Synchronization of *E. coli* O157 shedding in a grass-fed beef herd: a longitudinal study

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SUMMARY

This study aims to describe in detail the temporal dynamics of *E. coli* O157 shedding and risk factors for shedding in a grass-fed beef herd. During a 9-month period, 23 beef cows were sampled twice a week (58 sampling points) and *E. coli* O157 was enumerated from faecal samples. Isolates were screened by PCR for presence of *rfb*E, stx_1 and stx_2. The prevalence per sampling day ranged from 0% to 57%. This study demonstrates that many members of the herd were concurrently shedding *E. coli* O157. Occurrence of rainfall ($P < 0.01$), feeding silage ($P < 0.01$) and lactating ($P < 0.01$) were found to be predictors of shedding. Moving cattle to a new paddock had a negative effect on shedding. This approach, based on short-interval sampling, confirms the known variability of shedding within a herd and highlights that high shedding events are rare.

Key words: Cattle, enumeration, *Escherichia coli* O157, longitudinal, risk factors, shedding.

INTRODUCTION

Infection with *Escherichia coli* O157 in humans is uncommon but potentially fatal [1]. The organism is a common commensal in the gastrointestinal tract of cattle [2]. A great deal of research on *E. coli* O157 in cattle has been completed in the last 30 years [3–5], although it is uncertain whether this has resulted in lower rates of human illness. No decrease in reported clinical cases with *E. coli* O157 has been noted in Scotland over the last 10 years [6] and food-borne outbreaks still frequently occur in many countries. Whether humans become infected through foodborne or environmental pathways, cattle faeces are considered the primary source of infection [7]. Therefore understanding the dynamics of shedding of this organism in cattle and the factors that influence shedding are priorities for improving the prevention of human infection.

E. coli O157 is invariably detected in cattle populations that are subjected to sufficient sampling and testing. However, the prevalence of *E. coli* O157 within herds is variable, with estimates ranging from 0·7% to 28% in the USA [8, 9], 3·4–21·8% in the UK [6] and 1·9–13% in Australia [10, 11]. While some studies

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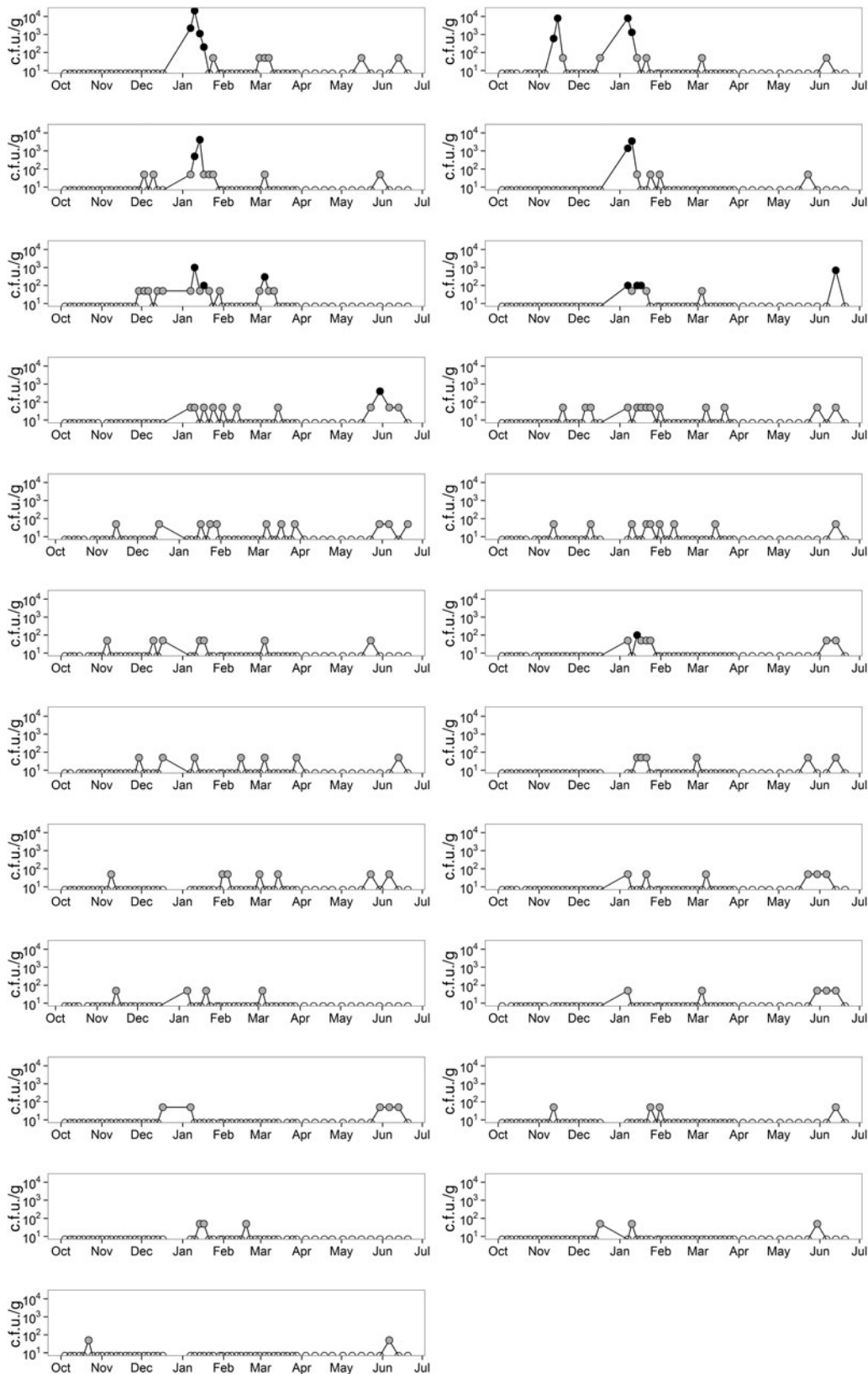(Email: glammers@csu.edu.au)

