

Anthropogenic Environmental Drivers of Antimicrobial Resistance in Wildlife

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20 **Abstract**

21 The isolation of antimicrobial resistant bacteria (ARB) from wildlife living adjacent to humans has led
22 to the suggestion that such antimicrobial resistance (AMR) is anthropogenically driven by exposure to
23 antimicrobials and ARB. However, ARB have also been detected in wildlife living in areas without
24 interaction with humans. Here, we investigated patterns of resistance in *Escherichia coli* isolated from
25 408 wild bird and mammal faecal samples. AMR and multi-drug resistance (MDR) prevalence in
26 wildlife samples differed significantly between a Sewage Treatment Plant (STP; wastes of antibiotic-
27 treated humans) and a Farm site (antibiotic-treated livestock wastes) and Central site (no sources of
28 wastes containing anthropogenic AMR or antimicrobials), but patterns of resistance also varied
29 significantly over time and between mammals and birds. Over 30% of AMR isolates were resistant to
30 colistin, a last-resort antibiotic, but resistance was not due to the *mcr-1* gene. ESBL and AmpC activity
31 were common in isolates from mammals. Wildlife were, therefore, harbouring resistance of clinical
32 relevance. AMR *E. coli*, including MDR, were found in diverse wildlife species, and the patterns and
33 prevalence of resistance were not consistently associated with site and therefore different exposure
34 risks. We conclude that AMR in commensal bacteria of wildlife is not driven simply by anthropogenic
35 factors, and, in practical terms, this may limit the utility of wildlife as sentinels of spatial variation in
36 the transmission of environmental AMR.

37

38 *Key words:* *E. coli*, Antimicrobial resistance, wildlife, birds, multi-drug resistance, wastewater
39 treatment

40 *Running head:* Wildlife and AMR

41

42 1. Introduction

43 Antimicrobial resistance (AMR) has existed for millions of years, and is an inevitable
44 evolutionary consequence of microbial competition in the environment (D'Costa et al 2011, Davies
45 and Davies 2010, Martinez 2009). While the increasing prevalence of AMR in clinically important and
46 commensal bacteria in both humans and livestock can be attributed largely to selection through the
47 use of antimicrobials (Ibrahim et al 2016, Karesh et al 2012), AMR has also been reported in the
48 commensal bacteria of wildlife (Arnold et al 2016). Commensal bacteria have the potential to act as
49 reservoirs of resistance genes, contributing to the development of AMR in pathogens by horizontal
50 transmission (Arnold et al 2016, Taylor et al 2011, von Wintersdorff et al 2016). AMR is a problem in
51 human and veterinary medicine worldwide, inhibiting the treatment of bacterial infections and is
52 estimated to be responsible for 25,000 preventable human deaths in Europe annually (Marston et al
53 2016) and an estimated global economic cost of 100 trillion USD by 2050 if not addressed (O'Neill
54 2016). Thus, there is increasing interest in the environment, including wildlife, as both a source of
55 clinically relevant AMR and in order to better understand the effects of anthropogenically-derived
56 antimicrobial pollution and resistance in ecosystems (Arnold et al 2016, Carroll et al 2015, Huijbers et
57 al 2015).

58 It is often assumed that antimicrobial-resistant bacteria (ARB) in wildlife result from contact
59 with anthropogenic sources such as farms and human waste that pollute the environment with AMR
60 bacteria and/or with antimicrobials (Allen et al 2010, Clarke and Smith 2011, Radhouani et al 2011).
61 Farms on which manure and slurry can be contaminated with ARB, antibiotics (or their metabolites)
62 and other selective drivers of AMR are important habitats for many small mammals and birds, as are
63 sewage treatment plants (STPs) where some birds and mammals feed directly from the bioprocessers
64 (reviewed in Arnold et al 2016). Run-off from farms, slurry tanks and manure-fertilised fields, along
65 with sewage effluent, can result in antimicrobial drug and ARB contamination of local water courses
66 and land (Fahrenfeld et al 2013). Consequently, it is unsurprising that ARB have been found in wild

67 animals in close contact with humans (Allen et al 2011, Bondo et al 2016, Furness et al 2017, Gilliver
68 et al 1999).

