	6/44	13.6
Deep (4-5cm)	1/44	2.3
Grass		
High traffic	0/4	0
Low traffic	1/20	5

4.1.4. Community diversity of *Fusobacterium necrophorum* in DNA from foot and mouth swabs from Study A

All three MLVA loci were amplified from 25/37 (68%) foot swabs and 15/16 (94%) mouth swabs. There were 7 unique community types, 4 of which contained more than 1 strain, these ranged from a minimum of 2 to a maximum of 4 strains. The *F. necrophorum* communities in mouth swabs were more complex than the communities in foot swabs (Figure 5). The same single strain of *F. necrophorum* was detected on 24/25 (96%) foot swabs. The three locus variants in this strain (13.2, 42.5 and 69.2) were also detected in mouths in sheep 5, 7 and 8, indicating that this strain was potentially present in mouths. There were, however, many more strain types in mouths than feet.

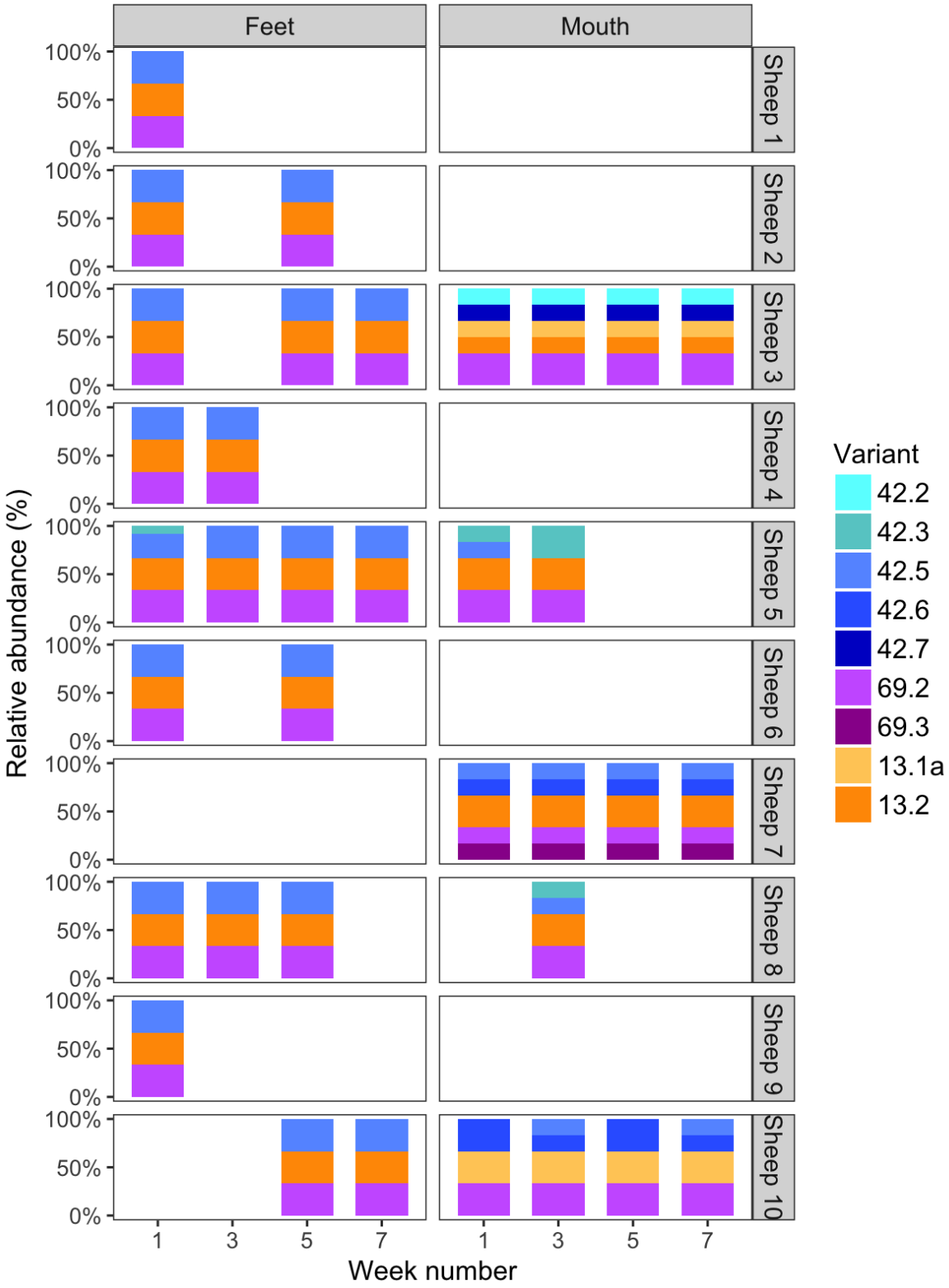

Figure 5 Relative abundance of locus variants in swab samples from Farm A
The ten sheep from Farm A are listed on the right of the figure. Results from all positive foot swabs from a sheep (sometimes >1 positive per sheep) are represented in the left-hand panels, and mouths in the right. Note, in all but one of the sheep (sheep 5, week 1) all positive samples contained the same community type, which was represented by a single strain type.

4.2. Results from Study B

4.2.1. Prevalence of footrot during Study B