Fig. 1. Plot of the individual shedding patterns. Samples in which *E. coli* O157 was detected only by immunomagnetic separation (grey dots) are represented as 50 c.f.u./g for graphical purposes. Sampling points for which enumeration was possible are indicated by black dots and samples in which no *E. coli* O157 was detected are represented by white dots.
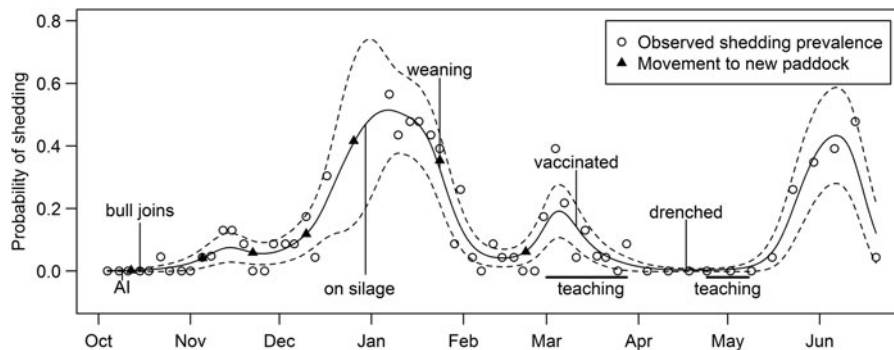
Fig. 2. Temporal change in probability of animals shedding *E. coli* O157 (and 95% confidence intervals), the timing of management variables and the timing of movement of animals between paddocks.

have described peaks in prevalence during late spring, summer and early autumn [12, 13], others, all carried out in Great Britain, report a lack of convincing seasonal pattern [14, 15]. Thus, whether or not shedding is a seasonal phenomenon is uncertain. In addition to variation in location, which may drive differences, most studies have not sampled for a sufficient length of time to allow them to conclude whether peaks in prevalence are part of an ongoing cyclical pattern or are simply a manifestation of the particular period of observation.

Describing the epidemiology of *E. coli* O157 in cattle is challenging because of the cost and inconvenience of measuring the concentration of the pathogen in faeces. Intermittent patterns of shedding have been shown in calves [16, 17], but little is known about the shedding pattern of live adult beef cattle. This is especially so for pasture-based production systems which are not well represented in the literature. While a few longitudinal studies have been performed in adult beef cattle [15, 18–22], data describing the shedding patterns of individual animals that have been repeatedly sampled at short time intervals (<7 days) are missing. Most longitudinal studies used fortnightly or monthly sampling intervals during a 3- to 12-month period [18, 19, 21]. Some studies have assessed the concentration of *E. coli* O157 in faeces and several others featured repeated observations on the same animals [15, 18, 19, 21]. A study that combines these aspects of repeated sampling and enumeration but with the repetition occurring at short time intervals will provide new and potentially useful information about shedding of *E. coli* O157 by cattle.

In this study 23 cattle were intensively studied over 9 months while managed in a temperate grazing system. They were repeatedly assessed at 3- to 7-day intervals for the presence and concentration of

E. coli O157 in faeces. The overall objective was to describe in detail the temporal dynamics of *E. coli* O157 shedding in individual cattle managed on pasture and to identify possible risk factors for shedding of this pathogen.