69 Assigning the source and directionality of AMR dissemination is challenging. Even within
70 wildlife populations living in close contact with humans or livestock, or at least their wastes, there is
71 little evidence directly linking an anthropogenic source of AMR with specific patterns of AMR and/or
72 resistance genes. For example, few overlaps in resistance patterns and AMR genes were found
73 between *E. coli* isolated from wildlife living on or near dairy farms and dairy cattle in England (Arnold
74 et al 2016, Wu et al 2018). Whereas wild rodents nearer to a river receiving sewage effluent excreted
75 more resistant *E. coli* than inland animals (Furness et al 2017), this was an association lacking evidence
76 of a clear transmission pathway. Moreover, other highly mobile taxa such as birds also carry ARB that
77 have not been attributed to any particular anthropogenic source (Guenther et al 2017, Schaufler et al
78 2016). Moreover, AMR has been detected in wildlife living in remote and isolated locations with no
79 obvious contact with the wastes of antimicrobial-treated humans or livestock (Cristobal-Azkarate et
80 al 2014). Thus, although transmission of AMR from humans or livestock to wildlife via direct contact
81 with sewage, slurry or faeces, has been suggested, the empirical evidence is lacking or contradictory.
82 Species or ecological guilds with different dispersal patterns, resource requirements and foraging
83 behaviours are likely to have different roles in the evolution and dispersal of AMR (Arnold et al 2016).
84 We argue that the efficacy of wildlife species as sentinels of environmental transmission of AMR will
85 vary depending on the spatial and temporal scales of interest.

86 In this study, three nearby communities of small wild rodents and birds were investigated for
87 evidence of AMR in faeces. The antimicrobials used to screen for resistance were chosen as they
88 represent a range of antibiotic classes of medical and veterinary interest. For example, cefpodoxime
89 resistance is seen as an indicator of extended spectrum beta-lactamase (ESBL) or AmpC beta-
90 lactamase producing bacteria which cause significant problems in human medicine especially with
91 urinary tract infections (Rawat and Nair 2010). Colistin resistance is also of relevance due to colistin
92 being an antibiotic of last resort. The sites for sampling were chosen to represent different exposures

93 to wastes and thus potentially different selection pressures for AMR: a dairy farm with antimicrobial-
94 treated livestock, a STP containing waste from humans treated by antimicrobials and an area of
95 parkland and neighbouring arable field edge with no obvious sources of waste containing
96 antimicrobials or ARB. We sampled wildlife species typical for small woodlands, farmland and
97 hedgerow habitats in the UK; small rodents including wood mice *Apodemus sylvaticus*, bank voles
98 *Myodes glareolus* and a number of bird species.

99 The overall aim of this study was to investigate the role of environmental contamination in
100 the patterns of AMR found in wildlife. We addressed whether the spatial location where wild birds
101 and mammals were sampled, including proximity to human and livestock wastes, explained variation
102 in: 1) prevalence and genomic diversity of AMR *E. coli* in birds and mammals; 2) patterns of AMR and
103 MDR prevalence in *E. coli* isolates; and 3) prevalence of phenotypic resistance to medically important
104 antimicrobials and the resistance genes responsible.

105

106 2. Material and Methods

107 2.1 Study sites

108 Three nearby study sites in the East Midlands of England, on a 1200m transect, were selected
109 (Figure S1), based on their differing potential exposure to human and livestock sources of AMR and
110 antimicrobial drugs. The 'Farm site' was a small woodland and hedgerows immediately adjacent to a
111 dairy farm that received run-off from farm buildings and livestock faeces potentially contaminated
112 with AMR bacteria and antimicrobials. The 'Central site', around 600m from the Farm site, comprised
113 an arboretum and neighbouring hedgerow edging an arable field. It was not adjacent to known
114 sources of human or livestock waste. The 'STP site' was a small sewage treatment plant around 450-
115 600m from the Central-site, comprising the land and hedgerows surrounding all the tanks and trickling
116 filters making up the STP and hedgerows adjacent to the pipe where treated water outflowed into a
117 local stream. All the sites were close enough to share common environmental traits and weather.

118 Conversely, the three sites were far enough apart, with physical barriers to dispersal (roads and a
119 railway line), such that most of the species sampled would not regularly move between the sites.

120 **2.2 Sampling wildlife**

121 All sampling took place between July and August ('Summer'), and October and November
122 ('Autumn') 2016 and was subject to full ethical review (see Supplementary Material). Sampling
123 occurred each week per month per site, but mammals and birds were not captured simultaneously to
124 avoid excessive disturbance. Small mammals were trapped in Longworth or similar live, small mammal
125 traps with shrew escape holes. The traps were sterilised between sites, filled with sterile hay as
126 bedding and mixed grain and carrot or apple as food and water sources. Traps were placed at 5m
127 intervals and checked daily. Faeces were collected with a sterile swab into a sterile sampling tube for
128 transport to the laboratory. The species of each rodent caught, the date and trap location were
129 recorded.

130 Wild birds were caught in mist nets, under licence from the British Trust for Ornithology (BTO),
131 located along and across hedgerows and patches of woodland within each study site. Each capture
132 location was selected to overlap with trapping sites for small mammals (above) and was pre-baited
133 for at least 3 days with bird feeders containing mixed seed. After capture, each bird was placed on its
134 own into a single use brown paper bag for up to 20 min in order to collect a faecal sample. The bird
135 was then fitted with a BTO leg ring, before being released. Sterile swabs were used to remove faeces
136 from the bags into sterile sampling tubes. If the same bird was caught more than once on the same
137 day the faecal samples were pooled. In addition, feral pigeons, which formed a large flock at the Farm-
138 site, were sampled for faeces post-mortem after shooting as part of pest control. Table S3 shows the
139 range of species caught. The foraging ecology of the species did not explain any of the patterns of
140 AMR or MDR observed (see Supplementary Material).