There were 3192 foot observations during the study. The scores for both ID and SFR ranged from 0 – 3, and the duration of footrot (number of consecutive weeks with lesions recorded) ranged from 1 to 8 weeks. The prevalence of footrot peaked in week 5, and there was no footrot recorded during week 11 (Figure 6).

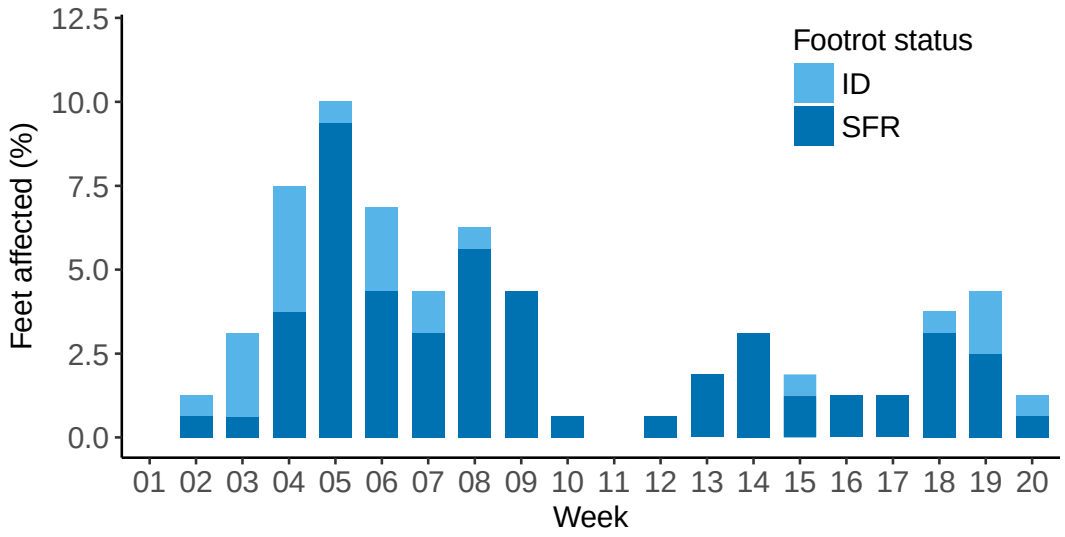

Figure 6 Footrot prevalence during Study B
The figure shows percentage feet affected with ID and SFR by week.

4.2.2. Climate data for Study B

Mean temperature during the study ranged from 0.7°C to 26.8°C and increased during the study period. The average weekly rainfall was 9.1mm, however, from week 7 to week 11 only 14.5mm of rain fell in total. This dry period preceded the period of lowest footrot prevalence (weeks 10-12).

4.2.3. Detection an quantification of *Fusobacterium necrophorum* for Study B

DNA was extracted from 1136 foot swabs, 284 mouth swabs and 283 faecal samples from 30 sheep from the study. Two DNA extraction batches (30 foot swabs) were excluded due to suspected contamination during the extraction process, giving a total of 1106 foot swabs. DNA was extracted from all 640 environmental samples.

Comparison of detection of Fusobacterium necrophorum between sample types

The distribution of positive samples across sample types was different than expected by chance ($p < 0.01$): there were more positive foot and mouth samples, and fewer positive environmental samples than expected (Table 12).

