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***Mycobacterium avium* subspecies *paratuberculosis* is widely distributed in British soils and waters: implications for animal and human health.**

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Running title

Mycobacterium avium subsp. *paratuberculosis* in GB

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21

22 **Summary**

23 In the first comprehensive geographical survey of distribution in Great Britain,

24 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) was detected in 115 of 1092 (10.5 %)

25 soil cores, in the range of 5 x 10^2 to 3 x 10^6 MAP cell equivalents (CE) g^{-1} wet weight soil

26 with the majority of the positive PCR reactions (n=75; 65 %) occurring around the limit of

27 detection (500-5000 CE g^{-1} wet weight soil). The distribution of MAP significantly increased

28 from North to South and was significantly correlated with increasing cattle numbers over the

29 same longitudinal axis. Similarly MAP occurrence significantly increased toward easterly

30 latitudes although none of the parameters measured were associated. Comparisons of land

31 use indicated that MAP was widely distributed in both farming and non-farming areas. Soil

32 core samples taken from the rivers Wyre and Douglas catchments (Lancashire, UK) and river

33 Tywi (South Wales) were negative for MAP. However, river monitoring showed a consistent

34 presence of MAP*s* throughout those catchments over a 6 month period. We concluded that:

35 MAP is widely distributed within and outside the confines of the farming environment; its

36 geographical distribution is wider than originally anticipated and; monitoring rivers describes

37 the MAP status of catchment better than individual soil samples.

38

39 **Introduction**

40 *Mycobacterium avium* subsp. *paratuberculosis* is a very slow growing mycobactin-dependent
41 member of the *M. avium* complex (Thorel *et al.*, 1990; Gerlach, 2002; Chacon *et al.*, 2004).
42 It has the specific ability to cause chronic inflammation of the intestine, or Johne's disease
43 (JD) (Buergelt *et al.*, 1978; Nielsen and Toft, 2009; Over *et al.*, 2011), which can affect many
44 animal species, including primates (McClure *et al.*, 1987; Clarke, 1997). This chronic enteric
45 pathogen is implicated in the causation of chronic inflammation of the intestine of the
46 Crohn's disease type (Chiodini *et al.*, 1986; Bull *et al.*, 2003; Hermon-Taylor, 2009; Chiodini
47 *et al.*, 2012). Despite its broad pathogenicity, *M. avium* subsp. *paratuberculosis* can persist in
48 animals for years without causing clinical disease. Johne's disease is chronic in nature with
49 multiple years separating the initial infection from clinical stages of disease (Wu *et al.*, 2007).
50 Subclinical infection is widespread in domestic livestock, especially cattle, sheep, and goats
51 (Nielsen and Toft, 2009). Europe and North America have been particularly affected
52 (Sorensen *et al.*, 2003; Nielsen and Toft, 2009), but infection and disease are now spreading
53 worldwide (Olsen *et al.*, 2002; Larsen *et al.*, 2012). It is estimated that the herd prevalence
54 for JD in cattle in the USA is 68% (USDA, 2008) and 32% in UK (Caldow *et al.*, 2007;
55 DEFRA, 2009; Nielsen and Toft, 2009). Both clinically and sub-clinically infected animals can
56 shed *M. avium* subsp. *paratuberculosis* in variable numbers on to pasture in their faeces,
57 depending on the animal, the pathogen strain and the disease characteristics) (Clarke, 1997).
58 Shedding into the environment may infect local stock or other animals such as deer (Fritsch
59 *et al.*, 2012). The organism can survive for many months in agricultural slurry and in the
60 wider environment (Larsen *et al.*, 1956; Pickup *et al.*, 2005; Pickup *et al.*, 2006), where it
61 also has the potential to persist within protists (Cirillo *et al.*, 1997; Mura *et al.*, 2006). Under
62 experimental conditions, we found *M. avium* subsp. *paratuberculosis* isolated from the
63 human intestine could survive within *Acanthamoeba polyphaga* for over 4 years of

64 incubation with several cycles of encystment and trophozoite activation (Mura *et al.*, 2006).

65 Similarly its survival in sterile lake water detected by culture for 632 days (Pickup *et al.*,

66 2005) and in animal trough water its survival exceeded 26 weeks (Whittington *et al.*, 2005).

67 Establishment and further environmental contamination is enhanced by cycling through wild

68 animal reservoirs (Judge *et al.*, 2006). The ingestion of pellets containing *M. avium* subsp.

69 *paratuberculosis* from infected rabbits has been shown to have the potential to establish a

70 cycle of re-infection for grazing livestock (Judge *et al.*, 2005; Judge *et al.*, 2006).

71 Furthermore, an ability to sporulate may also enhance survival in the environment (Lamont *et*

72 *al.*, 2012). As with other potentially zoonotic pathogens rain falling onto pastures

73 contaminated with *M. avium* subsp. *paratuberculosis* washes it into surface waters and rivers

74 (Pickup *et al.*, 2005; Pickup *et al.*, 2006). Previously, we showed that *M. avium* subsp.

75 *paratuberculosis* was present in 69% of samples taken from the river Tywi (South Wales,

76 UK) and its presence was entirely predictable from rain fall patterns and river flow (Pickup *et*

77 *al.*, 2006). Furthermore deposition and transport from the catchment was extensive in that

78 *M. avium* subsp. *paratuberculosis* was maintained in the river for several weeks at a time and

79 was a consequence of the endemic presence of *M. avium* subsp. *paratuberculosis* in cattle in

80 the Tywi catchment (Pickup *et al.*, 2006). Pickup and co-workers (2006) modelled the main

81 human exposure routes of *M. avium* subsp. *paratuberculosis* and suggested that although

82 driven by shedding from clinically and sub-clinically infected animals, the presence and

83 distribution of *M. avium* subsp. *paratuberculosis* in the environment may also be influenced

84 by other factors such as slurrying, soil redistribution from water treatment and aerosols and

85 that consequently the potential for human exposure was diverse (Pickup *et al.*, 2006).

86 In the present study we sought to extend on previously limited geographical studies to

87 determine the presence and number of *M. avium* subsp. *paratuberculosis* in over 1000 soils

88 collected across Great Britain as part of a nationwide monitoring scheme. The soils were

89 collected from the 2007 Countryside Survey (CS2007; www.countrysidesurvey.org.uk); a
90 long-term British monitoring programme incorporating extensive sampling of habitat and soil
91 characteristics at multiple spatial scales. Real time quantitative PCR (qPCR) was employed to
92 assess the distribution of *M. avium* subsp. *paratuberculosis* in soil with respect to two broadly
93 related aims: to address the environmental and spatial determinants of *M. avium* subsp.
94 *paratuberculosis* biogeography at multiple spatial scales across Britain and to generate a
95 broad-scale map of *M. avium* subsp. *paratuberculosis* distribution at a nationwide scale.
96

97 **Results**

98

99 **Analytical sensitivity of the real-time qPCR assays DH2 and DH3.** Using serially diluted
100 *M. avium* subspecies *paratuberculosis* K-10 genomic DNA in 5 repeat reactions at each
101 dilution, the limit of detection (LOD) of the DH2 (IS*900*) assay was demonstrated to mimic
102 that the original study of 0.1 genomes per µl (Herthnek and Bolske, 2006). However,
103 sensitivity of the DH3 assay was slightly different in that it was 1 genome μl^{-1} (data not
104 shown) compared with 0.3 genomes μl^{-1} originally described (Herthnek and Bolske, 2006). In
105 soil samples this limit of detection was 500-5000 CE g^{-1} wet weight soil and 5000-50000 CE
106 g^{-1} wet weight soil for the DH2 (IS*900*) and DH3 (F57) assays respectively. In river water
107 (where the DH2 assay was used solely) the detection limit was 10-100 CE 100 ml^{-1} water.
108

