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Antimicrobial resistant *Escherichia coli* in Scottish wild deer: Prevalence and risk factors☆

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ABSTRACT

Antimicrobial resistance (AMR) is a recognised threat to global health. Obtaining data on the prevalence of AMR in environmental bacteria is key to understanding drivers and routes of transmission. Here, 325 Shiga toxin negative deer faecal samples—gathered from across the Scottish mainland—were screened for the presence of AMR *Escherichia coli* and investigated for potential risk factors associated with AMR occurrence. *E. coli* with resistance to antimicrobials of clinical health concern, including carbapenems and 3rd generation cephalosporins, were targeted. Ninety-nine percent of samples yielded *E. coli*, and the prevalence of resistant *E. coli* at the level of faecal samples was 21.8% (n = 71) for tetracycline, 6.5% (n = 21) for cefpodoxime, 0.3% for ciprofloxacin (n = 1), with no recorded resistance to meropenem. Potential risk factors for tetracycline and cefpodoxime resistance were investigated. The presence of broadleaved woodlands was significantly associated with both AMR phenotypes, which may relate to land use within or around such woodlands. Associated risk factors varied across resistance phenotype and deer species, with proximity or density of horses an indicator of significantly decreased and increased risk, respectively, or tetracycline and cefpodoxime resistance in *E. coli* from roe deer, but not from red deer. Distance from wastewater treatment plants was a significant risk factor for tetracycline resistance in *E. coli* from red deer but not from roe deer. Data indicated that AMR *E. coli* can occur in wild deer populations that are not directly exposed to the selective pressure exerted by antimicrobial treatment. Overall, resistance to critically important antimicrobials was found to be low in the studied population, suggesting no immediate cause for concern regarding human health. Utilising existing culling frameworks, wild deer in Scotland could function well as a sentinel species for the surveillance of AMR in the Scottish environment.

1. Introduction

Antimicrobial resistant (AMR) bacteria are widely acknowledged as one of the most critical emerging threats to human wellbeing (Department of Health, 2018). In addition, AMR also has impacts in veterinary care, such as restricting access to certain antimicrobials, and occasionally necessitating euthanasia of animals with otherwise treatable infections (Bengtsson & Greko, 2014; Weese et al., 2015). Environmental AMR (including that within wildlife hosts) may arise as a result of multiple complex mechanisms (Arnold et al., 2016; Graham et al., 2019; Radhouani et al., 2014). This includes dissemination of resistant bacteria into the environment via anthropogenic waste (Literák et al., 2007; Pesapane et al., 2013), including waste specifically from sites of antimicrobial usage (Morris et al., 2015; Perry et al., 2019a), as well as dissemination of antimicrobials or antimicrobial residue into the environment, with subsequent selection for resistant bacteria within environmental bacterial populations (Guardabassi et al., 1998; Niemi et al., 2020). Surveillance of environmental AMR has been identified as a key area in which research is required in order to appropriately quantify and tackle the risks that AMR presents (O'Neill, 2016). While potential pathways for transmission of AMR bacteria between humans, animals and the environment have been identified, the origin of AMR bacteria in

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wildlife is largely unknown and specific risk factors for their presence are currently poorly characterised and understood (Laxminarayan et al., 2013). In order to fully assess these potential pathways or the risk posed by wildlife as a potential vector or reservoir for AMR bacteria, more data is required; understanding the extent and characteristics of AMR bacteria harboured by wildlife is a crucial step in this endeavour.

Background levels of AMR in environmental bacterial populations are influenced by the overall antimicrobial load in the environment, and by the effectiveness of legislative controls on antimicrobial usage (Gilliver et al., 1999; Österblad et al., 2001; Zaidi et al., 2015), but some bacterial populations possess a naturally higher prevalence of AMR than others (Burow et al., 2019). Wild animals are commonly sampled to obtain insights into the prevalence and spread of AMR bacteria in the environment, with birds and cervids most often utilised (Greig et al., 2015). For example, AMR bacteria have been identified in samples from wild deer (i.e., present within their faeces) in North America, Europe, the UK, and Asia (Bryan et al., 2004; Cenci Goga et al., 2009; Österblad et al., 2001; Sasaki et al., 2013), including multi-drug resistant (MDR) isolates (Lanthier et al., 2010; Lillehaug et al., 2005) and isolates with extended spectrum β-lactam (ESBL) resistance mechanisms (Alonso et al., 2016; Carrillo-Del Valle et al., 2016a; Costa et al., 2006; Literak et al., 2010).

In Scotland, the total wild deer population is currently estimated at around 1 million individuals and can be found across nearly the entire mainland. The population is primarily comprised of four species: red deer (*Cervus elaphus*), roe deer (*Capriolus capriolus*), fallow deer (*Cervus nippon*) and sika deer (*Dama dama*). Red and roe deer comprise the largest component, and their populations vary geographically, with red deer dominant in the Highlands of Scotland and roe deer more abundant in the lowlands (Scottish Natural Heritage, 2016a; Fig. S1). The red deer population in Scotland has gradually increased since the 1970s and currently constitutes the largest continuous population in Europe, numbering approximately 400,000 animals (Clutton-Brock et al., 2004; Edwards & Kenyon, 2013). Wild deer are also of increasing importance within the human food chain, as evidenced by increased UK sales of venison (from £32 million to £43 million between 2006 and 2009) and rising numbers of deer farms in Scotland (Scotland Food and Drink, 2018; Venison Advisory Service Ltd, 2016). Additionally, though rare, humans may become exposed to deer borne pathogens through faecal contamination of venison or wild grown foods (Laidler et al., 2013; Smith-Palmer et al., 2018). Hence, given their widespread distribution across Scotland, and their links with and exposure to human activity and livestock, wild deer are potentially an ideal candidate taxon to utilise for assessment of AMR bacteria within the Scottish environment.

Here, an assessment of the prevalence of AMR *Escherichia coli* within the Scottish wild deer population was made, utilising fresh deer faeces from culled animals. *E. coli* was targeted given (a) its ubiquitous nature in both the environment and the human and animal gut, (b) its common application as an indicator of faecal contamination (e.g., in human food/water), (c) its wide usage in previous and ongoing assessments of AMR, and (d) its importance as a pathogen in both animal and human disease; all of which make it a perfect target for AMR studies of this type (Bélanger et al., 2011; Health Protection Scotland, 2019; van den Bogaard & Stobberingh, 2000). This study sought to (1) establish a baseline for the prevalence of AMR *E. coli* within the faeces of wild deer within Scotland; (2) explore associations with wild deer host characteristics and environmental risk factors (i.e., variables or agents associated, either positively or negatively, with the carriage of AMR *E. coli*), including livestock presence, proximity to wastewater treatment facilities and health care facilities, land cover, etc.; and (3) investigate the suitability of these samples as source of *E. coli* for environmental AMR surveillance.