Table 1 Detection and load of *F. necrophorum* by sample type in Study B

Sample type	Frequency of detection		*rpoB* copies in positive samples	
	No.	%	Minimum	Maximum
Foot swabs	85/1106	7.7	1.03×10^2 swab^{-1}	8.50×10^7 swab^{-1}
Mouth swabs	21/284	7.4	1.82×10^2 swab^{-1}	1.67×10^6 swab^{-1}
Faeces	11/283	3.9	2.18×10^5 g^{-1} [a]	1.89×10^7 g^{-1}
Soil	4/462	0.9	6.52×10^2 g^{-1}	4.31×10^3 g^{-1}
Grass	0/178	0	NA	NA

[a] There was one rectal swab positive for *F. necrophorum* which had a load of 2.12×10^3 *rpoB* copies swab^{-1}

Detection of Fusobacterium necrophorum over time

F. necrophorum was detected at all time points except weeks 18 and 20. Foot swabs were the only samples where *F. necrophorum* was detected after week 10, with the exception of one positive mouth swab in week 17.

Load of Fusobacterium necrophorum on foot swabs

Foot swabs with higher loads of *F. necrophorum* were more likely to be from feet with ID or SFR than foot swabs with lower loads (OR 2.12, 95% CI 1.36-3.61; Figure 7).

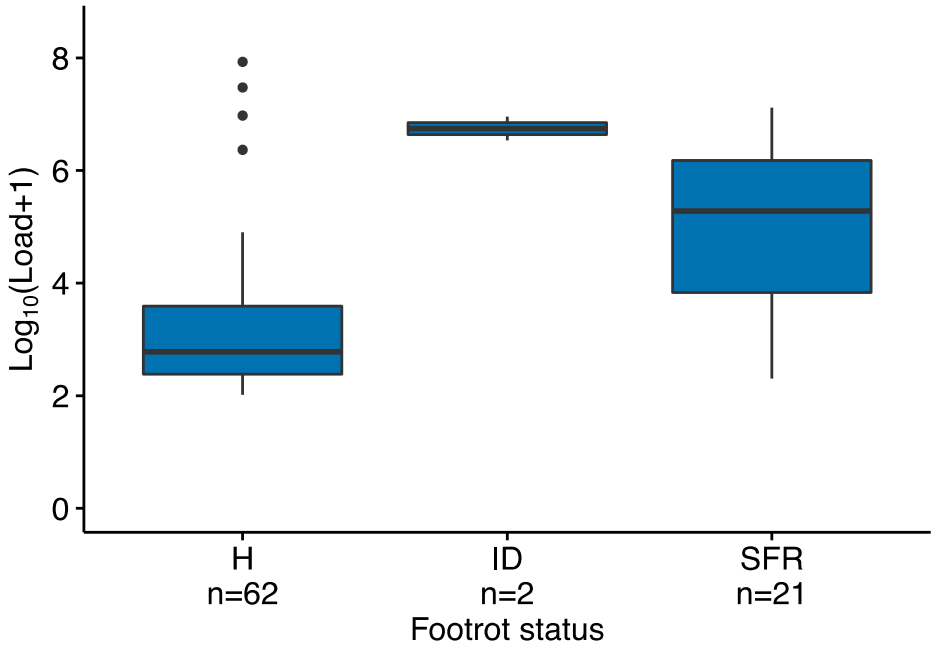

Figure 7 Load of *F. necrophorum* on foot swabs by footrot status for Study B

Persistence of Fusobacterium necrophorum on feet

F. necrophorum was detected on the same foot for between 1 and 12 consecutive weeks, and was more likely to persist on feet that had footrot than those that were healthy ($p < 0.01$; Figure 8).