109 **Real-time qPCR detection of *M. avium* subspecies *paratuberculosis* in soil cores**
110 Of the 233 1 km^2 squares available to this study, 209 were analysed for the presence of *M.*
111 *avium* subspecies *paratuberculosis*. This comprised 1114 individual soils cores of which
112 1092 were successfully subjected to DNA extraction. DNA yields ranged between 3.2 µg g^{-1}
113 and 440 µg g^{-1} wet soil and averaged 26 µg g^{-1} wet weight soil. Application of the DH2

114 (IS*900*) and DH3 (F57) qPCR assays on tcDNAs from 0.25 g soil samples detected *M. avium*
115 subsp. *paratuberculosis* in 115 out of 1092 samples (10.5 %). The DH2 assay detected IS*900*
116 in the range of 500 - 3x 10^6 *M. avium* subsp. *paratuberculosis* cell equivalents (CE) g^{-1} wet
117 soil with the soil assay LOD of 500-5000 CE g^{-1} wet weight soil being demonstrated for
118 majority (n=75; 65 %) of positive samples. However, detection of the *M. avium* subsp.
119 *paratuberculosis* F57 gene using the less sensitive DH3 assay was not observed in any
120 sample despite this PCR assay not being subject to inhibition (data not shown). Total or
121 partial inhibition of the DH2 IS*900* qPCR was confirmed in 34.3% (n=375) of all samples
122 and partial inhibition of the PCR reaction in 11 of the 115 positive samples (9.6%).
123

124 **Distribution of *M. avium* subspecies *paratuberculosis* in Great Britain**

125 The distribution of *M. avium* subspecies *paratuberculosis* in all soil cores at a resolution of 1
126 km^2 is shown in Fig. 1. *M. avium* subspecies *paratuberculosis* showed a widespread
127 longitudinal and latitudinal distribution across soils in England, Scotland and Wales. *M.*
128 *avium* subspecies *paratuberculosis* was detected in 79 of 209 1 km^2 squares (38%) and of
129 these, 19 of 209 1 km^2 squares (9.1%) contained between 2-4 *M. avium* subsp.
130 *paratuberculosis* positive core samples from within the 1 km^2 squares as shown in Fig. 1.
131 This indicates that *M. avium* subsp. *paratuberculosis* is widely distributed across British
132 soils.
133

134 **Occurrence of *M. avium* subspecies *paratuberculosis* at a single core resolution scale**

135 Using spatial analysis based on these data, the incidence of *M. avium* subspecies
136 *paratuberculosis,* in relation to its northerly and easterly position in Great Britain, to altitude,
137 and its relationship with the distribution of sheep and cattle (Figure 2a-e), was assessed. The
138 relationship between *M. avium* subspecies *paratuberculosis* and stocking densities of sheep

139 and cattle was assessed (Figs. 2a, 2b). No relationship was observed between sheep and *M.*
140 *avium* subspecies *paratuberculosis* (p=0.389, R^2=.06819, Fig. 2a) but a significant
141 association between cattle density and *M. avium* subspecies *paratuberculosis* was observed
142 (p=0.0251, R^2=0.3789, Fig 2b), although this trend was weaker than the relationship between
143 *M. avium* subspecies *paratuberculosis* and latitude indicating other factors also influence GB-
144 wide distributions. For the northing analysis, the incidence of *M. avium* subspecies
145 *paratuberculosis* increased significantly from northern to southern latitudes in Great Britain
146 (p=0.000112, R^2=0.7565; Fig. 2c). The density of sheep stocks showed no north-south
147 correlation (p=0.132425, R^2=0.1936; Fig. 2d) whereas the stocking densities of cattle
148 significantly increased towards southerly latitudes (p=0.00986, R^2=0.4685; Fig. 2e).

149 Spatial analysis was performed by easting (longitude) and a significant increase was observed
150 in the incidence *M. avium* subspecies *paratuberculosis* from west to east (p=0.00502, R^2=0
151 .5261). Sheep and cattle densities showed no significant trends with respect to longitude
152 (p=0.4023, R^2=0 .08906 and p=0.7719, R^2=0 .01112 respectively), nor did the relationship
153 between *M. avium* subspecies *paratuberculosis* and cattle or sheep distributions (p=0.5103,
154 R^2=0 .05103 and p=0.882, R^2=0 .0002925 respectively). Therefore, the significant increase
155 in *M. avium* subspecies *paratuberculosis* incidence with longitude was not a function of
156 sheep or cattle stocking density as was apparent with spatial analysis using latitude but
157 influenced by, as yet, unidentified factors.

158 With respect to altitude, spatial analysis showed that *M. avium* subspecies *paratuberculosis*
159 was significantly associated with decreasing altitude towards sea level (p= 0.0388,
160 R^2=0.3935). Sheep densities were not significantly associated with altitude (p=0.718, R^2=0
161 .01525) whereas for cattle, as with *M. avium* subspecies *paratuberculosis,* densities were
162 significantly associated with decreasing altitude (p=0.00431, R^2=0 .3808). By combining the
163 data with respect to altitude, *M. avium* subspecies *paratuberculosis* was significantly

164 associated with cattle densities (*p*= 0.00426, R^2=0 .6153) but not with sheep distribution

165 (*p*=0.122; R^2=0 .2447).

166

167

168 **Analysis of *M. avium* subspecies *paratuberculosis* at a resolution of 1km^2**

169 The previous section assessed the spatial analysis of all cores for the presence and absence of

170 *M. avium* subspecies *paratuberculosis*. The data were re-analysed for 1 km^2 squares that were

171 'unique' in that they were classed as positive regardless of multiple occurrences within 1 km^2

172 squares and would therefore represent a single point rather than multiple points within a 1

173 km^2. With this adjustment the northing analysis again indicated that *M. avium* subsp.

174 *paratuberculosis* incidence significantly increased towards the southerly latitudes but it

175 weakened the relationship with *M. avium* subspecies *paratuberculosis* distribution increasing

176 towards the east.

177

178 **Distribution of *M. avium* subspecies *paratuberculosis* in relation to habitat, soil chemical**

179 **and physical parameters and land use.**

180

181 Each soil core was analysed for its chemical and physical properties and was assigned a

182 habitat-type (e.g. pastureland, peat bog and so forth). We assessed the presence of *M. avium*

183 subspecies *paratuberculosis* in relation to pH (measured in water and in calcium chloride),

184 soil bulk density, soil loss on ignition, mean temperature and rainfall (Fig 3a-f). *M. avium*

185 subspecies *paratuberculosis* was shown to have highly significant associations with neutral to

186 alkaline pH (measured in water and in calcium chloride; *p*= <0.001and *p*= <0.001

187 respectively), higher mean temperatures (*p*=0.000008), low rainfall (*p*= 0.00003), high soil
188 bulk density (*p*=0.0000033) and lower loss on ignition values (*p*=0.0000003).
189
190 All samples were classified by land use according to the criteria laid down by Cs2007 (Smart
191 *et al.*, 2003). *M. avium* subspecies *paratuberculosis* incidence was then compared with land
192 use types (Fig. 4). *M. avium* subspecies *paratuberculosis* incidence was clearly associated
193 with broadleaved, mixed woodland, arable and horticultural, improved and natural grassland
194 which contrasted with its lower incidence in bog, fern, marsh and swamp, shrub heath and
195 bracken. For example the difference between bog and arable and horticultural and improved
196 grassland was significant at *p*=0.017 and *p*=0.003 respectively. Furthermore, the difference
197 between dwarf shrub heath and arable and horticultural descriptors was significant at
198 *p*=0.077. Two further features were apparent: Firstly, compared to improved and neutral
199 grassland, *M. avium* subsp. *paratuberculosis* had a lower association with acid grasslands;
200 secondly, the relatively lower incidence in coniferous woodland compared to grasslands may
201 be due to the co-location in both managed (e.g. grassland) and unmanaged areas (e.g.
202 bracken). However, when *M. avium* subspecies *paratuberculosis* incidence was assessed
203 with respect to agricultural use and/or management there was no significant difference
204 between those subjected to no agricultural use and those subjected to low, medium and high
205 intensity agricultural practices (*p*=0.3051).
206
207 **Distribution of *M. avium* subspecies *paratuberculosis* in two English rivers North West**
208 **England.**
209
210 In a comparative analysis with CS2007 soil samples, routine water samples were taken from
211 the River Douglas and River Wyre, both located in separate catchments in the Northwest of