2. Materials and methods

2.1. Sample collection

Sampling took place from March 2017 to October 2018. Faecal samples were harvested from deer across Scotland, culled as part of routine population management and sampled as part of a national deer health survey (DHS). Faecal samples were gathered from the rectum, via the anus, into sterile plastic pots, then sent via post to the Moredun Research Institute (MRI; Penicuik) for processing and analysis. Each sample was accompanied by metadata detailing sex, age, species, body condition score (BCS) and the location of the cull (six-Fig. Ordinance Survey National Grid-reference). Non-selective bacterial enrichments for each sample were prepared by MRI staff, by adding 3–5 pellets to 5 ml of brain heart infusion (BHI) broth and incubating overnight at 37 °C, then storing the broth as 15% glycerol stocks at −80 °C. Enrichments were screened for the presence of Shiga toxin genes *stx1* and *stx2*, and the intimin gene, *eae*, using a multiplex PCR using primers from Bai et al. (2010). Only samples which tested negative for all three genes were then analysed for AMR *E. coli*.

2.2. AMR E. coli detection

A 10 μl loop of each inoculum was streaked for isolation onto MacConkey agar (Ph. Eur., USP, JP; VWR Chemicals) without any antimicrobial supplement (designated MAC), as a positive control for the presence of *E. coli* (including fully susceptible strains) in the sample. Detection of AMR in *E. coli* was conducted for four antimicrobial classes, representing one of the most commonly used compounds in human and veterinary medicine (tetracycline; Public Health England, 2019; UK-VARRS, 2018); World Health Organisation-designated highest priority critically important antimicrobials (ciprofloxacin and cefpodoxime as representatives of the quinolones and 3rd or higher generation cephalosporins, respectively; https://www.who.int/publications/i/item/9789241515528); and drugs of last resort (meropenem as representative of the carbapenems; Armstrong et al., 2021).

Antimicrobial supplemented breakpoint plates were prepared using MacConkey Agar with EUCAST (European Committee for Antimicrobial Susceptibility Testing) clinical breakpoint (CBP) concentrations for ciprofloxacin (CIP; 0.5 mg/L), cefpodoxime (CPO; 1 mg/L) and meropenem (MER; 8 mg/L), and the epidemiological cut-off (ECOFF) concentration of 8 mg/L for tetracycline (TET) (European Committee on Antimicrobial Susceptibility Testing, 2020a; 2020b). Breakpoint plates for MER were also prepared at a lower concentration of 0.125 mg/L, as recommended for use in the detection of carbapenemase resistance mechanisms (Giske et al., 2017).

For each sample, 100 μl of inoculum (prepared from a 10 μl loop of glycerol stock diluted into 10 ml of buffered peptone water) was spread onto each breakpoint plate, allowed to dry, then incubated aerobically at 37 °C for 20–24 h. Following incubation, plates were examined for the presence of bacterial colonies exhibiting strong lactose fermentation. Where observed, a single colony for each such morphotype was harvested, stored as a glycerol stock, and PCR amplification of the *uidA* gene was used to confirm colonies as *E. coli* (Section SM1 in Supplemental Materials). Inoculations of *E. coli* ATCC® 25,922 were similarly prepared from glycerol stock and used as negative controls (confirmed inhibition) on breakpoint plates and positive controls for PCR and live culture. From these data, faecal samples were recorded as being positive or negative for *E. coli* classed as resistant to CIP; resistant to CPO; resistant to MER; or non-wild type (NWT) for TET.

All samples were tested against these CBPs for CIP and CPO, and the ECOFF for TET. For MER, a first batch of samples (n = 194) were tested using the higher CBP of 8 mg/L. Following no evidence of resistance at this concentration, remaining samples (n = 131) were assessed using the lower concentration (of 0.125 mg/L), on the condition that any sample exhibiting *E. coli* growth at 0.125 mg/L was then re-tested at 8 mg/L, to

assess growth at the CBP.

2.3. Data management and risk factor analysis

To illustrate the spatial distribution of AMR, the sample area of mainland Scotland was subdivided into six regions using council boundaries as a basis—Highlands, East, West, Central, Central Belt and South. Additionally, 15 km buffer zones were drawn around each sample, then merged (and anonymised to prevent identification of individual samples) to allow a visual comparison of resistance between different sample clusters. The 15 km distance was chosen as it is the likely extreme of any single deer's territorial range (Kamler et al., 2008), and for purposes of anonymisation. Where possible, any relationships between the prevalence of resistance phenotypes across sample clusters was investigated using Spearman's correlation, with clusters weighted by sample numbers.

Fifty-six independent variables that were deemed to be potential risk factors for presence of AMR *E. coli* were initially selected for investigation, guided by existing literature and available data. These included host characteristics, farming and livestock factors, human population density, proximity to potential sources of antimicrobial resistance genes (ARGs), and land cover local to deer cull locations (see supplementary materials, Table S3, for full list and data sources). All data was mapped using QGIS v. 3.4 (QGIS Development Team, 2018). Proximity data for potential ARG point sources, such as wastewater treatment plants (WWTP) or hospitals, was calculated as the straight-line distance from the cull location. Land cover data was calculated as the area of land cover class within a 2 km, 3 km, and 5 km radius of the sample, rounded up to the nearest km^2. Livestock or human population density was calculated as the number of individuals present within a 2 km, 3 km, and 5 km radius of the sample. These distances were chosen given existing literature indicating that typical home ranges for roe deer were smaller than for red deer (2–3 km or less), while a 5 km range would likely only apply to male red deer (Borkowski et al., 2016; Lovari et al., 2017). Ranges up to 5 km were explored for all deer in an effort to account for possible direct or indirect (via the environment) interactions between deer in close proximity to each other, since these are potential routes for ARG or ARB (antimicrobial resistant bacteria) transfer. Results were mapped using QGIS and statistically analysed using R-Studio (v. 1.1.463; RStudio Inc, 2015) and R (v. 3.6.0; R Core Team, 2018) as detailed in the next section (a full list of the R packages employed are listed in supplementary materials Table S4).