METHODS

Beef herd characteristics

A longitudinal study was performed from 4 October 2012 to 20 June 2013. The study subjects comprised 23 primiparous and multiparous Hereford cattle pastured at Charles Sturt University's beef farm, located at Wagga Wagga, NSW, Australia (latitude 35° S, longitude 147° E). Rainfall is distributed relatively evenly at 48 mm/month. Average daily minimum and average daily maximum temperatures for summer are 16·2 °C and 31·2 °C and for winter 2·7 °C and 12·5 °C, respectively. From October to 28 March the cattle were sampled twice a week, which was reduced to once a week during the months of April, May, and June because of a reduction in the number of cattle shedding (⩽1 animal per day) on three sampling occasions in the last weeks of March.

During the study period the cattle were grazed on different paddocks, varying between 3·5 ha and 7 ha in area, according to the availability of grass, herd nutritional requirements and environmental management considerations. The cattle were fed cereal and ryegrass silage every Monday, Wednesday and Friday when no fresh grass was available, starting from 30 December 2012, and continuing until the last sampling day (Fig. 2). The herd was used biweekly in March and bimonthly in April and May by veterinary students of the university for teaching purposes. Twenty-one cows and heifers calved in July 2012,

including two pairs of twins, resulting in 23 calves at foot and nursing until weaning on 24 January 2013. Thus the calves were only present for part of the study period.

Ethical standards

The use of animals in this study was approved by Charles Sturt University Animal Care and Ethics Committee Protocol number 12/060.

Sample collection

Animals

A minimum of 10 g of faeces were collected from each cow by rectal palpation or during defecation while the cows were restrained in a crush in the cattle yards. For each cow a new disposable sleeve glove was used and each faecal sample was individually placed into a Whirl-Pak bag (Nasco, Australia). Transportation occurred on ice within 2 h of the first sample being taken and samples were processed directly upon arrival at the laboratory.

Rectal temperature, faecal score and hide contamination were recorded for each cow at every sampling. Rectal temperature was measured after the faecal grab was taken. Faecal score was categorized from 1 to 4 (runny/loose/soft/dry) [23] and hide contamination from 1 to 5 (clean and dry/slightly dirty/dirty/very dirty/extremely dirty and wet) [24]. Weight (kg) and body condition score (score 1–5) [25] were recorded every 14 days. The first author undertook all sampling and data recording.

Environment

Rainfall (mm in 24 h prior to sampling), ambient temperature (mean °C in 24 h prior to sampling), day length (hours between sunrise and sunset on the day of sampling), humidity (% relative humidity at 09:00 hours) and hours of bright sunshine (bright sunshine in the 24 h to midnight prior to sampling) were obtained for each day throughout the study from the Bureau of Meteorology (BOM). Rainfall data was obtained from the BOM weather station located <2 km from the herd location, while all other data was retrieved from the BOM weather station located <15 km away. The quantity and quality of the pastures were recorded every 14 days. Quantity was measured by assessing the average height, using methods described by Morris & Kenyon [26]. Quality was

determined by the stage of pasture growth, e.g. short actively growing vegetative pasture (80%), tall actively growing vegetative pasture (75–80%), early flowering (70–75%) or late flowering (65–70%). Contamination of drinking water with *E. coli* O157 was analysed coinciding with the pasture estimates. Water samples, from all drinking-water troughs in the paddock used at the time of sampling, were collected into 120 ml sterile containers (Sarstedt, Australia), directly transported to the laboratory and analysed together with the faecal samples.

Management

Management variables that changed during the period of study were recorded. This included feeding of supplements, artificial insemination, joining with a bull, weaning of calves, spread of fertilizer on the pastures, relocation to other paddocks, medical treatments and vaccinations. The dates of veterinary teaching classes (teaching of cow handling, claw trimming and casting) were recorded.

Sample processing

Faecal samples

Ten grams of faeces from each sample were homogenized in 90 ml of sterile buffered peptone water (BPW) (Oxoid, Australia). For each homogenized broth 100 μl was plated directly on sorbitol MacConkey+5-bromo-4-chloro-3-indolyl-b-D-glucuronide agar (Oxoid) containing cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l) (Oxoid) (CT-SMAC+BCIG) and incubated for 18–24 h at 37 °C for direct culture [27]. Each homogenized broth was incubated at 37 °C for 6 h [28]. The enriched broth was stored at 4 °C until immunomagnetic separation (IMS) was performed the next day.