212 England, over a 6 month period and assessed for *M. avium* subspecies *paratuberculosis* using
213 the DH2 (IS*900*) assay (Table 1).
214
215 For the River Douglas, 42% of the samples were IS*900 M. avium* subsp. *paratuberculosis* -
216 positive with each site showing at least one positive during the sampling period (Table 1a).
217 Sites downstream in the catchment showed a higher number of positive samples than those in
218 the upstream region.
219
220 For the River Wyre, 36% of samples were positive for *M. avium* subspecies *paratuberculosis*.
221 All sites were positive on at least one occasion apart from at one site (Dolphinholme) which
222 was negative but only sampled once (Table 1b). At most sites in both River Douglas and
223 River Wyre samples *M. avium* subspecies *paratuberculosis* was detected at a concentration of
224 10-100 CE 100 ml^{-1} of river water. However, 4 samples from the River Wyre had detection
225 levels of 100-1000 CE 100 ml^{-1} with two of these being within the same sample date and
226 representing sites in the lower half of the catchment within the same temporal river
227 continuum (Table 1b).
228
229 The sizes of the sub-catchments of the Rivers Douglas and Wyre were calculated (Table 1a,
230 b). Each sample point receives an accumulation of water from all the sub-catchments above
231 that point on the river. As expected those lower down the river were *M. avium* subspecies
232 *paratuberculosis* positive (Table1) and for the lowest sampling points the water was derived
233 from > 300 km^2 of land. However, sufficient numbers of *M. avium* subspecies
234 *paratuberculosis* were detectable in the upstream regions that receive water from <50 km^2 of
235 land and in one case about 10 km^2 of catchment indicating high loads on the entire catchment.
236

237 Overall *M. avium* subspecies *paratuberculosis* was detected consistently in the rivers Douglas
238 and Wyre over a 21 week period in 2008 at concentrations of between 10 and 1000 CE per
239 100ml. Within one sampling date (19.06.08), *M. avium* subspecies *paratuberculosis* was
240 detected in the River Wyre at several sites on the river between and including both the
241 highest upstream sampling site at Tarnbrook and within the tidal reaches at Thornton.
242
243
244
245 **Discussion**
246
247 The data presented here were generated from samples collected from the Countryside Survey
248 (CS2007) of Great Britain (GB), which is a globally unique project to monitor ecological and
249 land use change over the whole nation (http://www.countrysidesurvey.org.uk/; (Emmett *et*
250 *al.*, 2010; Maskell *et al.*, 2010). The sample design is based on a series of stratified, randomly
251 selected 1 km^2 grid squares from a predefined group derived from a classification of all 1212
252 1 km^2 grid squares, comprising a grid covering Britain, based on their topographic, climatic
253 and geological attributes (Bunce *et al.*, 1996). We analysed 1092 soil samples from 209 1
254 km^2 grid squares across Great Britain for the presence of *M. avium* subspecies
255 *paratuberculosis*. The majority of surveys to date have focused on detecting *M. avium*
256 subspecies *paratuberculosis* in the farm environment, where it has been found in a number of
257 farms particularly where Johne's Disease is present (Raizman *et al.*, 2004; Berghaus *et al.*,
258 2006; Pillars *et al.*, 2009a; Pillars *et al.*, 2009b; Cook *et al.*, 2010; Eisenberg *et al.*, 2010b;
259 Eisenberg *et al.*, 2010a; Smith *et al.*, 2011). This is entirely predictable given the high herd
260 prevalence for the disease in GB, Europe and USA (Caldow *et al.*, 2007; Nielsen and Toft,

261 2009) and the high faecal shedding rates shown by clinically and sub-clinically infected
262 animals (Clarke, 1997). However, the strength of the CS2007 study was that the distribution
263 of the samples taken did not focus on agricultural land *per se* and each sample was classified
264 with a number of environmental, physical and chemical descriptors including land-use
265 (Bunce *et al.*, 1996; Smart *et al.*, 2003).

266 This study revealed a widespread distribution of *M. avium* subsp. *paratuberculosis* both in
267 areas where it would be predictable (areas of cattle farming) but also areas where such
268 practices were not carried out (e.g. broad leaf woodland). The distribution of *M. avium*
269 subspecies *paratuberculosis* showed no relationship with the distribution of sheep which are
270 also affected by Johne's Disease (Greig, 2000). However, the distribution of *M. avium*
271 subspecies *paratuberculosis* was significantly associated with cattle distribution along with a
272 number of parameters: including decreasing longitude and increasing latitude, decreasing
273 altitude, and soil characteristics such as pH, altitude, temperature and rainfall. Many of these
274 are inter-related. For instance, on a north to south transect of Great Britain soil pH decreases,
275 soil carbon increases and mean temperature increases (Emmett *et al.*, 2010). Land use
276 changes as consequence and hence cattle distribution, which increases down this transect
277 reflecting the increase in grasslands in southerly direction. Therefore the increase in
278 occurrence of *M. avium* subspecies *paratuberculosis* down the same transect is significantly
279 associated with the increase in cattle on the same transect and is only indirectly associated
280 with the other soil parameters. However, cattle distribution is influenced by these parameters.
281 Further strength to the relationship between *M. avium* subsp. *paratuberculosis* and cattle is
282 the significant association with low altitude which reflects the cattle grazing practices in GB.

283 Johne's disease in cattle which is endemic in GB clearly influences the distribution of *M.*
284 *avium* subspecies *paratuberculosis* as clinically and sub-clinically infected cattle shed *M.*
285 *avium* subspecies *paratuberculosis* on to the land (Clarke, 1997). Sheep, in contrast are

286 widely and evenly distributed across GB but show no influence over *M. avium* subspecies
287 *paratuberculosis* distribution despite being susceptible to Johne's disease. A trend of
288 increasing occurrence was shown on a west-east transect which was not associated with
289 either sheep or cattle distributions. In both cases the association was stronger with latitude
290 and longitude than with sheep and cattle distribution, indicating other factors, as yet
291 unknown, are influential.

292 The association of *M. avium* subspecies *paratuberculosis* with habitat showed that land use
293 for cattle grazing and dairy (e.g. grasslands) clearly influenced *M. avium* subspecies
294 *paratuberculosis* distributions. It is interesting to note that there was a significant decrease in
295 *M. avium* subspecies *paratuberculosis* occurrence on acid grassland when compared to
296 improved and neutral grasslands. This is likely to be a function of land use rather than pH *per*
297 *se* as acid grasslands are nutrient poor and are also conservation areas where management
298 practices often excludes sheep and cattle grazing. In Great Britain, pH decreases North to
299 South and follows the increasing trend of *M. avium* subspecies *paratuberculosis* distribution.
300 This would be supported by observations that *M. avium* subspecies *paratuberculosis* is more
301 frequently detected in water and sediment at lower pH (Whan *et al.*, 2005), indicating the
302 more acid soils may influence *M. avium* subspecies *paratuberculosis* survival. Low soil pH
303 has been suggested as a risk factor for Johne's diseases, through enhanced *M. avium*
304 subspecies *paratuberculosis* viability mechanisms related to iron availability (Johnson-
305 Ifearulundu and Kaneene, 1997) and better survival (Salgado *et al.*, 2011). However, in this
306 study the majority of the *M. avium* subspecies *paratuberculosis* positive samples lie in neutral
307 to alkaline soils where they pose a potential threat for re-infection of domestic and wild
308 animals. For our sampling regime, pH would appear to be disconnected from *M. avium*
309 subspecies *paratuberculosis* as a driver for this pathogen's distribution with animal
310 distribution remaining the strongest.