Generalised linear mixed models (GLMMs) with a logit link function were built to explore factors associated with AMR, using resistance (yes/no) to one antimicrobial as the binomial response variable. Prior to inclusion in models, factors were assessed for normal distribution and outliers and, where appropriate, transformations were applied. Factors that were present in less than 10% of samples were dropped, being deemed too rare to accurately assess. Livestock density and land cover factors were converted into presence/absence where present in association with less than 25% of samples. Where applicable, data was transformed to a mean of zero and standard deviation of 0.5, in order to avoid scale errors and allow easier comparisons between models. Correlation among factors was then assessed using paired panel correlation plots. Selection between any two strongly correlated factors ($r \geq \pm 0.6$) was made based on Akaike Information Criterion values (AIC) of corresponding univariate models, retaining the factor that produced a model with a lower AIC. 'Region' was included in all models as a random effect.

The final dataset was explored using automated model selection, utilising the dredge function of the MuMIn package in R. Those factors that consistently lent weight to the model (from the AIC), along with factors that were identified as statistically significant through univariate analysis, and those that were determined to be of specific interest (guided by existing literature on AMR), were then used to assemble multivariate GLMMs using both step-up and top-down approaches. A significance level of $p < 0.05$ was used to determine significance, but factors were also retained where p was <0.1 if the AIC indicated a better fitting model.

Results from initial models revealed a highly significant effect of species. Therefore, species-specific models, for roe and red deer, were created to examine risk factors other than species. Due to low sample numbers, models for sika and fallow deer were not considered viable. Final models were assessed for the amount of observed variability they explained by examining McFadden's pseudo-r-squared values and comparing loglikelihood values with null models.

3. Results

3.1. Deer sample cohort

In total, 325 deer faecal samples (from 1075 gathered) were negative for Shiga toxin and intimin genes and were analysed for AMR *E. coli*. Within these 325 samples, the distribution of deer species differed between regions, with red deer dominating in the Highlands and the West and roe dear in the Lowlands, whilst fallow deer and sika deer were limited to only two or three regions each (Fig. S1). Deer ranged in age from 0.5 to 13 years old, and samples were significantly skewed towards younger deer, almost certainly as a result of the longevity of red deer compared to the other species (Shapiro-Wilk, $W = 0.863$, $p = <0.001$; Fig. S2). The median age (and interquartile range (IQR)) was 3 years (2 years), with 76.5% of samples from deer aged 4 years and under. Age differed significantly between species (Kruskal-Wallis, $\chi^2 = 49.543$, $df = 3$, $p < 0.001$), whereby red deer were older on average than other deer species, which was expected since red deer are longer lived (Minami et al., 2009; Mitchell, 1977; Müller et al., 2010). BCS ranged from very poor (1) to very good (5), with a median (IQR) of 4 (±1), with 33.0% of deer scoring grade 4 and 21.2% at grade 5. No obvious trends were clear for sika or fallow deer, but for red and roe deer, BCS of culled deer increased with age, with the exception of red deer with the lowest BCS.

3.2. AMR prevalence

Growth of presumptive *E. coli* on MAC plates was observed in 99.1% ($n = 322/325$) of samples. Eighty-seven samples (26.8%) harboured *E. coli* resistant to at least one of the four antimicrobials tested based on growth on breakpoint plates. Prevalence by antimicrobial was 6.5% ($n = 21$) for CPO, 0.3% for CIP ($n = 1$), and 21.8% ($n = 71$) for TET. No resistance to MER was recorded in any samples at either the CBP 8 m/L concentration ($n = 194$) or the 0.125 mg/L carbapenemase screening concentration ($n = 131$; Fig. 1). Only six samples yielded *E. coli* on more than one of the breakpoint plates: 5 samples on CPO and TET, and one sample on CIP and TET, which is only slightly higher than what could be expected by chance under the assumption of independence of the resistance phenotypes (expectation 4.4 and 0.2 cases for CPO + TET or CIP + TET, respectively).

The Highlands region yielded significantly fewer wild deer faecal samples harbouring TET resistant *E. coli* ($\chi^2 = 12.951$, $df = 5$, $p = 0.024$), while no significant difference was observed between regions for prevalence of CPO resistance ($\chi^2 = 3.757$, $df = 5$, $p = 0.578$; Fig. 2A and B). Within regions, clusters were identified based on overlap of maximum home ranges, with heterogeneity between clusters observed for TET but not CPO (Fig. 2C and D). For roe deer, prevalence of TET-resistant *E. coli* and CPO-resistant *E. coli* were positively correlated within clusters when weighted by sample number ($r^2 = 0.94$), but the correlation was negative for red deer ($r^2 = -0.54$), or when considering all deer ($r^2 = -0.42$; Fig. S3).

3.3. Risk factor modelling

GLMMs used to explore associations between potential risk factors and the presence of TET or CPO resistant *E. coli* in wild deer faeces are

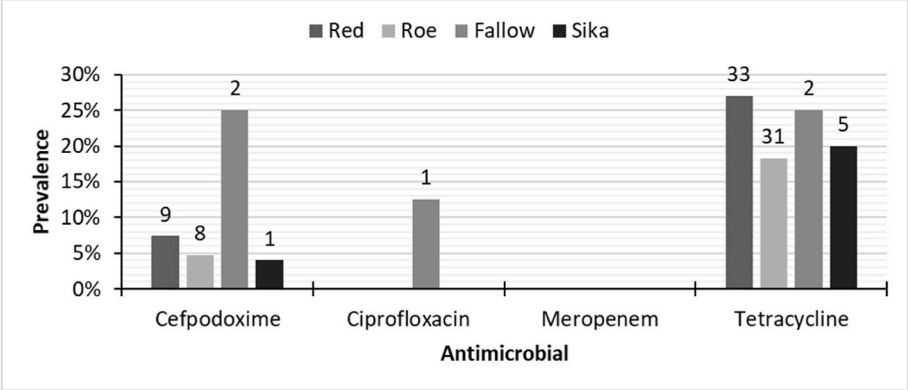

Fig. 1. Prevalence of antimicrobial resistant *Escherichia coli* harboured in faeces from Scottish wild deer species, as tested using breakpoint plate methods utilising clinical breakpoint concentrations for cefpodoxime, ciprofloxacin and meropenem, and an epidemiological cut-off concentration for tetracycline. Data labels indicate the number of positive samples in each category (total n values were red = 122, roe = 170, fallow = 8, sika = 25, and one unknown (not shown); total = 325).