Confirmation and storage

After incubation of the CT-SMAC+BCIG plates, all plates were screened for non-sorbitol fermenting and β-glucuronidase-negative colonies (straw-coloured colonies). Up to ten suspect colonies were tested using an *E. coli* O157 latex test (Oxoid) to confirm the colonies as O157 or otherwise [29]. When positive colonies on direct culture plates were found, the number of colony-forming units (c.f.u.) was counted and the c.f.u./g of faeces estimated after accounting for the dilution factor. Pure isolates were stored at −80 °C

Table 1. *Primers used for detection of* E. coli *O157 genes*

Primer	Sequence	Target gene	Amplicon size (bp)	Reference
rfbE_F	CTACAGGTGAAGGTGGAATGG	$rfbE_{O157}$	327	Wang *et al.* [30]
rfbE_R	ATTCCTCTCTTTCCTCTGCGG			
Stx1_F	ATAAATCGCCATTCGTTGACTAC	stx_1	180	Paton & Paton [31]
Stx1_R	AGAACGCCCACTGAGATCATC			
Stx2_F	GGCACTGTCTGAAACTGCTCC	stx_2 (including	255	
Stx2_R	TCGCCAGTTATCTGACATTCTG	stx_2 variants)		

in BPW with 20% glycerol for later analysis by polymerase chain reaction (PCR).

Water samples

The water samples were filtered with cellulose nitrate membrane filters with pore size 0.2 μm (Bio-Rad, Australia). The entire filters were added to 90 ml sterile BPW, direct plated and enriched as described for the faecal samples.

IMS

For all faecal and water samples that were negative after direct plating, manual IMS was performed. Two ml of each enriched sample was centrifuged for 2 min at 52 *g* prior to IMS. A 24-well plate was set up with 20 μl of anti *E. coli* O157 immunomagnetic beads (Invitrogen, Australia). One thousand μl of the supernatant of each enriched centrifuged sample was transferred to a well [11] and thoroughly mixed with the beads on a gyratory mixer (Ratek, Australia) for 1 h. The plate was then placed on a magnet for 2 min to immobilize the beads after which the enrichment solution was removed while the plate remained on the magnet. After removing the plate from the magnet, the beads were washed in 1000 μl of phosphate buffered saline (PBS) (MP Biomedicals, Australia) with 0·05% Tween 20 (Sigma, Australia) (PBST). The solution was mixed for 2 min and placed back on the magnet for 2 min to immobilize the beads. Again the wash solution was removed and the wash step repeated. After the second wash the beads were removed from the magnet and re-suspended in 100 μl PBST. The resulting solution was split and inoculated onto CT-SMAC + BCIG plates and incubated for 18–24 h at 37 °C [11, 28].

PCR

All isolates were screened by PCR for the presence of *rfb*E, which encodes the *E. coli* O157 serotype [30],

and for the virulence genes stx_1 and stx_2 [31] (Table 1). Colony PCR was performed on samples, using OneTaq DNA polymerase (New England Biolabs, USA). A multiplex PCR assay was set up for detection of the above-mentioned genes using a multiplex PCR plus kit (Qiagen, Australia). Thermocycling was performed in a Bio-Rad S1000 Thermal Cycler (Bio-Rad) following the cycling conditions from Paton & Paton [31]. PCR products were then visualized on a 2% agarose gel containing SYBR Safe DNA gel stain (Invitrogen).

Descriptive analysis

Graphical analysis was performed to assess relationships between the probability of cattle shedding *E. coli* O157 and a range of animal and environmental variables. Data describing each individual animal's shedding status at each sampling point was summarized for the herd to give the observed proportion of cattle shedding *E. coli* O157. These proportions were then fitted to a cubic spline model to provide a smoothed curve (and accompanying 95% confidence interval) defining the probability of shedding through time and providing a basis for visually assessing the relationship between various factors (animal, environment, management) and the occurrence of shedding (Figs 3–5). The graph displaying probability of shedding through time was annotated with the timing of key management events such as movement to new paddocks, weaning, vaccination and handling (Fig. 2).