311

312 The distribution of *M. avium* subspecies *paratuberculosis* with respect to land use showed
313 that it was evenly distributed between low, medium and intensive farming and those with no
314 current agricultural use. This study shows, that despite the close association of *M. avium*
315 subspecies *paratuberculosis* deposition with cattle, that it is no longer confined to areas
316 primarily of agricultural use. Wider distribution outside agricultural land use can be affected
317 by farm slurrying practices (Salgado *et al.*, 2011), soil movement (Pickup *et al.*, 2006; Dhand
318 *et al.*, 2009), rain and overland flow resulting runoff into rivers (Pickup *et al.*, 2005; Pickup
319 *et al.*, 2006), movement through aquifers (Bolster *et al.*, 2009), aerosols (Pickup *et al.*, 2005)
320 and movements with the animal reservoir (Simpson, 2002; Daniels *et al.*, 2003). With
321 respect to rainfall, this study showed that detection of *M. avium* subspecies *paratuberculosis*
322 was less likely in areas of higher rainfall. It is possible may be due to increased runoff
323 resulting in wash-out of *M. avium* subspecies *paratuberculosis* shed by animals at the time of
324 sampling by overland water flow (Tyrrel and Quinton, 2003). Previous work shows that
325 overland flow is a significant transport process that delivers *M. avium* subspecies
326 *paratuberculosis* into rivers (Pickup *et al.*, 2005; Pickup *et al.*, 2006).

327 We acknowledge a number of limitations to this study. These include single time point
328 samples and non-replicated sample cores. Replication was not a feasible option given the
329 scale of sample collection in CS2007 (Bunce *et al.*, 1996). In addition, across varied soil
330 types it is unlikely that DNA extraction efficiency was consistent and so 100 % bacterial cell
331 lysis or uniformity across samples cannot be guaranteed. We also acknowledge that the
332 analysis was performed at an area level and we should therefore not try to draw inference at
333 the point level or individual locations as the two scales are not necessarily equivalent. We
334 have tried to avoid this in our conclusions, but care should also be taken on the part of the
335 reader to not overly interpolate our results to a finer scale.

336 Performing the PCR reaction in duplicate and not triplicate was also a limitation. For the
337 majority of samples, amplification was at the limit of detection and detection was not always
338 shown in both duplicates of each reaction. For this reason we expressed our positive samples
339 in ranges to reflect this. We also assessed inhibition of the PCR reaction using *M. avium*
340 subsp. *paratuberculosis* K-10 genomic DNA in separate reactions rather than an internal
341 positive control. We feel that incorporation of genomic DNA into sample inhibition tests is
342 more representative than plasmid controls containing target regions. In PCR optimizations we
343 observed similar sensitivities of detection to the published assays (Herthnek and Bolske,
344 2006), with the exception that F57 detection using the DH3 assay was approximately one
345 order of magnitude lower than that for DH2 (IS*900*) assay. This, combined with the limited
346 number of replicates could explain why F57 was not detected in any samples, despite this
347 reaction not seeming to suffer from inhibition in the way that IS*900* amplification did. The
348 detection of *M. avium* subsp. *paratuberculosis* based solely on the amplification of regions on
349 IS*900* has been subject to criticism due to possible false positives (Cousins *et al.*, 1999).
350 However, the amplification of IS*900* due to false positives in the present study is considered
351 unlikely as the DH2 assay was shown to be highly specific to *M. avium* subsp.
352 *paratuberculosis* with the exception of one *M. kubicae* related strain which did not amplify
353 efficiently even in high DNA concentrations used in specificity testing of the original study
354 (Herthnek and Bolske, 2006). Inhibition testing showed that both number of positive samples
355 detected and the numbers of *M. avium* subsp. *paratuberculosis* in some positive samples were
356 likely to be underestimated. Added to the other limitations above, all of which reduce the
357 chances of detecting *M. avium* subsp. *paratuberculosis*, these findings support our previous
358 studies on the rivers Taff and Tywi (Wales) suggesting that the number of positives is an
359 underestimation, and that environmental distribution is probably much wider (Pickup *et al.*,
360 2005; Pickup *et al.*, 2006).

361 A further limitation is the imperfect land coverage in that only 233 1 km^2 grids were sampled
362 out of a potential 1212 as originally defined (Bunce *et al.*, 1996), therefore some areas are
363 underrepresented in our survey. Two local catchments were chosen that were sampled but
364 under-represented in the CS2007 survey. They both returned a low number of sample sites
365 and were negative for *M. avium* subspecies *paratuberculosis*. The Wyre catchment
366 (Lancashire, England) was represented by one negative sample square and the adjacent River
367 Douglas catchment was not sampled as part of CS2007. However, when river water was
368 sampled directly, both rivers showed a consistent presence of *M. avium* subsp.
369 *paratuberculosis* over a 6 month period indicating a high level of *M. avium* subsp.
370 *paratuberculosis* contamination of the catchment. Likewise, the river Tywi catchment in
371 South Wales showed no *M. avium* subsp. *paratuberculosis* through Cs2007 core analysis (one
372 core taken within the catchment), yet we have data showing that 69% of water samples were
373 *M. avium* subsp. *paratuberculosis* positive over several years (Pickup *et al.*, 2006). *M. avium*
374 subsp. *paratuberculosis* was detected in one of the two Taff catchment Cs2007 soil cores
375 which supported a previous but a more intensive study of the river Taff and its catchment
376 where *M. avium* subsp. *paratuberculosis* was detected in 39% of all samples (Pickup *et al.*,
377 2005). By comparing a nationally intensive study focussing on soil (this study) with local
378 intensive monitoring in rivers (Wyre and Douglas, this study and Taff/Tywi (Pickup *et al.*,
379 2005; Pickup *et al.*, 2006), we can conclude that river water sampling in a defined catchment
380 best describes the degree of endemic infection within those animals rather than randomised
381 soil sampling. Furthermore, Johne's disease in the Orkney Isles (GB) is highly prevalent
382 (Herd prevalence is 64.5% with an individual animal prevalence of 3.6% (Beasley *et al.*,
383 2011) yet the 4 out of 5 CS2007 samples were negative, therefore we would predict that river
384 sampling would be a more relevant predictor of *M. avium* subsp. *paratuberculosis*
385 contamination of the land. Quantification of *M. avium* subsp. *paratuberculosis* within the

386 river samples may therefore be a predictor of farm or herd prevalence of Johne's disease at a
387 catchment scale.