detailed in Table 1. Models for the entire sample set were less able to explain variation than those which included only roe or red deer (Table S5). Proximity of broadleaved woodlands was associated with increased likelihood of detecting TET resistant *E. coli* in faeces from red deer and roe deer (Fig. 3A and B), but not with CPO resistant *E. coli.* Proximity of coniferous woodland was associated with decreased likelihood of detecting CPO resistant *E. coli* across all species, but the effect was not significant for individual species. Increasing sheep density was associated with increased likelihood of detecting TET-resistant *E. coli* across all deer and in roe deer (Fig. 3B), but not in red deer. Detection of TET-resistant *E. coli* in red deer faeces was more likely than average in deer on mountains, heath and bog, and less likely in deer near farmed geese (Fig. 3A). In roe deer, horses were associated with decreased likelihood of detection of TET-resistant *E. coli* (based on presence/absence of horses) but an increased likelihood of CPO-resistant *E. coli* (based on horse density; Fig. 3C). Deer age was also positively associated with CPO resistant *E. coli* in roe deer faeces (Fig. 3C), but none of the factors examined were significantly associated with this phenotype in red deer faecal *E. coli.*

4. Discussion

4.1. AMR in Scottish wild deer

The WHO defines 3rd generation cephalosporins and fluoroquinolones as highest-priority critical antimicrobials, while MER is a critically important antimicrobial, considered to be the most reliable last-resort treatment for many bacterial infections (Meletis, 2015; World Health Organization, 2018). Therefore, the low levels of resistance to CPO and CIP and the lack of any observed resistance to MER, in the *E. coli* examined from the Scottish wild deer population, is encouraging. Only a single sample (0.3%), was found to harbour *E. coli* resistant to CIP. No samples harboured *E. coli* resistant to MER, at either the CBP or carbapenem resistance mechanism screening concentrations, indicating that resistance to carbapenems is not an issue at present in the Scottish wild deer population. This is encouraging given their importance as 'treatments of last resort' (Nicolau, 2008).

The deer samples used in this study should be viewed as representative of the typical culling practices currently carried out across Scotland, in areas where deer management regularly occurs (~57% of Scotland; Scottish Natural Heritage, 2016b). As these deer are associated with human activity, and often enter directly into the human food chain (as venison), they are arguably those most relevant for a study of AMR from a human health perspective. Additionally, only deer faecal samples negative for Shiga toxin and intimin genes were analysed in this work. Therefore, a similar study using/comparing samples positive for these genes would be desirable before the results here can be confidently applied to the wider deer population. However, some evidence exists to indicate that EHEC/EPEC gene prevalence is not associated with AMR prevalence (Assumpção et al., 2015).

E. coli resistance to CPO, a 3rd generation cephalosporin, was found in 6.5% (21 of 325) of samples. Although prevalence varied markedly between regions there was no statistically significant variation across the sample set. Cephalosporins are broad-spectrum antimicrobials important in both human and veterinary medicine. Resistance to cephalosporins has been detected in *E. coli* from wild deer populations in other countries, though detection methodology and occurrence vary across studies (Table 2). For example, no resistance to 3rd generation cephalosporins was detected amongst *E. coli* in wild deer faeces in Norway (n = 150, Lillehaug et al., 2005) or Slovakia (n = 52, Literak et al., 2010), while in Mexico, resistance to drugs from this family was recorded in 9.1% of samples (n = 22, Carrillo-Del Valle et al., 2016b). Carrillo-Del Valle et al. (2016a, 2016b) also recorded, in the same study, resistance to cefepime (a 4th generation cephalosporin) at 4.5% (n = 22), in wild red deer. In Portugal, no resistance to 2nd generation cephalosporins was detected from wild red deer (n = 46), while prevalence of resistance (1.1%) was detected for cefoxitin, a 1st generation cephalosporin (Dias et al., 2015). In farmed red deer in Spain, no resistance to 2nd or 3rd generation cephalosporins was detected (n = 122; Alonso et al., 2016). In summary, investigators in Mexico and Portugal — and here Scotland — found cephalosporin resistant *E. coli* in wild deer faeces, while Norway, Slovakia and Spain did not. However, without standardisation of compounds tested or methods used, it is difficult to discern why these differences may occur.

In the UK, the use of CPO is only authorised in human medicine, suggesting that veterinary application is unlikely to be a factor in the resistance observed here. However, the development of resistance to one cephalosporin can potentially impart resistance to others via the same mechanism (Pfeifer et al., 2010). For example, Markland et al. (2019), using a similar breakpoint plate method to the one used here, found an average of 47% prevalence of cefotaxime (another 3rd generation cephalosporin) resistant bacteria (CRB) in the faeces of Florida beef cattle – none of which were prescribed cephalosporin antimicrobials. Also, these authors found that CRB prevalence was higher in environmental samples than in cattle, concluding that the environment (soil, forage and drinking troughs) were the likely source of CRBs. Interestingly, in light of our findings that the 'mountain, heath and bog' land cover is associated with TET AMR, Markland et al. (2019) also reported that "wet and swampy" land was associated with an increased likelihood of CRB detection in faecal samples. Fallow deer seemed more likely to harbour CPO resistant *E. coli* here, but this could well be an artefact of the very low sample numbers tested (n = 8 of 325; Fig. 1). In roe deer, older deer were significantly more likely to harbour CPO resistant *E. coli* than younger deer (Fig. 3C). While this could be a result of age-related