141

142 **2.3 Isolation and AMR characterisation of presumptive *E. coli* isolates**

143 Phenotypic resistance to eight antibiotics was determined first by plating on antibiotic-
144 supplemented media or by disk diffusion. Faecal samples (0.5 g) were incubated in buffered peptone
145 water (BPW) at 37 °C for 18 h and 100 µl was spread onto Tryptone Bile X-Glucuronide Medium (TBX;
146 Oxoid, UK) agar supplemented with; ampicillin (10 µg/ml), apramycin (30 µg/ml), colistin (4 µg/ml) or
147 ciprofloxacin (1 µg/ml) or without antibiotics and incubated at 37°C for 18h. Presumptive *E. coli*
148 (blue/green) colonies were taken forward for further characterisation.

149 One presumptive antibiotic resistant *E. coli* colony per plate obtained from the initial
150 screening was then tested for resistance to other antibiotics using disc diffusion assays. Briefly, isolates
151 were cultured in BPW at 37 °C for 18 h. Samples (100 µl) were spread plated onto Muller-Hinton agar
152 (MH; Oxoid, UK) and left to dry. Six antibiotic discs impregnated with ampicillin (10 µg/ml), tetracycline
153 (3 µg/ml), apramycin (15 µg/ml), trimethoprim (2.5 µg/ml), imipenem (10 µg/ml) and cefpodoxime
154 (10 µg/ml), were placed on the agar and the plates were incubated for 18 h at 37 °C. After incubation
155 the diameter of the zone of clearance around each disc was measured and isolates were classified as
156 resistant if the zone was less than or equal to published breakpoints (EUCAST 2016).

157

158 **2.4 Characterisation and ERIC-PCR genotyping of *E. coli* isolates**

159 A randomly selected subsample of presumptive *E. coli* were subject to rRNA PCR and
160 sequencing (Srinivasan et al 2015). BLAST searches confirmed all were *Escherichia*, and the vast
161 majority clearly *E. coli*. In order to identify any patterns of genotypic similarity among *E. coli* by spatial
162 location or host (mammal/bird), we used ERIC-PCR. Twenty-four resistant *E. coli* isolates from
163 mammals at each sample site and all the resistant *E. coli* isolates from birds (total 91 samples) were
164 subjected to ERIC-PCR (Ibrahim et al 2016, Versalovic et al 1991). DNA (diluted 1:100) extracted from
165 the *E. coli* isolates, 12.5 µl of PCR Master Mix Plus (Qiagen, UK), 5 µM of the each ERIC primer (Table

166 S1), 2 µl of Coral Load Dye (Qiagen, UK) and sterile molecular grade water to 25 µl. The PCR parameters
167 for the ERIC-PCR are found in Table S1.

168

169 **2.5 Analysis of ESBL and AmpC resistance in cefpodoxime-resistant *E. coli***

170 Cefpodoxime resistant isolates were tested for ESBL or AmpC activity using the AmpC & ESBL
171 Detection Set (Mast Group, UK). Briefly, overnight liquid cultures of cefpodoxime resistant isolates
172 were spread plated onto MH agar and left to dry before discs containing cefpodoxime 10 µg (A),
173 cefpodoxime 10 µg + ESBL inhibitor (B), cefpodoxime 10 µg + AmpC inhibitor (C) and cefpodoxime 10
174 µg + ESBL and AmpC inhibitor (D) were added. Comparison of the zones of clearance enabled ESBL
175 and/or AmpC resistant bacteria to be identified using the manufacturer's calculator (Mast Group,
176 UK).

177

178 **2.6 DNA extraction and PCR parameters**

179 DNA was extracted from *E. coli* by heat-lysis. One colony was placed in 10 µl of sterile
180 molecular grade water and heated at 95° for 10 min. Samples were centrifuged (13000 *x g*; 3 min) and
181 the supernatant removed. The supernatant was stored at -20 °C until used as template DNA for
182 subsequent PCR reactions. PCR amplifications (apart from ERIC-PCR) were carried out in 20 µl reaction
183 mixtures comprising of 10 µl of PCR Master Mix Plus (Qiagen, UK): 0.5 µM of each primer, 2 µl of Coral
184 Loading Dye (Qiagen, UK) and molecular grade sterile water to 20 µl. See Table S1 for primers and PCR
185 cycling parameters.