388

389 This study represents the most comprehensive national survey of *M. avium* subsp.
390 *paratuberculosis* in the environment and it was found to be widespread. Despite the
391 limitations of the survey, this study would reinforce others (Pickup *et al.*, 2005; Pickup *et al.*,
392 2006; Smith *et al.*, 2011) in suggesting that the degree to which *M. avium* subsp.
393 *paratuberculosis* distributed is underestimated. Furthermore evidence presented here
394 suggests that rivers are a better mirror of catchment contamination than randomised soil
395 samples. The extensive land contamination by *M. avium* subsp. *paratuberculosis* has
396 implications on the animal welfare through re-infection and cross infection. Therefore, the
397 high herd prevalence found in GB, Europe and USA (Caldow *et al.*, 2007; Nielsen and Toft,
398 2009) needs to be addressed urgently particularly as this may have implications for human
399 health. Controversy still remains as to whether *M. avium* subsp. *paratuberculosis* is the
400 causative pathogen for Crohn's disease (Chiodini and Chamberlin, 2011; Over *et al.*, 2011;
401 Chiodini *et al.*, 2012) but it is now accepted that it is significantly associated with the disease
402 (Abubakar *et al.*, 2008). Inflammatory bowel diseases and particularly ulcerative colitis (UC)
403 and Crohn's disease (CD) are 'new' diseases which emerged perceptibly in Western Europe
404 and North America in the middle of the twentieth century. They have increased in incidence
405 and prevalence to become major healthcare and economic problems throughout Europe and
406 North America as well as in other countries such as New Zealand and Australia (Molodecky
407 *et al.*, 2012). CD is generally increasing in incidence and prevalence and is now rising in
408 former low incidence countries such as India, Korea, Japan and China (Economou and
409 Pappas, 2008). Genetic mutations conferring an increased susceptibility are clearly
410 indentified in a proportion of people with these diseases, as they are in other chronic

411 infections such as leprosy (Franke *et al.*, 2010) . The genetic data provide valuable insights
412 into disease mechanisms. The development of CD is known to involve one or more
413 environmental factors and exposure is a strong candidate. *M. avium* subsp. *paratuberculosis*
414 is a multi-host intracellular pathogen which can cause systemic infection and chronic
415 inflammation of the intestine in many species including primates (McClure *et al.*, 1987;
416 Nielsen and Toft, 2009). Given the rising incidence of CD in adults and children (Shen *et al.*,
417 2011; Henderson *et al.*, 2012; Hope *et al.*, 2012) and the far wider environmental distribution
418 of *M. avium* subspecies *paratuberculosis* in the environment suggested here, the environment
419 may have an increasingly significant role within the infection triangle. The environment
420 provides many pathways for human exposure both on the farm and outside its confines
421 (Pickup *et al.*, 2006) resulting in exposure through food pathways (NACMCF, 2010)
422 including milk (Millar *et al.*, 1996; O'Doherty *et al.*, 2002; Okura *et al.*, 2012) and potable
423 and finished drinking water (Whan *et al.*, 2005; Pickup *et al.*, 2006; Pierce, 2009; Beumer *et*
424 *al.*, 2010). The study shows that the potential for human exposure is higher than previously
425 expected and that interventions to reduce Crohn's Disease may have to be both at the animal
426 and human level and may require human vaccination.

427

428 **Experimental procedure**

429 *Sampling regime*

430 *Soil cores*: Soil samples were collected between May and November 2007 from 233 1 km^2
431 squares across the UK as part of the Countryside Survey 2007
432 (CS2007:http://www.countrysidesurvey.org.uk/; Fig 1). The locations of the 1km^2 sampling
433 units are chosen from the intersections of a 15km systematic grid with random start point and
434 are stratified according to the ITE Land Classification. Further detail of the design and

435 stratification can be found in (Norton *et al.*, 2012). Within each 1 km^2 sampling area, up to
436 five soil cores were sampled (5 cm diameter, 15 cm deep) from the centre of randomly
437 allocated 200 m^2 sub-plots used for the vegetation survey. For each soil core the location
438 (eastings, northings and altitude) was recorded along with field measures of flora and habitat.
439 A land use description was ascribed to each sample (Smart *et al.*, 2003). Soil physical and
440 chemical characteristics were determined from a duplicate 15 cm core taken adjacent to the
441 microbial core (Emmett *et al.*, 2008). These numeric variables included pH; % carbon (C), %
442 nitrogen (N), C:N Ratio, % organic matter (loss on ignition), phosphorous (Olsen P, mg kg^{-1});
443 and soil moisture content (% moisture). In addition we also mapped the distribution of sheep
444 and cattle across the GB using data obtained from the 2004 GB Agricultural Census data
445 (http://edina.ac.uk/agcensus/), which is conducted in June each year by the government
446 departments dealing with Agriculture and Rural Affairs for Scotland, England, and Wales.
447
448 *River water* : River water samples (100 ml) were taken twice monthly between June and
449 December 2008 from a number of samples sites (Table 1) from both the rivers Douglas and
450 Wyre, as described by Neal and co-workers (Neal *et al.*, 2011).
451
452 *Bacterial strains and culture: M. avium* subsp. *paratuberculosis* K-10 was used as the
453 positive control strain throughout and was cultured on Middlebrook 7H10 agar supplemented
454 with OADC (10 % v/v) (BD Biosciences, UK) and mycobactin J (2 µg ml^{-1}; Allied Monitor,
455 USA), or in Mycobacterial Growth Indicator Tubes (MGIT), supplemented with OADC
456 (10% v/v), mycobactin J (2 µg ml^{-1}) and the PANTA antibiotic mixture (BD Biosciences,
457 UK). Cultures were incubated at 37°C for up to 8 weeks before DNA extraction.
458

459 *Processing of samples and DNA extraction:* 100 ml river samples were concentrated by
460 filtration through sterile 0.2 µm (pore-size) membrane filters (Supor-200, Pall Corporation,
461 UK). Retained material was resuspended in 3 ml of 10 mM Tris-HCl (pH 7.4) by vortexing
462 and scraping with sterile plastic culture loops. The cleaned membranes were discarded, and
463 the remaining sample was aliquotted into 1.5 ml microfuge tubes and concentrated by
464 centrifugation at 13,000 x g for 20 min. The supernatant was removed, and the sample was
465 pooled in a final volume of 300 µl. Total community DNA (tcDNA) was then extracted using
466 the Ultraclean Soil DNA kit (Mo-Bio, USA) with one modification to the manufacturers
467 protocol whereby bead-beating tubes were replaced by the use of Lysing Matrix B tubes (MP
468 Biomedicals, UK). Samples were then disrupted in a FastPrep 24 machine (MP Biomedicals,
469 UK) at a setting of 6.5 m s^{-1} for 45 s. DNA was extracted directly from 300 µl *M. avium*
470 subsp. *paratuberculosis* K-10 cultures using the same extraction procedure.
471 Aliquots of DNAs extracted from 1092 soil cores from the CS2007 were provided for the
472 present study.For the preparation of each, 0.25 g soil had been aseptically handled and
473 homogenized and tcDNA extracted using a previously described method (Griffiths *et al.*,
474 2000; Griffiths *et al.*, 2011) modified to include a 30 min hexadecyltrimethylammonium
475 bromide (CTAB) freeze-thaw, soft-lysis stage. Once aliquotted, DNAs were maintained at -
476 80°C until required.