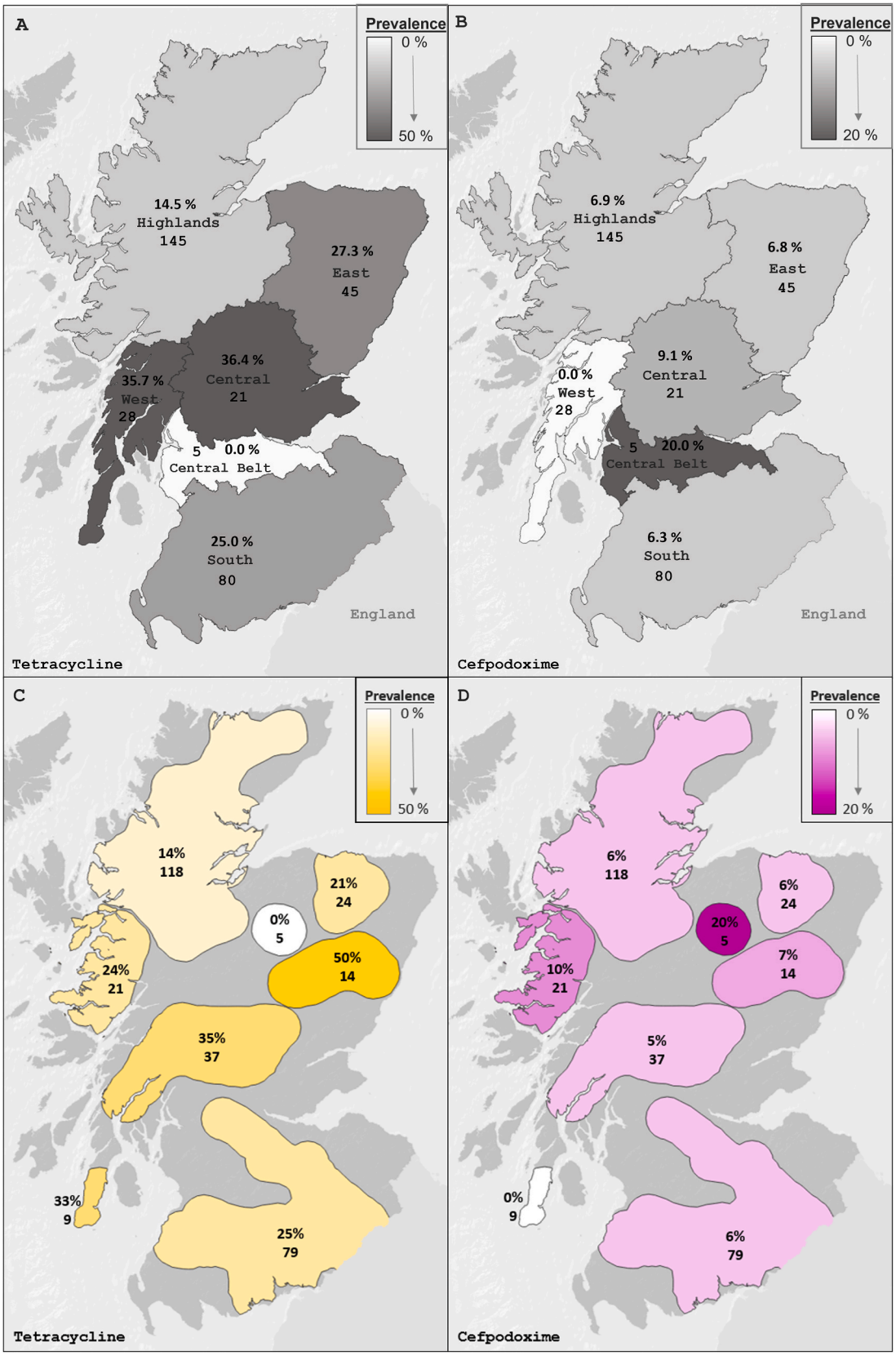

Fig. 2. Prevalence of tetracycline or cefpodoxime resistant *Escherichia coli* in wild deer faeces by region (A and B), and by sample clusters (C and D). Clusters are based on likely extreme range limits (15 km) for individual deer and do not imply distinct populations. Number of samples per region/cluster is shown. Shading indicates prevalence of resistant *E. coli* within the range recorded; note the different scales for prevalence range between the two antimicrobials.

Table 1
Generalised linear mixed models describing the association between host characteristics, livestock, land cover and proximity to potential sources of antimicrobial resistance genes upon the likelihood of *E. coli* in wild deer faeces harbouring resistance to tetracycline (TET) or cefpodoxime (CPO) (for land cover factors, P = Present, A = Absent; WWTW = wastewater treatment works).

Model	Factor	Estimate	Std. error	z-value	p-value
Model 1	Intercept	−2.1292	0.285	−7.471	<0.001***
Tetracycline	Species – Fallow	−0.1944	0.882	−0.220	0.826
All Deer	Species – Red	1.2895	0.456	2.893	0.004**
	Species – Sika	0.2046	0.757	0.270	0.787
	Sheep Density within 2 km	1.136	0.603	1.885	0.059
	Broadleaved woodland (P) within 2 km	1.061	0.379	2.802	0.005**
Model 2	Intercept	−2.8436	0.36473	−7.796	<0.001***
Cefpodoxime	Species – Fallow	1.82278	0.90473	2.015	0.044*
All Deer	Species – Red	0.05377	0.57697	0.093	0.9257
	Species – Sika	−0.32822	1.09070	−0.301	0.7635
	Coniferous woodland area within 3 km	−1.15846	0.61423	−1.886	0.059
Model 3	Intercept	−3.0838	0.9935	−3.104	<0.001***
Tetracycline	Geese (P) within 2 km	−1.0618	0.5241	−2.026	0.043*
Red Deer	Distance from WWTW (medium)[1]	−1.2180	0.6548	−1.860	0.063
	Mountain, heath and bog (P) within 2 km	2.8049	1.0480	2.676	0.007*
	Broadleaved woodland (P) within 2 km	1.9938	0.7786	2.561	0.010**
Model 5	Intercept	0.695	0.794	0.875	<0.381
Tetracycline	Distance to urban area	−0.957	0.574	−1.666	0.096
Roe Deer	Deer age	−0.887	0.486	−1.826	0.068
	Sheep density within 3 km	1.671	0.475	3.518	<0.001***
	Horses (P) within 2 km	−3.196	0.956	−3.337	0.001**
	Broadleaved woodland (P) within 2 km	2.019	0.588	3.434	<0.001***
Model 6	Intercept	−3.290	0.620	−5.306	<0.001***
Cefpodoxime	Deer age	2.470	0.909	2.719	0.007**
Roe Deer	Dairy cattle (P) within 2 km	−1.592	1.004	−1.585	0.113
	Horse density within 3 km	3.111	1.355	2.296	0.022*

* - p = 0.05; ** - p = 0.010; *** - p = 0.001.
[1] - As distance from WWTW increases, the likelihood of a sample testing positive for TET resistant *E. coli* decreases.

accumulation (of resistant bacteria, antimicrobial residues or other compounds that promote CPO resistance), the same affect is not seen with TET resistance, nor is any age-related increase observed in red deer. This might indicate that, compared to red deer, specific risk factors associated with CPO resistance are more frequently encountered by roe deer than those associated with TET resistance, which would go towards explaining the differences in correlations (Fig. S3). In roe deer, prevalence of CPO resistance in faecal *E. coli*, was significantly and positively associated with presence of horses. This could be an indication of increased human activity in association with horses. Horses can be classed as companion or leisure animals and as such can be stabled and taken out in areas associated with leisure activities, e.g., woodlands, thus exposing them to or making them into very different risk factors than livestock animals. Interestingly, the opposite effect (i.e., reduced prevalence in association with horse density) was observed for TET resistance.