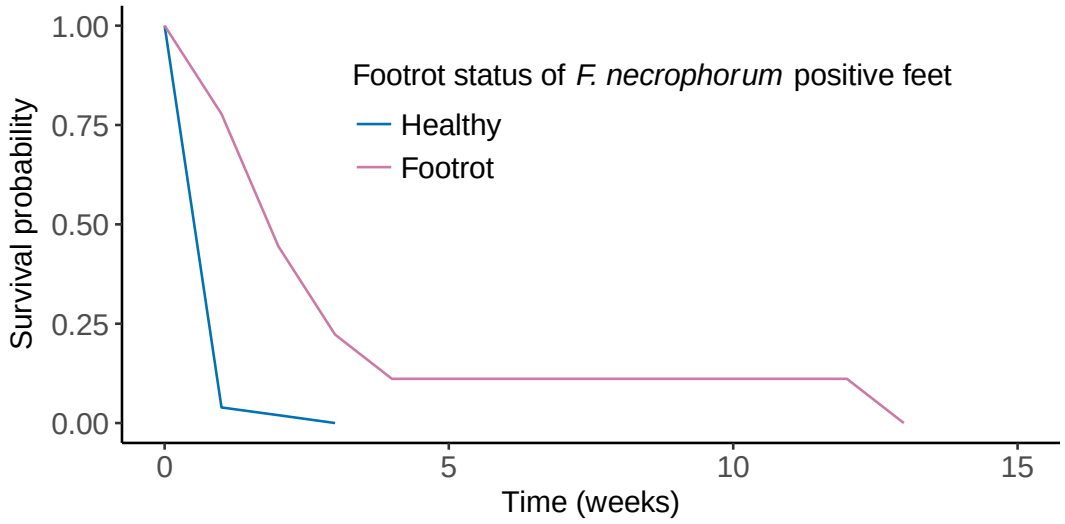

Figure 8 Survival probability of *F. necrophorum* on feet
The probability of feet positive for *F. necrophorum* remaining positive over time is plotted for feet that had footrot whilst positive and those that were healthy whilst positive.

Detection of Fusobacterium necrophorum on mouth swabs and in faecal samples

There was no association between load of *F. necrophorum* on mouth swabs or in faecal samples and footrot status. Eight sheep had positive mouth swabs and three sheep had positive faecal samples. The longest period of consecutive detection of *F. necrophorum* on mouth swabs was 6 weeks, and in faecal samples was 4 weeks.

Detection of Fusobacterium necrophorum in environmental samples

F. necrophorum was detected in soil but not grass, and this detection occurred only in the early part of the study: three of four positive soil samples were from the baseline samples taken 10 days before the sheep were moved onto the study pasture, and the fourth was from week 2. All positive soil samples were from the same high traffic area, a ring feeder; two were from surface soil (0-1cm) and two from deep soil (4-5cm).

4.2.4. Mixed effects models of *Fusobacterium necrophorum* on foot swabs from Study B

Binomial model of presence of Fusobacterium necrophorum on foot swabs

Four explanatory variables were retained in the multivariable model (Table 13). The odds of a foot being positive for *F. necrophorum* increased as the load of *D. nodosus* and *F. necrophorum* on that foot one week previously increased (OR 1.65, 95% CI 1.33 - 2.09 and OR 1.48, 95% CI 1.17 - 1.86 respectively). A foot was more likely to be positive for *F. necrophorum* as minimum temperature during the previous week increased and maximum temperature during the previous week decreased (OR 1.40, 95% CI 1.04 - 1.97 and OR 0.79, 95% CI 0.65 - 0.97 respectively).

Table 13 Multivariable binomial mixed effects regression model of presence of *F. necrophorum* on foot swabs as determined by qPCR for Study B

Value one-week previously	Odds ratio	Lower 95% CI	Upper 95% CI
Fixed effects			
Log_{10} (Dn[a] load + 1)	**1.65**	1.33	2.09
Log_{10} (Fn[a] load + 1)	**1.48**	1.17	1.86
Maximum temp (°C)	**0.79**	0.65	0.97
Minimum temp (°C)	**1.40**	1.04	1.97
Week[b]	0.53	0.04	5.99
$Week^2$	0.90	0.16	5.13
$Week^3$	2.67	0.47	16.1
$Week^4$	0.53	0.20	1.57
Random part			
Variance (foot level)	1.05		
Variance (sheep level)	0.36		

CI = confidence interval. Where odds ratios are in bold, they are statistically significant at 0.05 when CI do not include unity.
[a] Fn refers to *F. necrophorum* and Dn refers to *D. nodosus.* [b] Mean centred term for week

Linear model of load of Fusobacterium necrophorum on foot swabs

Two variables were retained in the final linear model (Table 14): feet with ID score 2 & 3 were more likely to have higher loads of *F. necrophorum* the following week than feet with ID0 & 1 (β = 2.41, 95% CI 0.93 – 3.88), and loads of *F. necrophorum* on feet increased with increasing maximum temperature during the previous week (β = 0.26, 95% CI 0.07 – 0.45).

Table 14 Multivariable linear mixed effects regression model of log_{10}(load + 1) of *F. necrophorum* on foot swabs as determined by qPCR for Study B

Value one-week previously	β	Lower 95% CI	Upper 95% CI
Fixed effects			
ID score 0&1	Ref		
ID score 2&3[a]	**2.35**	0.92	3.78
Maximum temp (°C)	**0.26**	0.07	0.44
Week[b]	-0.42	-2.37	1.54
Week^2	0.53	-1.24	2.30
Week^3	-0.12	-1.99	1.75
Week^4	-0.16	-1.29	0.97
Random part			
Variance (foot level)	0.38		
Variance (sheep level)	5.13×10^{-9}		

β = coefficient. CI = confidence interval. Ref = baseline category for comparison. Where odds ratios are in bold, they are statistically significant at 0.05 when CI do not include unity.
[a] Lesion scores 2 & 3 were grouped together due to low numbers of feet in each category. [b] Mean centered term for week.