477 *Real-time qPCR*: Where practicable all real time quantitative PCR amplifications were
478 carried and reported in accordance with the MIQE guidelines (Bustin *et al.*, 2009). All
479 amplifications were carried out on an ABI Prism 7000 Sequence Detection (Life
480 Technologies) in individual reactions using two previously verified *M. avium* subsp.
481 *paratuberculosis* assays (DH2 and DH3; (Herthnek and Bolske, 2006)) to amplify *M. avium*
482 subsp. *paratuberculosis*-specific regions IS*900* and F57, respectively. These assays
483 comprised primers and hydrolysis probes with the following sequences: forward primer

484 DH2F, 5'-GCC TTC GAC TAC AAC AAG AGC-3'; reverse primer DH2R, 5'-GCG TCG
485 GGA GTT TGG TAG-3'; DH2 hydrolysis probe 5'-6FAM-GCC GCG CTG ATC CTG CTT
486 ACT-TAMRA-3'; forward primer DH3F, 5'-AAC TAA GCG GAT CGA CAA TTC-3';
487 DH3R, 5'-TGG TGT ACC GAA TGT TGT TG-3'; DH3 hydrolysis probe 5'-6FAM-TGC
488 AAC TCG AAC ACA CCT GGG A-TAMRA-3' (Life Technologies, UK). Reactions were
489 performed in optical 96-well reaction plates (P/N N801-0560, Life Technologies). Primer and
490 hydrolysis probe concentrations were optimized according to protocols specific to the
491 TaqMan Universal PCR Master Mix (Life Technologies, UK). Each reaction (25 µl)
492 contained the following: 12.5 µl of 2x TaqMan Universal Master Mix (P/N 4318157, Life
493 Technologies, UK) 2 µl (300 nM) of each primer (P/N 4304972, Life Technologies, UK) and
494 2 µl (250 nM) of each probe (P/N 450003, Life Technologies, UK); 1.5 µl sterile PCR grade
495 water (Sigma-Aldrich, UK) and 5 µl DNA. No-template controls received sterilised PCR
496 grade water instead of DNA. The following cycling profile was used: 1 cycle of 50°C for 2
497 min (for optimal uracyl-*N*-glycosylase activity), 1 cycle of 95°C for 10 min (for activation of
498 the AmpliTaq Gold enzyme), and 40 cycles of 95°C for 15 s and 60°C for 1 min.

499 To construct standard curves, the number *M. avium* subsp. *paratuberculosis* cell equivalents
500 (CE) in a given volume of DNA was estimated due to typical cell clumping in cultures. The
501 estimation was based upon a genome size of 4.83 Mbp for *M. avium* subsp. *paratuberculosis*
502 K-10 (GenBank accession no. NC002944) and an average number of gene copies per genome
503 of 17 (IS*900*) and 1 (F57) (Poupart *et al.*, 1993; Li *et al.*, 2005). Control *M. avium* subsp.
504 *paratuberculosis* K-10 DNA was quantified using a Nanodrop ND-1000 spectrophotometer
505 (Labtech, UK) and serially diluted for use in standard curves. Sensitivity of the DH2 and
506 DH3 assays was determined for the present study using serially diluted K-10 DNA. The
507 sensitivity of detection of DH2 (IS*900*) was demonstrated to mimic that of the Herthnek and

508 Bölske (2006) study of 0.1 genomes per µl. However, sensitivity of the DH3 assay was
509 slightly lower in that it could not detect less than 10 genomes per µl (not shown).

510 For the assessment of *M. avium* subsp. *paratuberculosis* in river water samples,
511 amplifications were carried out in triplicate using the DH2 qPCR assay solely. Due to the
512 number of samples and separate inhibition testing, only duplicate reactions were carried out
513 for the assessment of *M. avium* subsp. *paratuberculosis* in British soils. The exception was
514 standards which were carried out in triplicate. Inhibition of the PCR reaction was tested by
515 the addition of 2 ng of *M. avium* subsp. *paratuberculosis* K-10 DNA (approximately 4×10^5
516 genomes) to all samples and to control reactions where no sample DNA was added. No
517 amplification, or a shift to a higher quantification cycle value (C_q; (Bustin *et al.*, 2009)) when
518 compared to the control reactions, was interpreted as inhibition.

519 *Statistical analysis*

520

521 For broad scale spatial analysis of *M. avium* subsp. *paratuberculosis* and correlations with
522 cattle and sheep density, we calculated the incidence of the species in spatially segregated
523 bins. The use of spatially segregated bins as opposed to more complex spatial clustering
524 analyses was necessary due to the systematic grid design of the Countryside Survey meaning
525 that the data do not conform to a stochastic spatial process. Furthermore, species incidence
526 was used because the number of samples taken within any particular 1km varied from 0 to 5.
527 It was therefore important to have these on a common scale and not allow unequal sampling
528 be a factor in the analysis. Using incidence also smooths out any confounding affect of
529 competing risks and ensures that interpretation is straight forward for the reader. Because of
530 the nature of this spatial analysis, the bins were defined in three distinct ways: according to
531 northing; easting; and altitude. Average cattle and sheep densities were also calculated in the
532 associated bins enabling us to test correlations between the two variables. Having derived the

533 incidence data in the respective bins, relationships with spatial variables and cattle and sheep
534 densities were examined using simple regression based techniques. Associated p-values,
535 indicating the significance of the fitted model, and R^2 values, indicating the goodness of fit,
536 were stored from the model output enabling intuitive understanding of the results. Plots of the
537 derived incidence data against explanatory variables were drawn and, in the case of
538 significant results, regression lines added. Because of the derivation of the data into three bin
539 categories, assessment of *M. avium* subsp. *paratuberculosis* occurrence against cattle and
540 sheep density is carried out three times – once for each bin category. This process makes it
541 easier to separate out spatial effects from direct cattle/sheep effects and is more conservative
542 in suggesting correlations rather than direct causative links.
543 To assess the relationships between *M. avium* subsp. *paratuberculosis* and soil and
544 environmental variables we adopted generalised linear model based approach (McCullagh
545 and Nelder, 1989) implemented in the R statistical programming environment (R:
546 Development Core Team, 2010). This approach is similar to standard linear regression but
547 with the added ability to include response variables with different distributions to the normal
548 distribution. That was essential in this analysis as we modelled the presence/absence data of
549 *M. avium* subsp. *Para*tuberculosis recorded in all the soil cores from CS2007. Hence a model
550 capable of handling binomially distributed data was required. Results from each of these
551 models provided us with significance terms representing the relationship with *M. avium*
552 subsp. *paratuberculosis* occurrence and the soil/environmental variable in question. Boxplots
553 of the raw data provide a visual representation to assess the significant results against and to
554 aid interpretation.
555
556 Finally, similar generalised linear models were adopted to examine the effects that different
557 habitat and land use intensity categories have on *M. avium* subsp. *paratuberculosis*

558 distribution. The slight difference between this and the previous analysis being that the
559 explanatory variables are categorical as opposed to continuous numerical variables. A simple
560 analysis of variance was performed to examine if the overall term (e.g. habitat) was a
561 significant factor in the occurrence of *M. avium* subsp. *paratuberculosis* and if the term was
562 significant a Tukey honest significant difference test (Miller, 1981), which corrects for
563 multiple testing, was performed to see which of the levels within the term (e.g. which specific
564 habitats) were significantly different. Simple barplots provide a visual reference with letters
565 associated with each bar defining whether two levels are significantly different (no letters in
566 common) or are not (at least one letter in common) at the 5% significance level.