Resistance to TET was the most prevalent phenotype identified here, found in 21.8% (n = 71/325) of samples. The historic and ongoing widespread use of TET, in both human and veterinary medicine, is a likely cause of the comparatively higher prevalence of TET resistance overall (as observed in other wild deer AMR studies; Table 2). Of the four antimicrobials assessed, only TET is authorised for veterinary use. In livestock, TET is authorised for use in cattle, pigs and chickens, but not in sheep. However, oxytetracycline, a variant of TET, is authorised for use in sheep, and resistance to TET is widely used to indicate resistance to oxytetracycline, and vice versa (UK Veterinary Antibiotic Resistance and Sales Surveillance report (UK-VARSS), 2019). Further, high levels of TET resistance in faecal *E. coli* have been recorded in modern pigs even before administration of any antimicrobial treatment (48%, n = 403, Burow et al., 2019), suggesting that a higher prevalence of TET resistance may now be commonplace (as a natural 'background' level) in some *E. coli* populations. The prevalence of TET resistance recorded here in Scottish deer is higher than that reported in other wild deer studies (Alonso et al., 2016; Bryan et al., 2004; Carrillo-Del Valle et al., 2016b; Cenci Goga et al., 2009; Dias et al., 2015; Lillehaug et al., 2005; Literak et al., 2010; Smith et al., 2014). However, ours is the only study to date to use sample-based rather than isolate-based prevalence estimation, leading to significantly higher prevalence estimates (Humphry et al., 2018) and precluding direct comparison. The breakpoint plate method used in this study permitted detection of the presence of AMR *E. coli* in a matrix (faeces), even if resistant isolates constituted a minority of the total *E. coli* population in the sample. This is a much more sensitive, but a less specific method, than that utilised by the vast majority of studies on AMR in wildlife and livestock, where isolate (rather than whole sample) based assessments are the norm. For example, Humphry et al. (2018) showed that only 16% of faecal samples that tested positive for ampicillin resistant *E. coli* using a breakpoint plate method were likely to also test positive if using a single isolate approach.

The presence of broadleaved woodland (within 2 km of the deer cull site) was consistently and significantly positively associated with an increased likelihood of a sample harbouring TET resistant *E. coli*. There is some evidence to suggest that other wildlife associated with this habitat are (potentially for reasons unrelated to wild deer) more likely to harbour AMR bacteria (Furness et al., 2017; Gilliver et al., 1999). For example, rooks (*Corvus frugilegus*), commonly nest in broadleaved trees, are known to forage in both agricultural and urban areas, and have been shown to harbour AMR bacteria, including *E. coli* (Brenchley, 1986; Literák et al., 2007; Oravcova et al., 2013). In England, wild rodents from mixed woodlands have also been repeatedly shown to harbour AMR *E. coli* (Gilliver et al., 1999; Williams et al., 2011). In addition, broadleaved woodlands can commonly be associated with companion animal access and human leisure activities (Martin, 2007), thus potentially increasing the occurrence of antimicrobial residues, ARGs or AMR bacteria in these habitats (through human and companion animal presence, activity and waste (Scott Weese, 2008);).

In red deer, TET resistant *E. coli* prevalence was also significantly positively associated with the presence of 'mountain, heath and bog' habitat (Table 1, Model 3). This is the most abundant land cover type

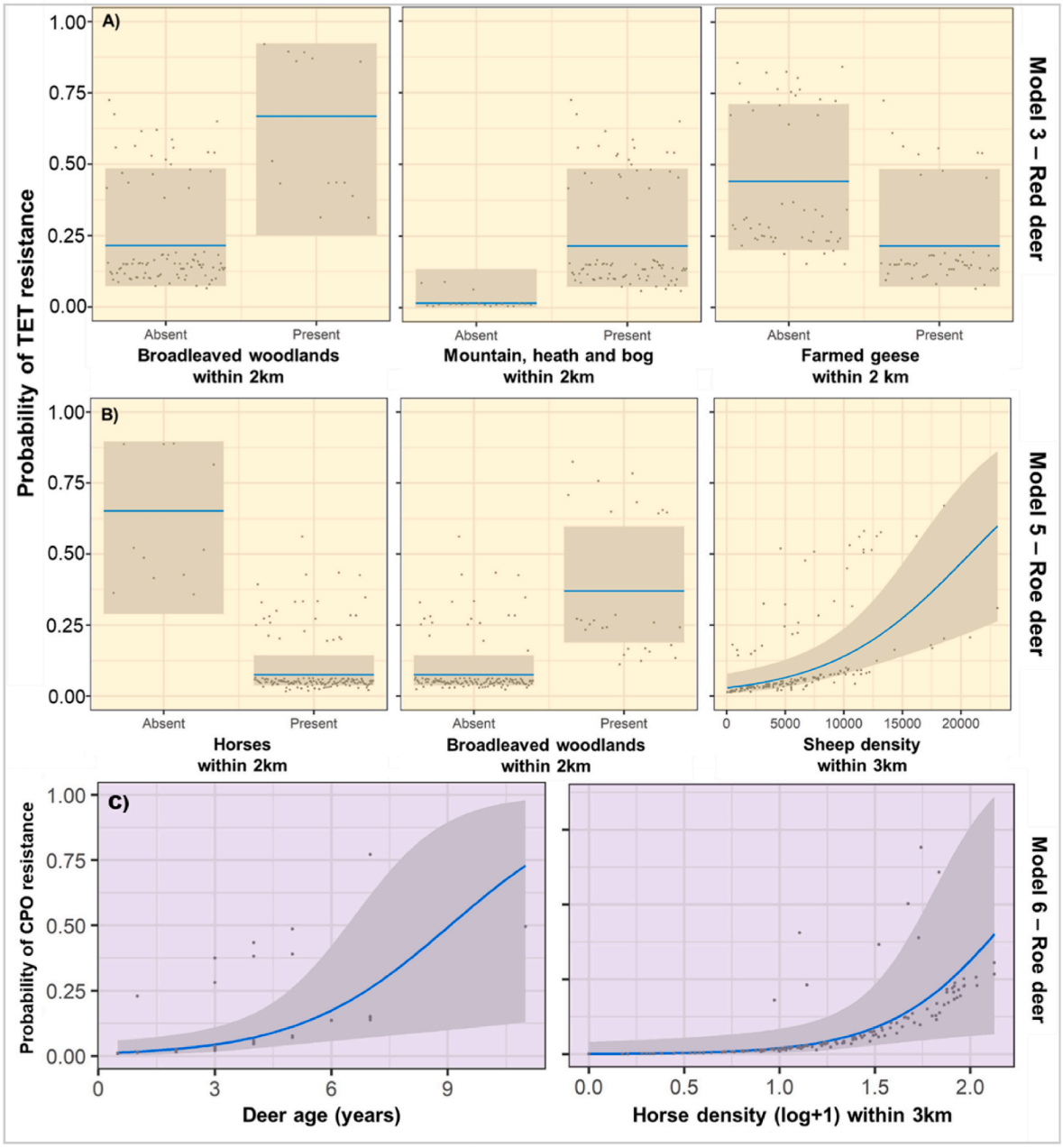

Fig. 3. *Significant associations between independent variables and prevalence of tetracycline (TET) resistant Escherichia coli in faeces of red deer (A) or roe deer (B) and prevalence of cefpodoxime (CPO) resistant E. coli in faeces of roe deer (C). No significant associations were identified for CPO resistant E. coli in red deer.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