Statistical analysis

Herd level

Trend in the proportion of animals testing positive to *E. coli* O157 on each sampling day was estimated by a cubic spline model. Visual assessment of this curve, overlaid with animal, environment and management variables, was performed to explore any putative
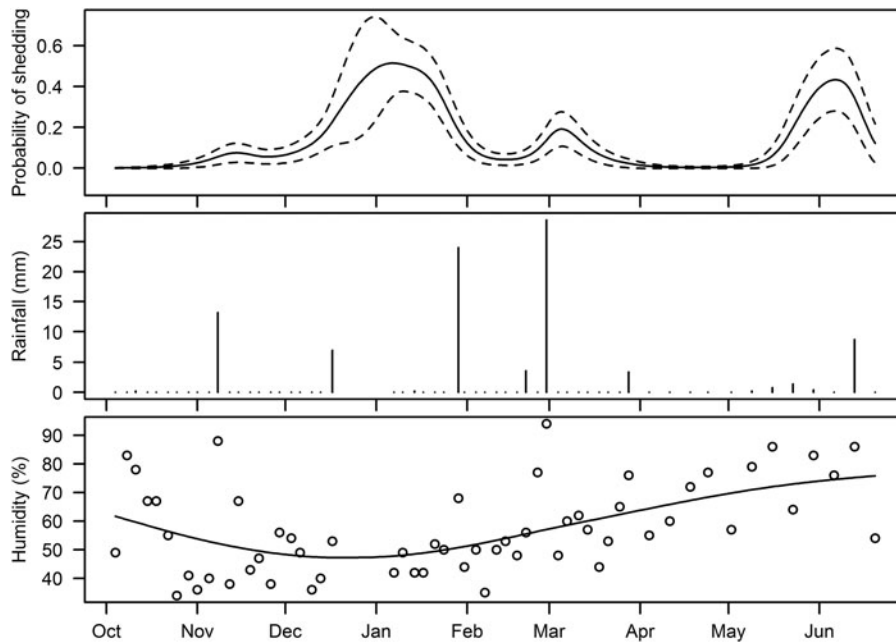
Fig. 3. Temporal change in probability of animals shedding *E. coli* O157, rainfall (mm) in the 24 h prior to sampling, and relative humidity (%) at 09:00 hours on the day of sampling.
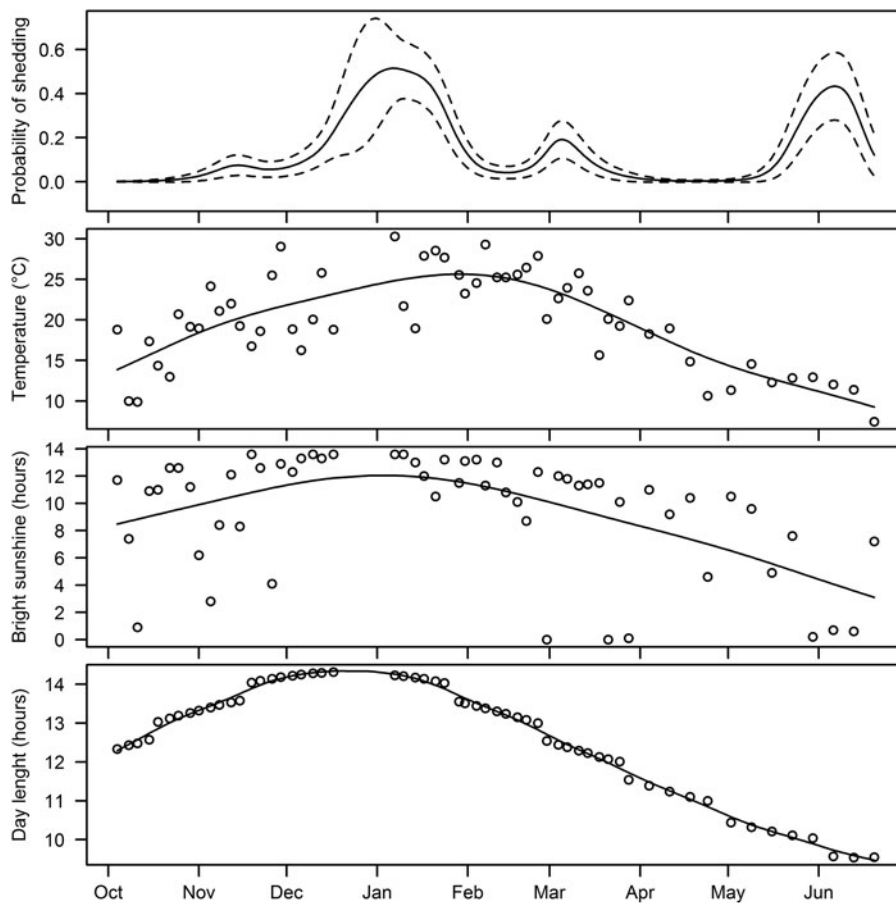