186

187 **2.7 Molecular characterisation of colistin and ciprofloxacin resistant *E. coli***

188 *E. coli* isolates with phenotypic colistin and ciprofloxacin resistance were further
189 characterised. DNA from ciprofloxacin and colistin-resistant colonies was diluted 1:100 and used as
190 template DNA for PCR to amplify the *gyrA* and if present the transposable *mcr-1* gene (Liu et al 2016).
191 For ciprofloxacin resistant isolates DNA was purified from agarose gels using a Gel DNA Extraction Kit
192 (ZymoResearch, UK) and sequenced. The sequences were aligned and compared against *E. coli* K12
193 using CLC SequenceCe Viewer (Qiagen) to identify specific point mutations in *gyrA* associated with
194 ciprofloxacin resistance. As a positive control for colistin resistance, DNA harbouring the *mcr-1* gene
195 was used.

196

197 **2.8 Statistical analyses**

198 Binomial logistic regression models were used to ascertain the effects of site (Farm, Central
199 and STP), season (Summer = Jul/Aug, Autumn = Oct/Nov,) and taxa (bird or mammal) on the
200 prevalence of *E. coli* in faecal samples and prevalence of resistance, i.e. if *E.coli* were resistant to one
201 or more antibiotic ('AMR ≥ 1 antibiotic') or MDR (resistant to three or more antibiotics). All of these
202 analyses were carried out using SPSS v.24.

203 The ERIC-PCR gel image was analysed using a Gel-Doc XR system (Bio-Rad, UK)(Ibrahim et al
204 2016). Using GelCompar II (Applied Maths) a dendrogram was generated from the comparison of ERIC-
205 PCR profiles, using the Dice coefficient, and clustered by the unweighted pair group method with
206 arithmetic averages (UPGMA) with 1.5% of optimization and 1.5% of tolerance. Molecular variance
207 framework analysis (AMOVA) (Excoffier et al 1992) was used to analyse the confidence of the selected
208 similarity threshold and the significance of clusters. The AMOVA calculation was carried out using
209 GenALEx v 6.5b5 software (Peakall and Smouse 2006). The significance was examined with the
210 calculation of Φ_{PT} , a measure of population differentiation that suppresses intra-individual variation.
211 In the case of AMOVA, the null hypothesis (H_0 ; $\Phi_{PT} = 0$) meant that there was no genetic difference

212 among the populations and the alternative hypothesis ($H_1; \Phi_{PT} > 0$) meant there were genetic
213 differences amongst the populations.

214

215

216 3. Results

217 3.1 *E. coli* in rodent and avian samples

218 In total, 125 faecal samples from bank voles, 15 from field voles and 89 from wood mice were
219 collected. A further 96 faecal samples were collected from traps in which small rodents had escaped,
220 and were recorded as 'unknown' (see Table S2). We collected 84 avian faecal samples from 18
221 different species, but one sample did not yield an isolate.

222 Overall *E. coli* were isolated from 66 % (269/408) of faecal samples (Figure 1). The prevalence
223 of *E. coli* was explained by site, season and taxa (Table 1a). Samples collected from the Central (63%;
224 n= 145) and STP sites (64%; n= 125) did not differ significantly. Samples collected from the Farm Site
225 (prevalence = 71 %; n = 138) were significantly more likely to contain *E. coli* than those from the Central
226 Site (Table 1a; Figure 1). Mammalian samples were significantly more likely to contain *E. coli*
227 (prevalence = 74%; n = 325) than avian samples (33%; n = 83)(Table 1a). Samples collected in Summer
228 (prevalence = 73%; n = 227) were significantly more likely to contain *E.coli* than those collected in
229 Autumn (57%; n = 181)(Table 1a).

230

231 3.2 Genotyping of *E. coli* isolates by ERIC-PCR

232 A selection of AMR *E. coli* representing different hosts and sites were compared by ERIC-PCR
233 (Figure 2). Cluster analysis suggested five main groups of isolates at a 50 % similarity threshold
234 (indicated as I-V in Figure 2). Cluster significance analysis demonstrated these were non-overlapping
235 and hence genomically independent groups (cluster significance $\Phi_{PT} = 0.036$; $p < 0.001$). Each larger
236 cluster (II-V) contained *E. coli* from a range of hosts and sites with no obvious association between
237 their AMR pattern and which cluster the isolates resided in. However, there was a tendency towards
238 certain clusters containing isolates from predominantly one site: cluster II with Farm Site, cluster III
239 with Central Site and cluster V with STP Site. Given an expected probability of 0.33, binomial tests

240 indicated that the proportion (0.69) of Farm Site samples in Cluster II was significantly higher than
241 expected ($p = 0.0002$), as was the proportion of Central Site samples (0.62) in Cluster III ($p = 0.033$)
242 and the proportion of Farm Site samples (0.75) in Cluster V ($p = 0.0006$).