across Scotland (34% cover) and dominates the Highland region (62% of the region), however, this classification covers a wide range of different habitat types. Some of these habitats may promote or support AMR phenotypes locally, either through dietary impact on the deer gut microbiome, or, as a result of distinct soil and water chemistry in the environment. For example, this classification includes extensive peatlands, a habitat type that has recently been shown capable of harbouring novel ARGs even in pristine conditions (Obermeier et al., 2019). Some wetlands in this category might also be vulnerable to contamination from rivers (e.g., in floodplains; Henriot et al., 2019), and of course, wetlands are important habitats for both resident and migratory birds. However, given the resolution of the available data, these explanations are purely speculative.

Transmission of bacteria between wild deer and livestock is most likely to occur through faecal contamination of shared foraging areas or via vectors such as invertebrates, e.g., ticks (Böhm et al., 2007). Sheep are the most prolific livestock in Scotland, present across the near entirety of the sample area (Fig. S4) and all 325 samples analysed came from areas where sheep density was >1 animal within 2 km of the cull site. Roe deer samples came predominantly from areas where sheep density was comparatively higher, and sheep density was significantly positively associated with TET resistant *E. coli* in wild roe deer faeces (Fig. 3B). In the UK, roe deer have been strongly implicated as a reservoir for the bacterial pathogen which causes tick-borne fever in sheep (Alberdi et al., 2000), which shows evidence of existing links between these two species. However, sheep density alone would dictate that the prevalence of TET resistance should be highest in the south Scotland region, where sheep density is highest, and this is not the case. The south region does however possess the lowest density of broadleaved woodland cover, which, given the observed positive association with this land

Table 2
Results from studies investigating AMR in faecal *E. coli* from wild deer across the world. All papers listed used an isolate based approach to determine AMR. Results presented in this work (which used a breakpoint plate assessment) are included for comparison (shaded cells) (CBP = clinical breakpoint).

Country	Host	Class	Antimicrobial	n	R%	Method	Source
Mexico	Red Deer	1st Gen. Cephalosporins	Cefazolin	22	4.5%	CLSI	Carrillo-Del Valle et al., 2016a, 2016b
Slovakia	Red Deer		Cephalothin	53	0.0%	CLSI	Literak et al. (2010)
Spain	Red Deer (Farmed)	2nd Gen. Cephalosporins	Cefoxitin	122	0.0%	CLSI	Alonso et al. (2016)
Portugal	Red Deer		Cefoxitin	46	1.1%	CLSI	Dias et al. (2015)
Spain	Red Deer (Farmed)	3rd Gen. Cephalosporins	Cefotaxime	122	0.0%	CLSI	Alonso et al. (2016)
Spain	Red Deer (Farmed)		Ceftazidime	122	0.0%	CLSI	Alonso et al. (2016)
Mexico	Red Deer		Ceftazidime	22	9.1%	CLSI	Carrillo-Del Valle et al., 2016a, 2016b
Mexico	Red Deer		Ceftriaxone	22	4.5%	CLSI	Carrillo-Del Valle et al., 2016a, 2016b
Portugal	Red Deer		Cefotaxime	46	0.0%	CLSI	Dias et al. (2015)
Portugal	Red Deer		Ceftazidime	46	0.0%	CLSI	Dias et al. (2015)
Norway	Wild Deer		Ceftiofur	150	0.0%	VetMIC	Lillehaug et al. (2005)
Slovakia	Red Deer		Ceftazidime	53	0.0%	CLSI	Literak et al. (2010)
Scotland	Wild Deer		Cefpodoxime	325	6.5%	CBP Agar	This study
Mexico	Red Deer	4th Gen. Cephalosporins	Cefepime	22	4.5%	CLSI	Carrillo-Del Valle et al., 2016a, 2016b
Spain	Red Deer (Farmed)	Carbapenems	Imipenem	122	0.0%	CLSI	Alonso et al. (2016)
Scotland	Wild Deer		Meropenem	325	0.0%	CBP Agar	This study
Spain	Red Deer (Farmed)	Fluoroquinolones	Ciprofloxacin	122	1.3%	CLSI	Alonso et al. (2016)
Ireland	Red/Sika Hybrid		Ciprofloxacin	30	0.0%	EUCAST	Carroll et al. (2015)
Norway	Wild Deer		Enrofloxacin	150	0.0%	VetMIC	Lillehaug et al. (2005)
Slovakia	Red Deer		Ciprofloxacin	53	0.0%	CLSI	Literak et al. (2010)
Ireland	Red/Sika Hybrid		Ciprofloxacin	30	0.0%	CLSI	Smith et al. (2014)
Scotland	Wild Deer		Ciprofloxacin	325	0.3%	CBP Agar	This study
Spain	Red Deer	Tetracyclines	Tetracycline	122	5.2%	CLSI	Alonso et al. (2016)
USA (MN)	Wild Deer		Tetracycline	74	4.0%	MIC	Bryan et al. (2004)
Ireland	Red/Sika Hybrid		Tetracycline	30	6.7%	EUCAST	Carroll et al. (2015)
Italy	Red Deer		Tetracycline	39	12.8%	CLSI	Cenci Goga et al., 2009
Portugal	Red Deer		Tetracycline	46	7.8%	CLSI	Dias et al. (2015)
Norway	Wild Deer		Oxytetracycline	150	3.3%	VetMIC	Lillehaug et al. (2005)
Slovakia	Red Deer		Tetracycline	53	7.5%	CLSI	Literak et al. (2010)
Ireland	Red/Sika Hybrid		Tetracycline	30	3.3%	CLSI	Smith et al. (2014)
Scotland	Wild Deer		Tetracycline	325	21.8%	CBP Agar	This study

type (with TET resistance), may partly explain this variation. For comparison, the Highlands region, with the lowest prevalence of TET resistance, possesses both the lowest overall sheep density and the second lowest broadleaved woodland density of all regions.