567

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573

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872

873 **Figure legends**
874
875 **Figure 1**
876
877 Distribution of *M. avium subsp. paratuberculosis* over Great Britain at a resolution of 1km^2.
878 Each block represents a 1 km^2 Ordnance Survey GB (OSGB) grid square although for clarity
879 it has been expanded to a scale of 10km^2.
880 Fig. 1a. Distribution of all the sampling sites in this study.
881 Fig. 1b. Presence of *M. avium* subsp. *paratuberculosis* within each 1 km^2
882 (■Number of *M. avium* subsp. *paratuberculosis* positive within each 1 km^2
883 cores squares (see scale) □ *M. avium* subsp. *paratuberculosis* negative squares).
884
885 **Figure 2**
886
887 The relationship between *M. avium* subsp .*paratuberculosis*, sheep, cattle and their
888 distribution over Great Britain at a resolution of 1km^2. (a, *M. avium* subsp. *paratuberculosis*
889 incidence as a function of sheep density (p =0.389 R^2=0.0681); b, *M. avium* subsp.
890 *paratuberculosis* incidence as a function of cattle density (p = 0.0251 R^2= 0.3789); c, *M.*
891 *avium* subsp. *paratuberculosis* incidence as a function of northing p = 0.000112: R2
892 =0.7565); d, Sheep density as a function of northing (p=0.132425: R^2=0.1936); e, Cattle
893 density as a function of northing (p =0.00986: R^2= 0.4685)).
894
895 MAP incidence is the proportion of all the plots tested in which MAP was detected.
896
897 **Figure 3**

898 Boxplots showing the association between the presence and absence of *M. avium* subsp.
899 *paratuberculosis* (MAP) and soil and environmental parameters. (a, Soil pH in CaCl2; b,
900 Soil pH in Water; c, Mean temperature; d, Rainfall; e, Soil loss on ignition; f, Soil bulk
901 density; – denotes MAP absent; + denotes MAP present)

902 MAP incidence is the proportion of all the plots tested in which MAP was detected.
903
904 **Figure 4**

905 Barplot showing the incidence of *M. avium* subsp. *paratuberculosis* in differing habitat types.
906 (BMYW – Broadleaf, mixed, Yew woodland; CW – Coniferous woodland; AH- Arable and
907 horticultural; IG-Improved grassland; NG- Neutral grassland; AG- Acid grassland; Bn-
908 Bracken; DSH-Dwarf shrub heath; FMS- Fern, marsh and swamp; Bg – Bog). Bars sharing a
909 common letter are not statistically significantly different from each other, whereas bars with
910 no letter in common are significantly different at the 5% level.

911 MAP incidence is the proportion of all the plots tested in which MAP was detected.
912
913
914
915

Table 1 RT-qPCR detection of *M. avium* subsp. *paratuberculosis* (CE 100ml^{-1}) in the River Douglas and River Wyre from June to November 2008 (sites are ordered with respect to river flow from upstream (east) to estuary (west). (nd - not detected; ns - not sampled;)

River Douglas (a)	GBOS	Catchment size (km^2)	26-08-08	08-09-08	22-09-08	06-10-08	20-10-08	03-11-08
Adlington	SD60201262	38.80	nd	10-100	nd	nd	10-100	nd
Standish	SD57910988	59.95	nd	nd	nd	nd	10-100	nd
Parbold	SD48941030	151.01	nd	nd	nd	10-100	10-100	10-100
Grimshaw Green	SD47641250	187.38	10-100	10-100	10-100	nd	nd	10-100
Upto Tidal reach		22						

River Wyre (b)	GBOS	Catchment size (km^2)	19-06-08	15-09-08	29-09-08	13-10-08	27-10-08
Tarnbrook	SD58815559	10.68	10-100	ns	ns	ns	ns
Marshaw Bridge	SD56475421	18.87	ns	10-100	10-100	nd	nd
Stoops Bridge	SD56335438	27.58	ns	10-100	nd	10-100	10-100
Abbeystead	SD55575382	48.80	10-100	10-100	nd	100-1000	10-100
Dolphinholme	SD51925343	75.34	nd	ns	ns	ns	ns
Garstang Bridge	SD49324496	113.36	ns	10-100	nd	10-100	100-1000
Scorton	SD49984826	94.41	10-100	ns	ns	ns	ns
Bowgreave	SD48814300	143.10	10-100	ns	ns	ns	ns
St. Michaels	SD46164110	272.87	10-100	nd	nd	10-100	100-1000
Rawcliffe	SD40804127	317.80	10-100	ns	ns	ns	ns
Thornton	SD36954102	Tidal area	100-1000	ns	ns	ns	ns

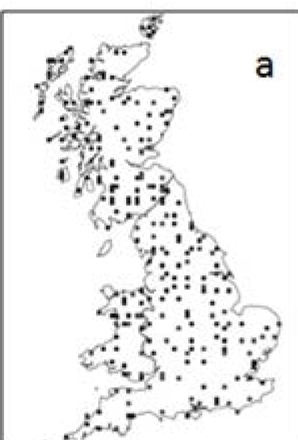
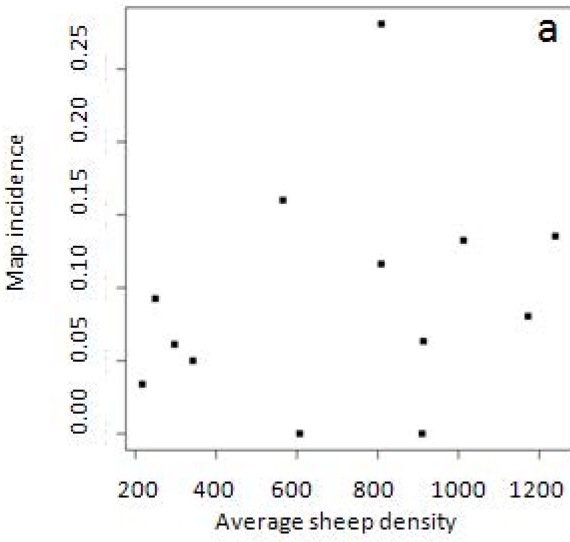
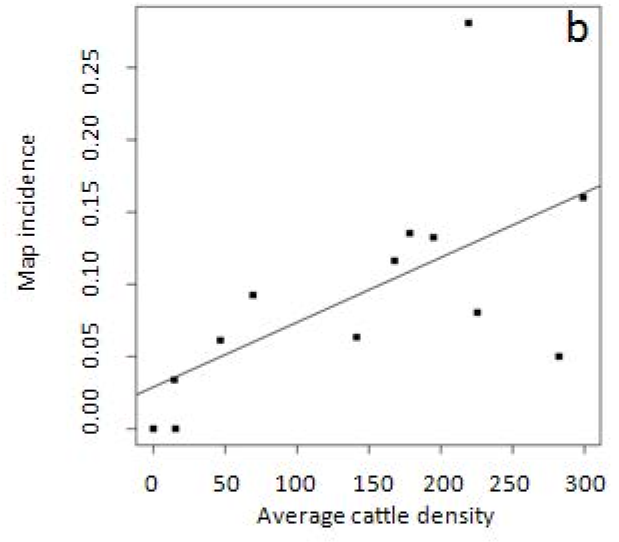