Fig. 4. Temporal change in probability of animals shedding *E. coli* O157, mean environmental temperature (°C) in the 24 h to 09:00 hours, bright sunshine (hours) in the 24 h to midnight prior to sampling, and day length on the day of sampling.
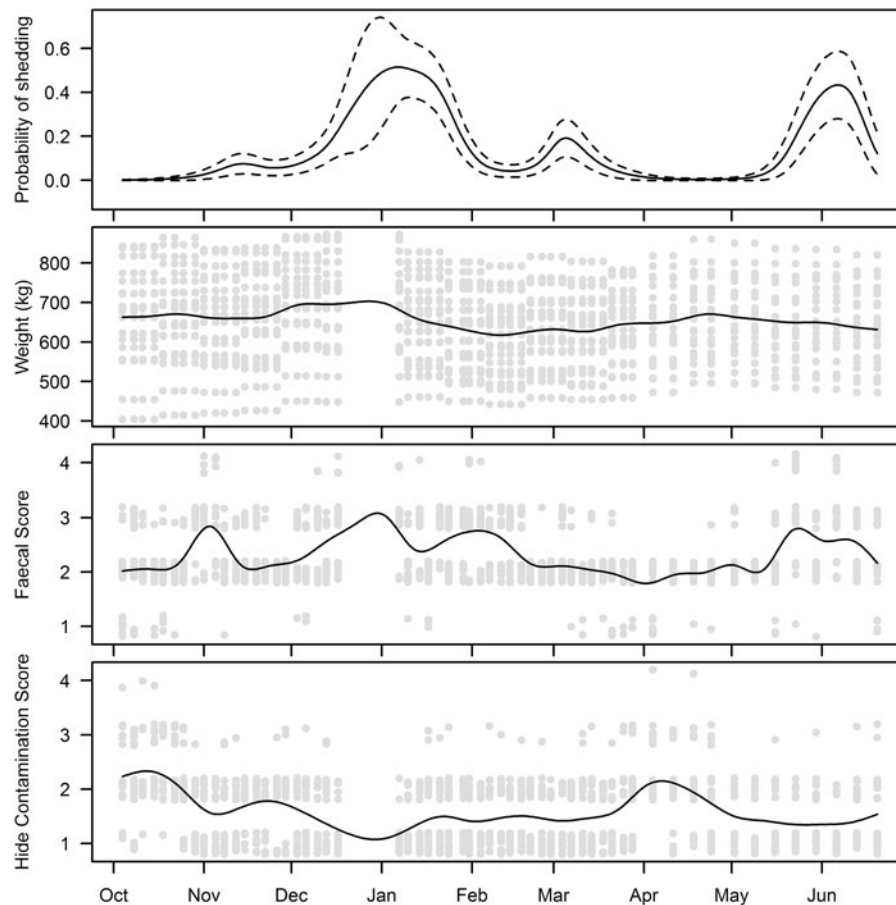
figure

Individual animal level

Logistic regression analysis was used to explore the effect of potential animal-, environmental- and management-level risk factors on individual animal shedding. First, univariable analyses were performed. Variables with $P < 0.05$ were jointly analysed using a forward selection procedure in multiple regressions. As repeated observations of individual animals may not be independent, a random effect of cow identifier was included.

Based on the estimated counts from faecal samples, animals were categorized as negative, low-level ($<10^3$ c.f.u./g) or high-level ($\geqslant 10^3$ c.f.u./g) shedders of *E. coli* O157. These data were analysed by using a generalized linear model with presence of a high shedding as the binomial outcome, low shedding as a fixed effect and cow identifier as a random effect.

RESULTS

Descriptive and statistical analysis

During the 9-month study period, a total of 1323 faecal samples were collected from 23 Hereford cows over 58 sampling days. *E. coli* O157 was isolated from 168 (12·7%) samples with 21 (1·6%) positive on direct enumeration plates and 147 (11·1%) positive only through IMS. The amount of *E. coli* O157 shed in faeces ranged from <100 to 20 700 c.f.u./g faeces. All cows within the herd shed *E. coli* O157 at least once during this study (Fig. 1). A large variation in the frequency of shedding among animals was identified. *E. coli* O157 was shed intermittently by all animals and the number of occasions on which an individual cow was identified as shedding *E. coli* O157 ranged between 2 and 15 (out of 58 sampling points in total). The maximum number of consecutive sampling points that an animal was found to be positive (by either direct culture or IMS) was seven (3·5 weeks). The prevalence per sampling day ranged from 0% to 56·5% (Fig. 2). Out of 168 isolates screened by PCR, all were positive for the O157 *rfb* gene, 166 (98·8%) were positive for stx_1 and stx_2, and two (1·2%) were positive for stx_1 only.

E. coli O157 was isolated from 1 of 62 water samples that were collected during the study. The isolate identified within water tested positive for the O157 *rfb*, stx_1 and stx_2 genes by PCR. This positive water sample was found on 11 November 2012 when none of the cows were positive for *E. coli* O157.

Temporal data on shedding events and management events are shown in Figure 2. This figure shows three distinct peaks in probability of shedding. There was a major peak in mid-summer (mid-December to late January), a minor peak in autumn (late February to mid-March) and a second major peak in early winter (late May to late June). None of the management variables appeared to be associated with any of the peaks in shedding.