In contrast to the positive association identified with sheep, the presence of farmed geese (with red deer) or horses (with roe deer) was negatively associated with TET resistant faecal *E. coli*. While usage of tetracyclines is much lower in horses than in food producing animals (UK-VARSS, 2019), which could in part account for the lack of a positive association in this case, the result here is based on rather limited data (Fig. 3B), and so should be treated with caution. Without specific data on prescribing or management practices for geese, it is also difficult to comment on how geese may affect antimicrobial loads in the local environment. Importantly, horse and geese are present in the landscape at orders of magnitude below that of sheep, i.e., average animal density across the sample area is 89 animals per 1 km^2 for sheep, but only 0.6 and 0.08 for horses and geese, respectively. While this could suggest a greater effect, it also warrants significant caution when seeking to interpret the effect of such a comparatively small number of animals.

4.2. Other risk factors of interest

Amongst the potential risk factors examined, it is interesting to note some did not appear to be associated with AMR prevalence. For example, while modelling did indicate that a closer proximity to WWTWs was associated with an increased probability of finding TET resistance, the statistical significance of this finding ($p = 0.063$) depends on the chosen cut-off value for significance, and hence on the balance between risk of type I error and risk of type II error. The WWTW sites referred to here are those with a processing capacity of up to 10,000 population equivalents (PE) and are thus those most associated with larger towns/settlements. As such these sites also act as a proxy for a likely increase in other anthropogenic factors, e.g., other forms of pollution. While there is extensive evidence indicating that WWTWs are viable sources of ARGs (Huijbers et al., 2015; Harris et al., 2014), such installations typically discharge into nearby rivers and streams or (commonly in Scotland) directly into the sea. As such, their potential impacts on any AMR signal are unlikely to be a simple function of proximity, but rather, more broadly related to watercourses and receiving catchments. Nevertheless, antimicrobial residues, ARGs, or AMR bacteria may be transferred directly to the environment by birds (and other animals) foraging around and within WWTWs and in water in proximity to discharges and human habitation (Marcelino et al., 2019; Nelson et al., 2008).

Proximity to arable land was not found to be significant risk factors for AMR in this study. It has been suggested that arable land may be linked with a higher prevalence of AMR through certain agricultural practices, such as the application of antimicrobials against plant pathogenic bacteria, or, the spreading of animal manure as fertiliser (Cytryn, 2013). In Scotland, and other parts of the world, applications of sewage sludge or 'biosolids' (a by-product of human wastewater treatment) to arable land is widely carried out (which we were unable to account for in this study due to a lack of open-source geographical data on this activity). Both treatments may increase or introduce antimicrobial residues, AMR bacteria or ARGs into the environment (Cytryn, 2013; Graham et al., 2019; Munir & Xagoraraki, 2011), though this is not always the case (Brooks et al., 2007). Arable land is also a recognised important foraging ground for birds (Robinson et al., 2002), and links between birds and AMR bacteria are well documented (Greig et al., 2015).

Finally, human population density and urban land cover was not significantly associated with AMR. However, as with land cover, these categories cannot be used to infer specific activities or land use at sites. Links between anthropogenic activity and AMR are well documented, but the results here underline that specific activities, rather than simply the presence of a human population, are likely more broadly responsible. Human population density and settlements were included in this study under the inference that such areas might serve as a proxy for AMR associated activities. However, these factors alone clearly do not

represent a suitable proxy for the diverse range of human activities that could be associated with environmental AMR. There is a wealth of evidence showing that health care application of antimicrobials and wastewater treatment — both linked directly with human settlements — are known activities that can concentrate or drive AMR in the environment (Perry et al., 2019b; Pruden et al., 2012). However, there are many other anthropogenic activities, not directly linked to human population density or urban land cover, that may be responsible risk factors relevant to environmental AMR. For example, mine water effluents have been shown to harbour AMR bacteria (Mathiyazhagan, 2011), and such sites are not typically in direct proximity to population centres. It should also be noted that Harris et al. (2014) found that hospital effluents were not the primary factor influencing AMR within receiving WWTWs, suggesting there are other factors within such populated areas that are still to be understood.

5. Conclusions

This work provides a baseline for future investigation of AMR bacteria in the of Scottish wild deer population and in the wider Scottish environment. Of the four antimicrobial compounds tested, only resistance to MER, a critically important antimicrobial 'of last resort', was not detected. Resistance to TET was identified as the most prevalent phenotype, a finding in line with existing literature on AMR in wild deer. However, the detection of *E. coli* resistant to other critically important antimicrobials (CIP and CPO) indicate the potential for wild deer to act as widespread biomonitoring species for these potentially concerning phenotypes in the wider environment. Models built to broadly investigate potential risk factors associated with the presence of TET and CPO identified factors associated with human leisure activities, companion animals, and other wildlife, however, there was considerable variation in associated factors between AMR phenotypes, and between deer species within a phenotype. Therefore, future biomonitoring efforts for AMR should look to take specific host species ecology and behaviours into account, and may wish to incorporate environmental samples for context/comparison, or tailor the range of antimicrobial compounds tested to a specific goal, e.g., AMR within cephalosporins only.

Finally, a primary aim of this study was to assess the use of deer faeces as a Scotland wide AMR biomonitoring tool. The work carried out demonstrates the successful detection and investigation of the prevalence of AMR bacteria across Scotland amongst samples which are routinely available within the existing deer management framework.

CRediT author statement

Derek Elsby: Conceptualisation, Data Curation, Formal Analysis, Investigation, Methodology, Project administration, Visualisation, Writing (original draft), Writing (reviewing and editing); **Ruth Zadoks**: Conceptualisation, Funding acquisition, Supervision, Writing (original draft), Writing (reviewing and editing); **Kenneth Boyd**: Supervision, Writing (reviewing and editing); **Nuno Silva**: Investigation, Writing (reviewing and editing), **Margo Chase-Topping**: Formal Analysis, Methodology (model development), Writing (reviewing and editing), **Mairi Mitchell**: Investigation, Writing (reviewing and editing); **Carol Currie**: Investigation, Writing (reviewing and editing); **Mark Taggart**: Conceptualisation, Funding acquisition, Project administration, Supervision, Writing (original draft), Writing (reviewing and editing).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120129.

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