Associations and correlations between explanatory variables

Among explanatory variables in the final model, the load of *F. necrophorum* and *D. nodosus* were significantly positively correlated, and the load of *D. nodosus* was significantly positively correlated with minimum temperature. Minimum and maximum temperature were strongly positively correlated.

All variables in the final model were positively correlated with the foot having footrot, and increasing SFR score. Minimum and maximum temperature were both positively correlated with the percentage of feet with footrot in the flock, and negatively correlated with the percentage of mouths and faeces positive for *F. necrophorum* in the flock. Maximum temperature was negatively correlated with the percentage of feet positive for *F. necrophorum* in the flock.

There was a strong positive correlation between both minimum and maximum temperature and soil temperature. Maximum temperature was negatively correlated with soil moisture. Load of *F. necrophorum* was positively correlated with weekly rainfall and soil moisture, and negatively correlated with mean temperature. Load of *D. nodosus* was positively correlated with weekly rainfall.

4.2.5. Community diversity of *Fusobacterium necrophorum* in DNA from foot swabs, mouth swabs and faecal samples from Study B

A full MLVA profile was obtained from 24/79 (30%) foot swabs, 4/19 (21%) mouth swabs and 2/11 (18%) faecal samples. Partial profiles were generated from 16 foot swabs, 13 mouth swabs and 9 faecal samples. From the 30 samples with a full MLVA profile, 6 community types were identified.

From samples analysed, there were 2 variants detected at locus Fn13, and 4 variants at each of loci Fn42 and Fn69 (Table 15). At locus Fn13 and locus Fn42 one variant was found on 94% (31/33) and 95% (54/57) of samples respectively, whereas at locus Fn69 the most frequently detected variant was found on 55% (31/56) of samples (Table 15). Only 1 faecal and 1 foot sample had > 1 strain.

Table 15 Frequency of detection of locus variants at sample sites in Study B

MLVA Variant	Foot swabs (n=40)	Mouth swabs (n=17)	Faecal samples (n=11)	Total
Locus Fn13				
13.1a	1 (0)[a]	1	0	2
13.2	23 (15)	5	3	31
Locus Fn42				
42.4	1 (0)	0	0	1
42.5	32 (16)	13	9	54
42.6	0	1	0	1
42.7	1 (0)	0	0	1
Locus Fn69				
69.1	0	1	0	1
69.2	0	9	5	14
69.3	24 (14)	1	6	31
69.4	8 (0)	2	0	10

[a] Number detected (number from feet with footrot)

Variation at locus Fn69

The majority of variation occurred at locus Fn69 for all sites, therefore this locus was used to study variation between feet, sheep and over time (Figure 9).

Variant Fn69.3 and Fn69.4 were detected on foot swabs (Table 15). Fn69.4 was detected on feet from weeks 1 – 3 but never for more than one week. Fn69.3 was detected on feet from week 4 and

was detected on consecutive weeks on 6 feet. The same variant was detected on multiple feet of the same sheep on 5 occasions; these were variant Fn69.4 in sheep 03463 week 2, and variant Fn69.3 in sheep 03468 week 13, sheep 03478 week 7, and sheep 03535 weeks 8 and 9.

All 4 variants were detected from mouth swabs and variants Fn69.2 and Fn69.3 were detected from faeces (Table 15). Variants Fn69.1 and Fn69.4 were only detected for one week but variant Fn69.2 was detected on consecutive weeks from both faeces and mouth swabs, and variant Fn69.3 was detected on consecutive weeks from faeces. Fn69.2 was detected in the mouth and faeces of the same sheep on one occasion; this was sheep 03463 in week 1. Fn69.4 was detected in the mouth and on a foot of the same sheep on one occasion; this was sheep 03547 in week 1. The same variant was not detected in the faeces and on a foot of a sheep at any time point.