Time-based change in the probability of shedding is also shown in Figure 3 accompanied by the temporal patterns of rainfall and humidity. Although all of the peaks in shedding are immediately preceded by rainfall this can also be said for many periods where shedding was not detected. Thus, descriptive evidence alone does not support a relationship between variation in rainfall and the presence or absence of a 'herd-shedding event'. However, the generalized linear mixed model showed that rainfall is a risk factor ($P = 0.03$): the mean of rainfall in the 24 h prior to sampling during herd-shedding events (3·4 mm) differed significantly from that for non-shedding events (0·6 mm) (Table 2). Visually, there was no apparent association between humidity and shedding, which was confirmed by statistical analysis.

Temporal change in the probability of shedding in Figure 4 is accompanied by the temporal patterns of temperature, hours of bright sunshine and day length. All three variables showed a similar temporal curve. Based on this descriptive evidence the variation in the environmental variables could not be explained by the presence or absence of a 'herd-shedding event'. This was supported by statistical analysis in which none of these variables was a significant risk factor (Table 2).

Time-based change in the probability of shedding is shown in Figure 5 together with the temporal patterns of the individual animal variables: body weight, faecal consistency and hide contamination. Visually, there was no apparent association between any of the three variables and the probability of shedding *E. coli* O157. Statistical analysis of the animal variables in a generalized linear mixed model showed that neither the variation in live weight nor in faecal consistency was associated with the presence or absence of a 'herd-shedding event' (Table 2). The average level of hide contamination score during shedding events (1·4) did differ from non-shedding events (1·7) ($P = 0.04$).

Univariable analysis to explore potential risk factors associated with individual animal shedding resulted in eight variables that were jointly analysed using multiple regression. The final logistic model is

Table 2. *Variables relating animal, management and environmental factors with the mean and standard error (S.E.) for shedding vs. non-shedding events and the P value*

Variable	Shedding events mean (S.E.)	Non-shedding events mean (S.E.)	P value
Animal			
Hide contamination (1/2/3/4/5)	1·39 (0·08)	1·69 (0·08)	0·04
Faecal score (1/2/3/4)	2·42 (0·15)	2·21 (0·14)	0·22
Temperature	37·95 (0·06)	37·97 (0·06)	0·81
Pregnant (yes/no)	1·61 (0·06)	1·60 (0·06)	0·39
Lactating (yes/no)	0·25 (0·28)	0·30 (0·28)	0·88
Weight	644·53 (12·28)	648·05 (12·26)	0·77
Body condition score (1/2/3/4/5)	3·87 (0·08)	3·89 (0·08)	0·63
Management			
Move paddock (yes/no)	0·09 (0·07)	0·11 (0·05)	0·88
Silage (yes/no)	0·91 (0·25)	0·66 (0·25)	0·43
Handled by students (yes/no)	0·13 (0·16)	0·20 (0·15)	0·69
Calves at foot (yes/no)	0·27 (0·30)	0·33 (0·30)	0·88
Pasture quality (0/65/80)	11·32 (16·21)	22·00 (16·01)	0·50
Pasture quantity	107·70 (221·93)	305·73 (219·66)	0·43
Environment			
Rainfall previous 24 h (mm)	3·35 (1·12)	0·58 (0·84)	0·03
Mean environmental temperature	19·72 (3·64)	20·34 (3·59)	0·73
Day length	12·09 (1·02)	12·62 (1·02)	0·96
Sun	8·61 (2·12)	9·48 (2·06)	0·90
Humidity	60·38 (6·25)	57·64 (5·90)	0·99

Table 3. *Final model from multiple regression on individual animal level*

Variable	Estimated effect (S.E.)	OR	95% CI	P value
Silage (yes/no)	2·36 (0·273)	10·56	6·18–18·03	<0·001
Lactating (yes/no)	2·07 (0·225)	7·89	5·08–12·26	<0·001
Rainfall previous week (mm)	0·67 (0·214)	1·95	1·28–2·97	0·002
Move paddock (yes/no)	−1·12 (0·531)	0·33	0·12–0·92	0·035

OR, Odds ratio; CI, confidence interval; S.E., standard error.

shown in Table 3. Four variables remained in the final model. In order of statistical significance the risk factors were: feeding of silage ($P < 0.01$), lactating ($P < 0.01$), rainfall in the week prior to sampling ($P < 0.01$) and movement between paddocks ($P = 0.04$).

In total, *E. coli* O157 was isolated from 168 samples on 38 of the 58 sampling days. Analysis showed that the presence of a high-level shedder was significantly ($P = 0.04$) associated with a higher proportion of low-shedding animals on the same day (odds ratio 1·3, standard error 0·14).

DISCUSSION

The current study focused on the temporal dynamics of *E. coli* O157 shedding in adult grass-fed beef cattle.