243

244 **3.3. Antimicrobial resistance**

245 The prevalence of AMR was expressed as the percentage of samples from which *E. coli* was
246 isolated (on the TBX plate without antibiotics) that also contained at least one isolate resistant to at
247 least one of the antibiotics tested ($AMR \geq 1$). The overall prevalence of AMR *E. coli* was 54 % ($n =$
248 262) and was significantly explained by a model that included season, taxa and site (Table 1b). AMR
249 prevalence in samples from the STP was 61.3 % ($n = 80$) which was significantly higher than the
250 prevalence of resistance in samples from the Central Site (50.0 %; $n = 86$) (Table 1b; Figure 3a).
251 Prevalence in samples from the Farm site was 52.1 % ($n = 96$) and did not significantly differ from
252 that in Central Site samples (Table 1b).

253 *E. coli* from samples collected in Summer (prevalence = 65.4 %; $n = 159$) were significantly
254 more likely to be resistant than those collected in Autumn (36.9 %; $n = 103$) (Table 1b). There was a
255 tendency ($p = 0.056$; Table 1b) for mammalian faecal samples to have a higher prevalence (55.7 %; n
256 = 235) of resistant *E. coli* than avian samples (40.7 %; $n = 27$).

257

258 **3.4 Multi-drug resistance (MDR)**

259 For the purpose of this study MDR was defined as resistance to three or more of the eight classes of
260 antibiotics tested. Overall, 80.3 % ($n = 142$) of the AMR *E. coli* were MDR. A model including taxa
261 and site significantly explained MDR prevalence (Table 1c). Prevalence in samples from the Farm site
262 (66.0 %; $n = 50$), was significantly lower than from the Central site (83.7 %; $n = 43$). Prevalence of
263 MDR in samples from the Central and STP sites (91.8 %; $n = 49$) did not differ significantly (Fig. 3b;
264 Table 1c). *E. coli* from samples collected from mammals (prevalence = 84.7 %; $n = 131$) were

265 significantly more likely to be MDR than those collected from birds (27.3%; n = 11) (Table 1c).
266 Season (MDR prevalence in Summer = 77.9 %; n = 104 and in Autumn = 86.8 %; n=38) was non-
267 significant so was excluded from the model.

268 Individual *E. coli* isolates were resistant to up to seven different antibiotics (Figure 3c). There
269 was no obvious difference in MDR profiles between the different sites tested (Table 2).

270 **3.5 Prevalence of ESBL or AmpC producing *E. coli***

271 All isolates resistant to cefpodoxime were further investigated for ESBL or AmpC production.
272 From the 53 cefpodoxime resistant *E. coli*, six were ESBL, 22 were AmpC and six were positive for both
273 ESBL and AmpC production (Table 3). Across all samples, there was a significant difference between
274 the sites in the number of isolates testing positive for AMPC and/or ESBL, with the highest number at
275 the STP site ($\chi^2(2) = 6.59, p = 0.034$; Table 3).

276

277 **3.6 Genotypic analysis of ciprofloxacin and colistin resistant isolates**

278 Ciprofloxacin resistant *E. coli* were further characterised by sequence comparison with a
279 known sensitive strain of *E. coli* (K-12) and four of amino acid changes were observed (Figure 4). All
280 colistin resistant isolates were subjected to *mcr-1* PCR and none were found to be positive for this
281 gene, suggesting resistance is derived from other ARGs.

282

283

284 4. Discussion

285 AMR, including MDR, was common among the commensal *E. coli* of the wildlife studied, but
286 clear patterns in resistance were not seen in terms of spatial proximity to anthropogenic sources of
287 waste containing antimicrobials and ARB. Previous studies have suggested that wildlife could be
288 used as sentinels of environmental AMR (Furness et al 2017, Vittecoq et al 2016). Our study supports
289 this to some extent, although as with previous work by ourselves and others (Arnold et al 2016,
290 Bondo et al 2016, Gilliver et al 1999, Literak et al 2010, Williams et al 2011), factors other than
291 geographic distance from the wastes of antibiotic treated animals or humans clearly influence AMR.
292 This is also demonstrated by the wide variations in MDR profiles within and between sites suggesting
293 other factors affecting AMR in these animals (Table 2). Host taxonomic differences, as well as spatial
294 and temporal factors, seemed to influence AMR prevalence. Moreover, our models explained about
295 20% of the variance in AMR and MDR, indicating that other, unmeasured factors, were also
296 important in determining prevalence. Thus, there are significant caveats to using wildlife as sentinels
297 of environmental transmission of AMR due to antimicrobials and ARB in anthropogenic wastes.