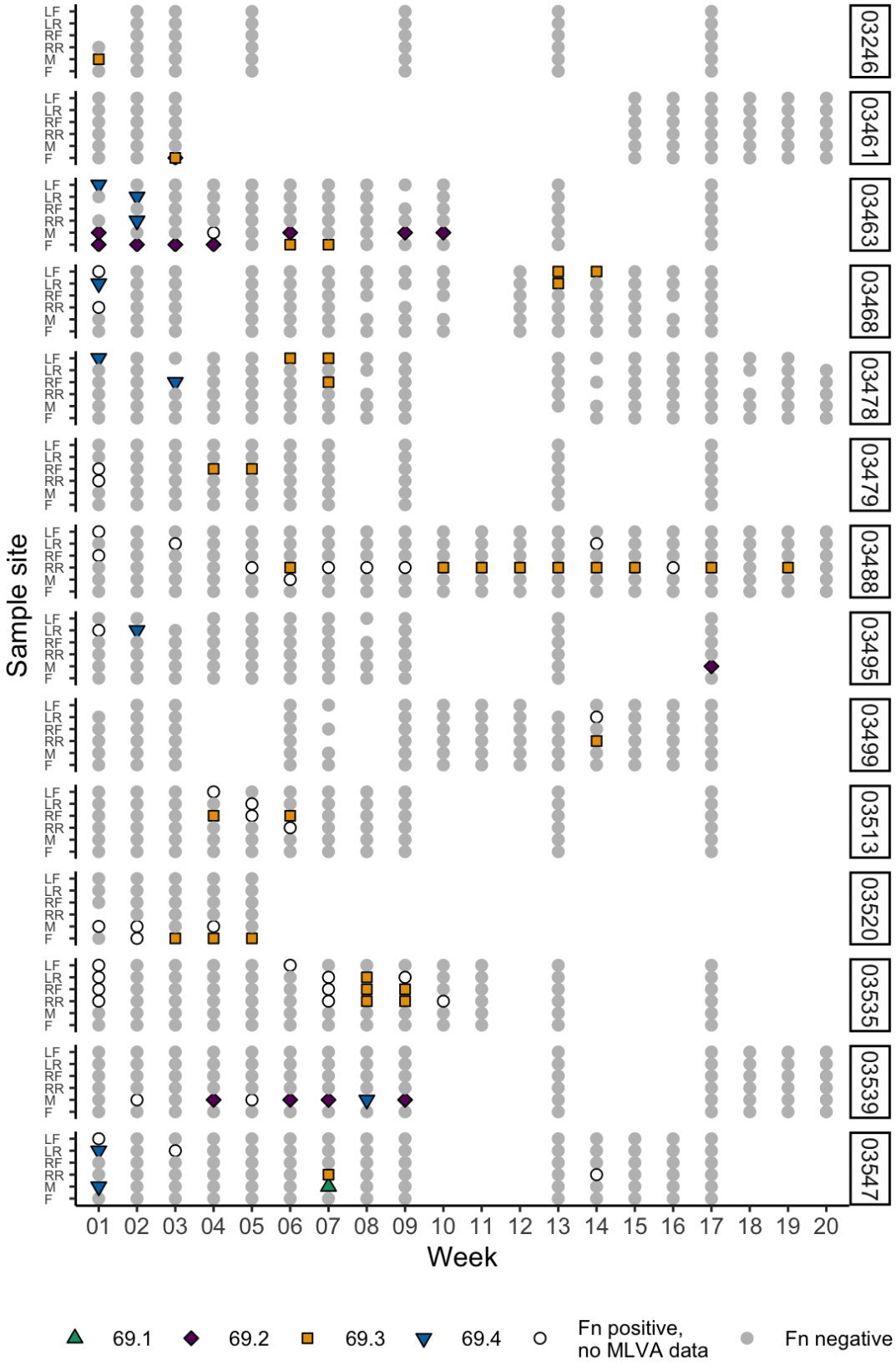

Figure 9 Detection of locus Fn69 variants over time and by sheep
Sheep ID number right hand panel, sample site on the *y* axis (LF = left fore, LR = left rear, RF = right fore, RR = right rear, M = mouth, F = faeces). Fn = *F. necrophorum*. The faeces sample from sheep 03461 in week 3 contained variants 69.2 and 69.3.

4.2.6. Comparison of community types detected on foot swabs between Study A and Study B

Given the predominance of single strain communities on the feet of sheep (Sections 4.1.4 and 4.2.5), the MLVA strains detected in Study B were compared with those in Study A. There were four strains detected across the two farms, three of which varied from each other only at locus Fn69 (Table 16).

Table 16 Strains of *F. necrophorum* detected on foot swabs from Study A and Study B

Community type number[a]	Variant (number of repeats) by locus			Number of times detected	
	Fn13	Fn42	Fn69	Study A	Study B
1	2	5	2	24	0
8	2	5	3	0	21
17	2	5	4	0	1
18	1	5	4	0	1

Only foot swabs with a complete MLVA profile are included.