Although marked variation of shedding between and within individuals occurred, an obvious pattern in shedding over time was identified. The outstanding feature of the variation in the probability of shedding was the three distinctive peaks indicating the herd experienced discrete shedding events where many animals were concurrently shedding, referred to as synchronization of shedding. Previous studies have reported peaks of shedding in summer and early autumn, which is in agreement with the first two peaks in shedding in the current study; however, the third peak of shedding occurred in winter. Most longitudinal studies performed previously sampled only once a month [18, 20], which only provides crude information on temporal shedding patterns within a herd. Our findings suggest that if more frequent

sampling had been used in earlier studies, e.g. twice a week, a different seasonal pattern might have been detected. Moreover, with the use of individual animal data it is evident that the synchronization of shedding in cows is accompanied by a marked elevation in the concentration of the pathogen in faeces. This indicates that shedding at high levels does not occur independently of other animals shedding low levels of the same pathogen within the herd. Previous studies have also observed an association between the presence of a high-level shedder in the herd and a high proportion of low shedders [32, 33]. Whether high-level shedding is the cause of low-level *E. coli* O157 shedding, or vice versa, is not known.

Intermittent shedding patterns have also been reported previously in calves [16, 17]. However, in this study very few cattle shed at high levels and the duration was very short. Robinson and colleagues [16] observed animals shedding at high levels for extended periods but this study looked at a group of calves purposely selected because they were known to be shedding. The current study is unique because it studies shedding and non-shedding animals in detail over time in a natural setting. There are two other notable explanations for the lower frequency in high-level shedding and the lower concentrations found in this study compared to other studies. One is an effect due to the age of the animals, since younger animals (aged 2–6 months) are associated with a higher prevalence of *E. coli* O157 than adults [34]. Second, the majority of previous studies involved feedlot cattle, where the high density of housing is likely to increase animal-to-animal transmission [35], compared to animals at pasture. Next to this, *E. coli* O157 in cattle faeces is not evenly distributed and its density can vary between sites in a faecal pat [36], which might influence the concentration of the organism.

Previously, only Williams *et al.* [37] identified an association between increasing rainfall and increased shedding of *E. coli* O157 in calves in Sydney, Australia (a higher rainfall environment compared to the present study). Rainfall events could feasibly promote shedding by making conditions beneficial for multiplication of the bacteria in the environment, changing pasture conditions to increase exposure of cattle to *E. coli* O157, or by somehow affecting forage composition and nutrition leading to alterations in the gut favouring proliferation of *E. coli* O157. Previous research in other countries has not found a relationship between rainfall and shedding. However, these studies did not rely on short-interval sampling and

were conducted in different climates and production systems.

The negative association between hide score and probability of shedding in this study was unexpected and does not appear to be biologically plausible. It is very likely that this association is a spurious finding arising from either chance or measurement error.

Feeding silage was demonstrated to be associated with *E. coli* O157 shedding at the individual animal level. Many studies on diet and *E. coli* O157 shedding have been performed, often presenting contradictory results. Whether the significant association with silage found in the present study is due to a direct effect of silage on the microbiome or due to changes in the environment (e.g. drought) which were the reason for feeding silage, is not known.

Lactating was also positively associated with shedding of the pathogen. This effect has not been reported in literature before. It is possible that stress or hormonal changes influence *E. coli* O157 shedding cattle.

This study found that when animals were moved to another paddock in the week prior to sampling they were less likely to shed *E. coli* O157. This might have resulted from the absence of fresh faecal pats in a new paddock and reduced *E. coli* O157 contamination.

The advantage of using a smoothed curve (rather than raw data on animal shedding) is that it takes account of noise due to sampling variation arising from the small number of individuals, and, at each time point it uses data from proximal time points to support the estimation of probability of shedding [38]. In this way, it was possible to avoid potential false-positive significant correlations due to chance associations that frequently occur when comparing two time series of events [39]. It must be noted that when analysis of variance was applied on score variables, such as hide contamination score, that these scores should not be seen as 'ranks', but as proxy measurements for an underlying true scale of dirtiness, and averages and standard errors do supply useful information.

Because this study was based on a single herd, care must be taken when extrapolating interpretation to the broader population of cattle. Nevertheless, the major findings are helpful because, for example, they suggest that shedding patterns vary substantially between production systems (e.g. pasture beef, dairy, feedlot beef), and that animals within a herd can be synchronized in their shedding (and non-shedding) which may have

practical implications for the risk of human exposure to *E. coli* O157. Moreover, the extent of individual animal variability revealed in this work and in previous studies [16, 40] casts doubt on findings of risk factor and intervention studies where outcomes are based on single assessment of dichotomous shedding status. Findings here have also highlighted the lack of precision in the present understanding of shedding patterns in beef cattle and indicate the need to now focus on describing the extent of daily variation in shedding within individual animals.

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DECLARATION OF INTEREST

None.

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