298 Some studies have reported relatively high AMR prevalence in wildlife collected near AMR
299 sources such as water bodies receiving sewage effluent or agricultural wastes, compared with more
300 pristine sites (Bonedahl et al 2009, Furness et al 2017). In our study, a significantly higher
301 prevalence of AMR was observed at the STP (61%) compared with the other two sites (<53%). That
302 site and site-specific environments might be drivers of exposure is supported by the ERIC analysis
303 that found that genotypes of *E. coli* showed spatial- rather than host-specific clustering (VanderWaal
304 et al 2014). Multidrug resistance prevalence showed somewhat different patterns with the STP
305 (92%) again having a significantly higher MDR prevalence than the farm (66%), but a similar
306 prevalence to the Central site (84%). If the prevalence and patterns of resistance were driven by
307 exposure to either anthropogenic antimicrobials or ARB from humans and/or livestock, a higher
308 prevalence of resistance would have been expected at the Farm Site as well as the STP Site, and the

309 prevalence at the Central site might have been expected to be lower than both of the other two
310 sites. However, this was not the case (see also (Carter et al 2018)).

311

312 **4.1 Host taxa and temporal variation**

313 Taxonomic differences in both the prevalence of samples containing *E. coli* and the
314 prevalence of AMR and MDR were observed. Mammals (74%) were significantly more likely to be
315 carrying *E. coli* than birds (33%), with a prevalence of 66% overall. Host taxonomic differences in *E.*
316 *coli* may reflect the relatively small size of faecal samples from birds and their tendency to dry out,
317 but might also simply reflect the relative contribution of *E. coli* to the normal gut biota of very
318 different taxa. The prevalence of phenotypic AMR (expressed as the percentage of samples that
319 contained resistant *E. coli*) was 54% overall, with a marginally higher prevalence in mammalian
320 (56%) than avian (41%) samples ($p = 0.056$). Our prevalence of ARB in mammals was similar to that
321 previously reported in the UK (35% and 79% for inland and coastal populations respectively of small
322 mammals (Furness et al 2017), but higher than that reported in similar species from mainland
323 Europe (for example 5.5% AMR in *E. coli* from rural small mammals in Germany (Guenther et al
324 2010) and 2 – 12% in a range of wild mammals the Czech Republic (Literak et al 2010). Reported
325 AMR prevalence in wild birds is similarly diverse, varying both by species and geography (Carter et al
326 2018). For example, a study of AMR in *E. coli* from gulls across Europe found a prevalence of 32%
327 overall, but with considerable geographic variation, from 61% in Spain to 8% in Denmark (Stedt et al
328 2014). Notably, a larger number of avian than mammal species were sampled, so differences in
329 ecology and diet among species might obfuscate comparisons of the relative roles of mammals and
330 birds in AMR dispersal.

331 Furthermore, in our study, as in others (Ahammad et al 2014, Bondo et al 2016, Sun et al
332 2012, Williams et al 2011), *E. coli*, AMR and MDR patterns and prevalence varied over time.
333 Temporal variation in *E. coli* and resistance patterns might reflect changing environmental
334 conditions (temperature and rainfall), selective drivers (e.g. patterns in antibiotic usage) and/or food

335 availability (and changing gut biota) for wildlife as well as differences between the species'
336 population dynamics (Waite and Taylor 2014, Williams et al 2011). Since sampling took place during
337 only two seasons, temporal and seasonal patterns in AMR evolution and dispersal need further
338 study. Despite some limitations, our study lays the foundations for future studies looking a larger
339 numbers of animals at a wider variety of sites and, ideally, longitudinally, along with direct sampling
340 of the environment for antibiotics and ARB.

341

342 **4.2 MDR prevalence and resistance profiles**

343 As described in other studies (Arnold et al 2016, Williams et al 2011), many AMR isolates
344 from mammalian wildlife were multidrug-resistant (MDR). This was likely an outcome of prevalent
345 mobile genetic elements such as plasmids and transposons (Carroll et al 2015), but chromosomal
346 mutations are also common. The prevalence of MDR (AMR ≥ 3), like overall AMR (AMR ≥ 1) was
347 higher in mammal (85%) than in bird samples (27%). On the other hand, the large diversity of MDR
348 profiles found (Table 2) suggests only limited MDR transmission between individuals. Some of these
349 resistances (ciprofloxacin) were found to be derived from point mutations and therefore are not
350 necessarily linked to the other resistances carried by the individual bacterium. Moreover, MDR
351 prevalence was highest at the STP. It is tempting, therefore, to speculate that animals at the STP Site
352 were exposed to a wider range of MDR bacteria, plasmids, or antimicrobials, than animals at other
353 sites. This in turn would fit well with a hypothesis that these animals had exposure to sewage
354 derived from many different people, with different histories of antimicrobial exposure, whereas
355 wildlife at the Central and Farm Sites would have exposure to less varied sources and drivers. This
356 would still, however, leave unanswered the questions of what might be the drivers that led to such
357 high MDR prevalence overall, why different animals in the same population might have such
358 different exposure histories and why the Farm Site and not the Central Site had the lowest MDR
359 prevalence.