5. Discussion

The aim of the current study was to identify reservoir sites of *F. necrophorum* in sheep and their environment, and to understand their relevance for ovine footrot. This is the first longitudinal study of presence and load of *F. necrophorum* in sheep and their environment, and included the development of a novel MLVA typing scheme for *F. necrophorum*.

5.1. *Fusobacterium necrophorum* presence and persistence in the environment of sheep

F. necrophorum was detected at low frequency in soil in both Study A and Study B (8% and 0.9% respectively), and was only detected on one occasion on grass. This overturns the belief that *F. necrophorum* is ubiquitous on sheep pasture.This belief was based on evidence from soil microcosm experiments which demonstrated that *F. necrophorum* could survive in soil under laboratory conditions (Garcia *et al.*, 1971). However, Witcomb (2012) failed to detect *F. necrophorum* in soil taken from sheep pasture; this supports the evidence from the studies presented here, and suggests that soil is not a significant reservoir for *F. necrophorum* in sheep flocks.

Across the two studies detection of *F. necrophorum* in soil occurred mostly in high traffic areas, suggesting contamination of the environment by sheep. Detection levels in feet and the environment decreased concurrently in Study A, and in Study B detection occurred in soil in week 2 following high levels of detection on feet in week 1. Outbreaks of necrobacillosis in other ungulates are reported in

connection with animals gathering at feed or watering stations during periods of high rainfall (Monrad *et al.*, 1983; Edwards *et al.*, 2001; Handeland *et al.*, 2010), suggesting that the environment supports transient presence of *F. necrophorum* when conditions are favourable for transmission. Further study of high traffic areas during periods of high rainfall would be necessary to determine if the environment has a significant role in transmission of *F. necrophorum* between sheep in wet conditions.

5.2. *Fusobacterium necrophorum* presence and persistence in sheep

5.2.1. Variation in strains of *Fusobacterium necrophorum* between sites

F. necrophorum was detected on foot swabs, mouth swabs and in faecal samples from both Study A and Study B. The single strains of *F. necrophorum* detected on foot swabs from Studies A and B were closely related by MLVA profile. There were strains of *F. necrophorum* detected in mouths and faeces that were never detected on feet. These findings suggest that the strains detected on feet may share characteristics that make them well adapted to this site and that some of the strains in mouths and faeces may not survive on feet. Strain by site variation may occur because of differences in pathogenicity or the different environment on feet compared with mouths and faeces, for example, differences in temperature, moisture, and pH. Further research using sequencing methods to analyse individual isolates of the strains found on feet and in mouths would improve our understanding of the similarities and differences between the strains identified.

5.2.2. *Fusobacterium necrophorum* presence and persistence on feet

Feet were the only site where *F. necrophorum* was consistently detected over the entire study period for Study B, suggesting that feet were the primary site for persistence of *F. necrophorum* within this flock. Using survival analysis, *F. necrophorum* was more likely to persist on feet with footrot than healthy feet, and the majority of healthy feet were only positive for 1 week. This suggests that although *F. necrophorum* can be detected on healthy feet, they are only transiently positive and therefore unlikely to represent a significant site of persistence. This is a key finding as it provides the first evidence for the role of footrot in maintenance of *F. necrophorum* populations within a flock.

5.2.3. *Fusobacterium necrophorum* presence and persistence in mouths and faeces

This study provided the first evidence that *F. necrophorum* could be shed in sheep faeces, but suggests that shedding is not widespread amongst sheep. Further study would be needed to understand if shedding is a transient property, as suggested by Spencer *et al.* (2015), that could occur in any individual, or if it is specific to certain individuals based on *F. necrophorum* being a stable member of the GI microbiota in these sheep and not others.

In both studies presented here there was evidence that *F. necrophorum* could persist in the mouths of sheep, and in Study A complex communities persisted in the mouths of sheep however only single strains were detected in mouths in Study B. It is unclear why *F. necrophorum* communities in the mouths of sheep were different between the two flocks. There is no evidence from existing literature on the acquisition and development of the microbial community in the oral cavity of sheep. In humans the oral cavity microbial community is mainly derived from the microbial communities of the mother, and is then modified by factors including diet and the external environment (Gomez & Nelson, 2017). If the same is true for sheep, it is possible that differences between farms in diet and bacteria present in the farm environment could influence the oral cavity community in sheep on that farm; this requires further investigation.