Figure 1





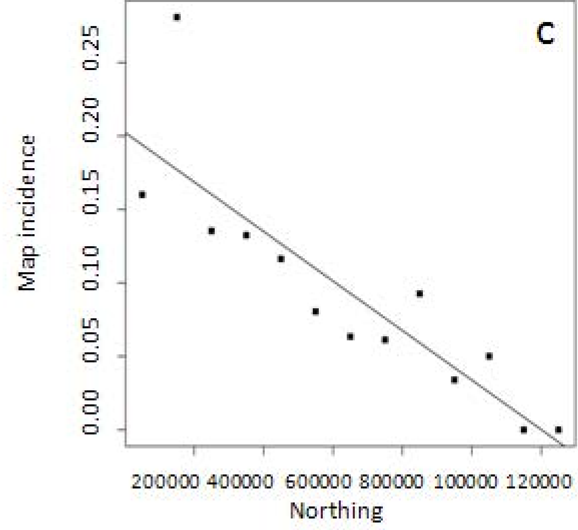
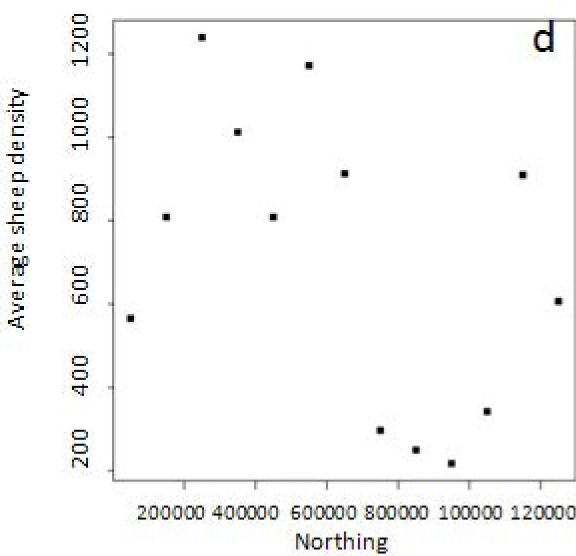
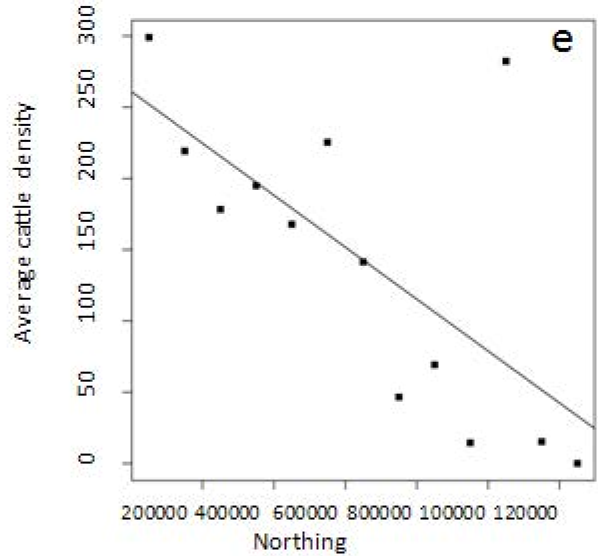
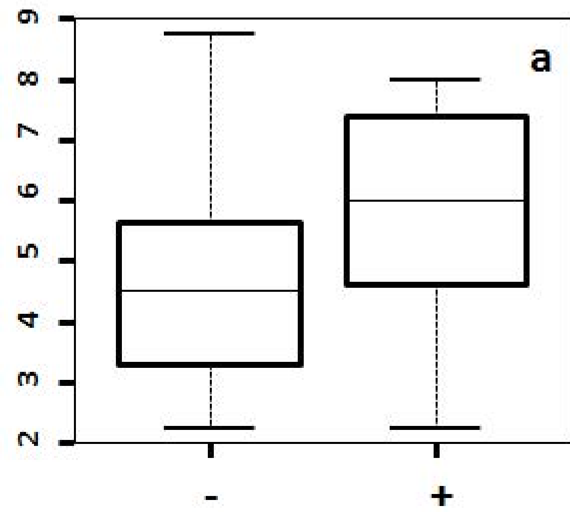


Figure 2







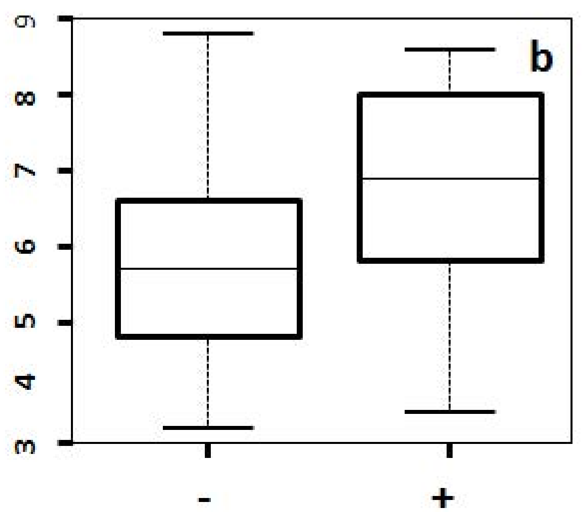
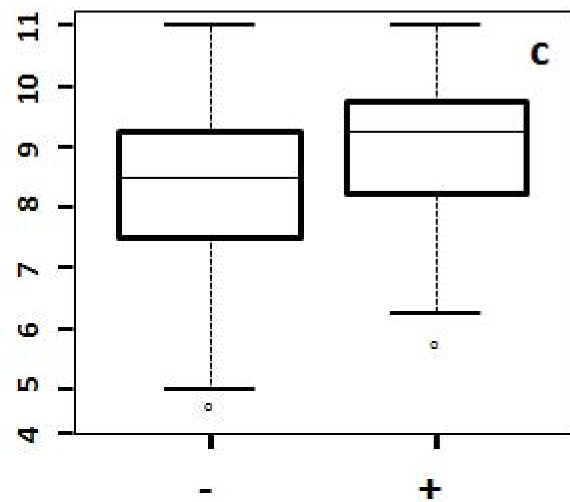
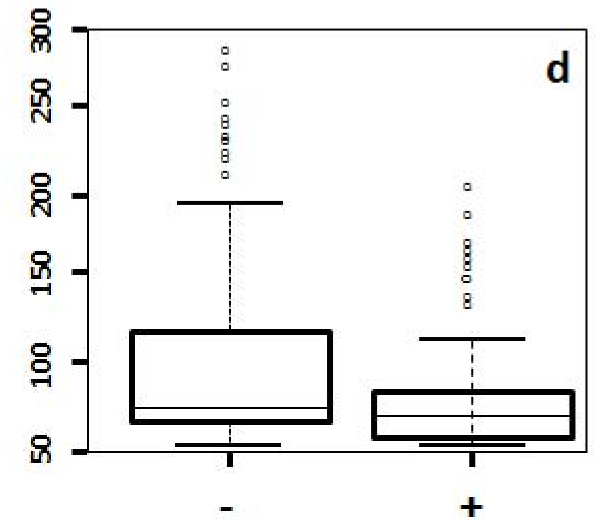
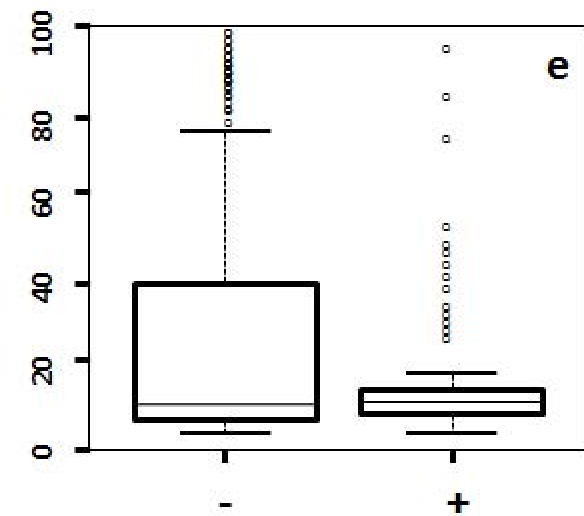


Figure 3







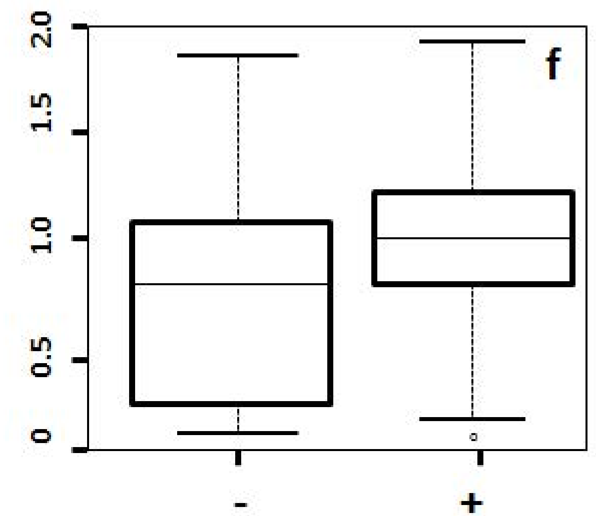


Figure 4