360 The most common MDR resistance profile encountered in this study was combined
361 resistance to ampicillin, colistin and ciprofloxacin (Table 2). A high prevalence of resistance to
362 ampicillin was expected as this beta-lactam antibiotic is frequently used in both human and
363 veterinary medicine and resistance is common not only in clinical samples (Briñas et al 2002) but has
364 also been described previously in wild rodents (Arnold et al 2016, Williams et al 2011). It is
365 commonly plasmid-encoded and associated with MDR, as in this study where 83% of the ampicillin
366 resistant isolates were resistant to three or more antibiotics and 23% to five or more antibiotics
367 (Table 2). A high prevalence of phenotypic resistance to colistin was neither expected nor has been
368 described previously in wild rodents, although colistin-resistant *E. coli* strains have been isolated
369 from waterbird faeces (Wu et al 2018). Colistin resistance genes have been demonstrated in waste-
370 impacted river water (Wu et al 2018), and especially at STPs (Hembach et al 2017). Although
371 chromosomally-encoded colistin resistance has been described for many years, its prevalence was
372 historically generally low. The recent discovery of the *mcr-1* gene, that confers colistin resistance
373 and is plasmid encoded, enabling rapid horizontal transmission of resistance, (Liu and Wong 2013) is
374 of great clinical concern as colistin is now a 'last line' antibiotic used for treating MDR infections in
375 humans (Velkov et al 2013). The high prevalence of colistin resistance found in our study (35-40%),
376 along with most colistin resistant isolates being MDR (87% resistant to three or more antibiotics and
377 26% to five or more antibiotics) is suggestive of horizontal transmission although screening for the
378 *mcr-1* gene by PCR was negative. However, other plasmid-encoded genes for colistin resistance
379 have been subsequently described (Xavier et al 2016), and further characterisation of the underlying
380 mechanism of the colistin resistance found in in our study is underway. Seven out of the nine
381 ciprofloxacin resistant isolates contained four nonsynonymous mutations in the *gyrase A* gene
382 (Figure 4), which had been reported previously, and two had mutations that have not previously
383 been reported in *E. coli*. Wildlife can. Therefore, harbour and disperse novel and/ or clinically
384 important ARGs in the environment.

385 In terms of other clinically important resistances, cefpodoxime resistance is a common
386 indicator of ESBL production (Oliver *et al.*, 2002), also of major concern in human medicine. From
387 the 53 cefpodoxime resistant *E. coli* isolated from wildlife, six were ESBL producers, 22 were AmpC
388 and six were positive for both ESBL and AmpC production (Table 3). ESBLs have previously been
389 detected in *E. coli* isolates from a range of wildlife taxa, for example, 32% of *E. coli* isolates obtained
390 from gulls' faeces (Simões *et al.* 2010), and such findings have been ascribed to contact with human
391 waste. In our study, significantly more ESBL and/or AmpC – producing *E. coli* were found in wildlife
392 samples collected from the STP Site, which suggests that human waste may be a factor driving
393 ESBL/AmpC resistance in the environment.

394

395 **4.3. Conclusions**

396 Taken together, the results of this study support those of previous studies in that they
397 confirm that wildlife commonly harbour ARB. Whether or not wildlife might be a source for onward
398 transmission to domestic animals or to humans has not been directly examined. Our study was more
399 concerned with beginning to investigate the drivers of AMR in wildlife, and in particular the role that
400 anthropogenic waste, whether of directly human or domestic animal origin, might play in developing
401 and maintaining that resistance. Diverse patterns of resistance were found in *E. coli* from wildlife in
402 this study, suggesting variation within and between host taxa, between individuals, and over time.
403 Overall, study site was not associated clearly with AMR, MDR or resistance patterns. However,
404 resistance to antibiotics used only in human medicine was more prevalent at the STP site than the
405 Farm and Central sites. Thus, the drivers of AMR in wildlife appear to be more complex than simple
406 anthropogenic causes. Consequently, care needs to be taken if wildlife are to be used as sentinels of
407 environmental AMR or pollution.

408

409

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706 **Figure Legends**

707 **Figure 1:** Inter-site variation in the percentage prevalence of faecal samples testing positive (solid
708 blue bars) or negative (orange hatched bars) for a) *E. coli*. Boxes on the bars show the number of
709 samples in each category.

710

711 **Figure 2.** ERIC profile of *E. coli* isolated from both small mammals and birds at Farm site (light green,
712 mammals; dark green birds) Central site (red, mammals; dark red, birds) and STP site (light purple,
713 mammals; dark purple, birds). Horizontal lines demonstrate significant clusters (I - V) based on 50 %
714 cut-off (vertical line). Red cells demonstrate resistance to each antibiotic: Amp – ampicillin; Cef –
715 cefpodoxime; Col – colistin; Apra – apramycin; Imi – imipenem; Trim – trimethoprim; Tet –
716 tetracycline; Cip – ciprofloxacin

717

718 **Figure 3:** Site-specific patterns of resistance in *E. coli* isolates: a) AMR: The percentage of faecal
719 samples which contained *E. coli* susceptible to ≥ 1 antimicrobial (negative = orange hatched bars) or
720 resistant to one or more antimicrobial drugs (positive = solid blue bars); b) MDR - The percentage of
721 samples containing *E. coli* that were resistant to ≥ 3 antibiotics (positive = resistant = solid blue
722 bars); c) Prevalence of resistance to 1 – 7 different antibiotics. The sites were Farm, Central and STP.