The strains of *F. necrophorum* involved in footrot were intermittently present in mouths and in faeces, suggesting that these could be transient reservoir sites of *F. necrophorum* in footrot. It is possible that these sites, along with healthy feet, could be relevant for *F. necrophorum* persistence within a flock in the absence of footrot, but further investigation would be necessary to confirm this.

5.2.4. Transmission of *Fusobacterium necrophorum* within and between flocks

Increased loads of *F. necrophorum* were found in feet with footrot in both studies and in Study B feet with ID were more likely to have higher loads of *F. necrophorum* the next week. This supports previous evidence from Witcomb *et al.* (2014), that *F. necrophorum* load increases once footrot has occurred and not before. This is an important distinction, as it implies that footrot facilitates *F. necrophorum* growth, rather than *F. necrophorum* facilitating footrot as previously suggested (Roberts & Egerton, 1969).

Individuals shedding levels of an organism above a certain threshold are referred to as “super shedders” (Omisakin *et al.*, 2003; Cobbold *et al.*, 2007). Feet (and therefore sheep) with footrot have high loads of *F. necrophorum* for extended periods of time, and could therefore be considered as super shedders. It has generally been assumed that super shedders are important for pathogen transmission, however, recent work for both *E. coli* O157:H7 and *Mycobacterium avium* subsp. *paratuberculosis* in cattle faeces suggests that for these bacteria super shedders have minimal effects on transmission (2-3 fold increase in risk of transmission) despite shedding quantities of bacteria several orders of magnitude higher than low shedding animals (Spencer *et al.*, 2015; Slater *et al.*, 2016). Further work would be necessary to understand the effect of sheep with footrot on *F. necrophorum* transmission within a flock.

Regression analysis of foot swab data from Study B indicated that there was an association between environmental temperature and both the likelihood of a foot being positive for *F. necrophorum* and

load of *F. necrophorum* on positive feet. The load of *F. necrophorum* on feet increased as maximum temperature increased, and this may reflect an effect of temperature on the growth rate of *F. necrophorum*. Feet were more likely to be positive for *F. necrophorum* at higher minimum temperatures but at lower maximum temperatures. This implies that *F. necrophorum* survival and transmission increased when temperatures were less extreme. Evidence from Australia showed that footrot transmission did not occur below 50°F (10°C), or during hot dry periods (Graham & Egerton, 1968), however evidence from the UK suggests that footrot transmission can occur year round despite temperatures frequently falling below 10°C (Ridler *et al.*, 2009). It should be noted that there were only 19 values present in the regression models for each temperature variable, and this may have reduced the reliability of the parameter estimates for these variables.

The findings discussed so far refer to the transmission of *F. necrophorum* within a sheep flock. In the UK, sheep farmers frequently buy in new stock from other farms, and therefore transmission of *F. necrophorum* between flocks is also possible. The evidence from the studies presented here highlights that healthy sheep may be transiently carrying strains of *F. necrophorum* capable of causing footrot, either in the mouth, on healthy feet or in faeces. These sites may therefore be significant for transmission of *F. necrophorum* between flocks.

5.3. Conclusions

Contrary to previous assumption, the environment was not a significant reservoir for *F. necrophorum*. The feet of sheep were the primary site of persistence for *F. necrophorum*, with footrot facilitating persistence. This study provided the first evidence that *F. necrophorum* could be shed in sheep faeces, and both faeces and the mouths of sheep may be transient reservoirs for *F. necrophorum* in footrot. There was evidence that *F. necrophorum* formed part of a microbial community in the mouths of sheep, but strains in this community were frequently different to those involved in footrot.

6. Industry messages

Fusobacterium necrophorum is an opportunistic pathogen that increases the severity of footrot. It was previously believed that the main sources of *F. necrophorum* in footrot were the environment and sheep faeces. This study provided evidence that *F. necrophorum* is rarely present on sheep pasture. There was evidence that different strains of *F. necrophorum* could be found in sheep, and only some of these were involved in footrot. The strains involved in footrot were most often found on the feet of sheep, and feet with footrot had higher numbers of *F. necrophorum* for longer periods of time. Feet with footrot may therefore be important for transmission of *F. necrophorum* within the flock. The strains of *F. necrophorum* involved in footrot were transiently present in the mouths of sheep and were intermittently shed in faeces of a small proportion of sheep. Mouths and faeces of

healthy sheep may be a potential route for *F. necrophorum* to spread between flocks when sheep are bought and sold.

7. References

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