723

724 **Figure 4.** Mutations of ciprofloxacin-resistant *E. coli* isolated from small mammals (blue boxes).
725 Translated sequences of *gyraseA* gene from ciprofloxacin resistant *E. coli* isolates compared to the
726 known sensitive reference strain K-12.

727

728 Table 1: Final binomial logistic regression model outputs explaining prevalence of a) *E. coli*; b)
 729 AMR ≥ 1 antibiotic; c) MDR (AMR ≥ 3 antibiotics). The coefficients for the Site variable are compared to
 730 the Central Site, for the Taxa variable was compared to birds and for the Season variable was
 731 compared to Autumn.

	Nagelkerke R ²	χ^2 (df)	Wald (df)	p-value	Odds ratio	95% C.I.
a) <i>E. coli</i>	21%	67.50 (4)		< 0.0001		
Site:			16.21 (2)	< 0.0001		
Farm			15.07 (1)	< 0.0001	3.51	1.86 – 6.60
STP			0.23 (1)	0.63	1.14	0.67 – 1.93
Taxa			45.75 (1)	< 0.0001	9.26	4.86 - 17.66
Season			3.89 (1)	0.048	1.57	1.00 - 2.46
b) AMR	14.4%	29.97 (4)		< 0.0001		
Site:			4.75 (2)	0.093		
Farm			1.17 (1)	0.28	1.44	0.74 - 2.79
STP			4.742 (1)	0.029	2.11	1.08 - 4.73
Taxa			3.64 (1)	0.056	2.48	0.98 - 6.32
Season			23.93 (1)	< 0.0001	3.96	2.28 - 6.89
c) MDR	25.9%	40.91 (4)		< 0.0001		
Site:			8.02 (2)	0.018		
Farm			0.05 (1)	0.82	1.09	0.51 - 2.34
STP			7.07 (1)	0.008	3.37	1.38 - 8.26
Taxa			14.30 (1)	< 0.0001	12.53	3.38 – 46.43
Season			0.57 (1)	0.45	1.34	0.63 - 2.84

732

733 **Table 2:** Frequencies of MDR profiles for combinations of antibiotics to which *E. coli* isolates were
734 resistant for faecal samples collected from birds and mammals captured at the STP, Central and Farm
735 sites. Only profiles that were found at two or more individuals are presented. Amp – ampicillin; Cef –
736 cefpodoxime; Col – colistin; Apra – apramycin; Imi – imipenem; Trim – trimethoprim; Tet –
737 tetracycline; Cip – ciprofloxacin

738

Antibiotics	Farm	Central	STP	Totals
Amp Tet Col	7	8	8	23
Apra Col Tet	2	3	2	7
Amp Cip Tet	5	0	0	5
Amp Tet Cef	0	1	3	4
Amp Tet Trim	0	2	2	4
Amp Apra Tet	1	2	1	4
Col Cef Tet	0	1	2	3
Apra Trim Col	0	0	2	2
Amp Apra Cef	1	1	0	2
Amp Apra Col Tet	2	5	2	9
Amp Tet Trim Col	2	1	3	6
Col Trim Cef Tet	0	1	2	3
Amp Tet Cef Col	0	0	3	3
Amp Cef Trim Col	1	0	2	3
Apra Tetra Cef Col	1	1	0	2
Amp Apra Trim Col	0	2	0	2
Amp Apra Cef Trim Col	3	2	3	8
Amp Col Trim Cef Tet	1	2	4	7
Amp Apra Tet Trim Col	2	0	0	2
Amp Apra Tet Cef Trim Col	1	2	1	4

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741 **Table 3:** Number of AmpC and ESBL producing *E. coli* isolates for bird and mammal samples collected
 742 at the Farm (livestock waste dominated), Central (no waste source) and STP (human waste dominated)
 743 sites. The percentages in brackets were calculated across all 53 cefpodoxime resistant isolates that
 744 were tested for AmpC and ESBL activity.

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Site	Mammal				Bird			
	AmpC	ESBL	AmpC & ESBL	Negative	AmpC	ESBL	AmpC & ESBL	Negative
Farm	4 (8%)	0	2 (4%)	5 (9%)	0	0	0	2 (4%)
Central	6 (11%)	2 (4%)	1 (2%)	4 (8%)	1 (2%)	0	0	0
STP	7 (13%)	4 (8%)	3 (6%)	7 (13%)	4 (8%)	0	0	1 (2%)
Total	17 (32%)	6 (12%)	6 (12%)	16 (30%)	5 (9%)	0	0	3 (6%)

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