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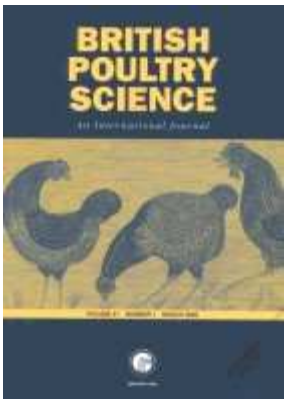

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Note to scientific Editor. Et al needs italicising. Journal titles need formatting. The Tables need some tidying up. There is just one Figure, which is a combined electrophoretic gel sequence and associated text – the Legend strikes me as a bit cursory – if you agree then I guess you have to consult the authors and get it expanded.

Influence of production system on the rate of onset of *Campylobacter* colonisation in chicken flocks reared extensively in the United Kingdom

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Short title: - *CAMPYLOBACTER* IN EXTENSIVE FLOCKS

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Abstract.

1. Because thermophilic *Campylobacter* spp. are common in chicken flocks reared extensively, cross-sectional and longitudinal studies were carried out on organic and free-range farms to determine the onset of colonisation (lag phase) and likely sources of flock infection.

2. For 14 organic and 14 free range flocks, there was a difference in lag phases, with the former being colonised at a mean of 14.1 d in comparison with 31.6 d for the latter. Whereas most free-range flocks became colonised when released on to pasture, those reared organically were usually colonised at the housed brooding stage.

3. Further study of organic flocks on three farms over 7 successive crop cycles confirmed that colonisation was strongly influenced by the prevailing husbandry conditions and was not a consequence of the length of the rearing period.

4. Molecular epidemiological investigations on a farm showing the shortest lag phase, using PFGE typing with two different restriction enzymes (*Sma*I and *Kpn*I) and *fla*A SVR sequence typing, revealed that potential sources of colonisation for organic chickens were already present on the farm at the time of chick placement. Such sources included the ante area of the brooding house, surrounding pasture and other livestock being kept on the farm.

5. Overall, the study demonstrated that, under UK conditions, the prevalence of colonisation was greater in extensive flocks (95 – 100%)

than it was for conventional broilers (55%), similar to the situation in other countries, but all three management systems showed comparable levels of caecal carriage in positive birds (log_{10}/g 6.2 – 6.7).

INTRODUCTION

Thermophilic *Campylobacter* spp. are environmentally widespread and a major cause of acute bacterial gastro-intestinal infection in man (Kramer et al., 2000; Newell and Fearnley, 2003). During 1992 – 2000, these organisms were responsible for an estimated 359 000 cases in England and Wales (Adak *et al.,* 2002). Although more than 90% of cases of campylobacteriosis are attributed to *Campylobacter jejuni*, there is increasing recognition of the role of *C. coli* in this disease (Tam *et al.,* 2003). In the developed world, most *Campylobacter* infections result from the ingestion of contaminated food, including raw milk (Fahey ei1995) and red meat (Kramer *et al.,* 2000); however, raw and undercooked poultry meat are considered to be the principal sources of human campylobacteriosis (Rivoal *et al*., 1999; Pearson *et al.,* 2000; Adak *et al.,* 2002) and approximately 80% of raw poultry being sold in the United Kingdom (UK) was found to be contaminated with *Campylobacter* (Jørgensen *et al.,* 2002).

In the UK, most poultry meat comes from birds that are reared in controlled-environment houses and at relatively high stocking densities. Such birds are usually slaughtered at 35 – 42 d of age (El-Shibiny *et al.,* 2005). Nevertheless, there is a growing market for free-range and organically-produced food products (Sato *et al.,* 2004). Poultry flocks of this

type are grown at lower stocking densities and, for the organic birds, there are strict rules governing the use of antimicrobials and organically-produced feedstuffs. Both types of extensively-reared birds are relatively slow-growing and are given low-energy, low-protein feeds; consequently, they are often slaughtered in the UK at around 56 and 73 d for free-range and organic flocks respectively. During the brooding period of up to 24-28 d, the flocks are continuously housed. However a key feature of the production systems used is the access given to pasture land, so that a proportion of the rearing period in each case is spent outside the house (El-Shibiny et al., 2005).

Conventionally-reared poultry have been studied widely in relation to colonisation by *Campylobacter* spp., which are generally carried asymptomatically, mainly in the caeca and colon (Corry and Atabay, 2001; Newell and Fearnley, 2003). However, several studies have shown a significantly higher prevalence of *Campylobacter* infection in organic chicken flocks than in those reared conventionally (Heuer et al., 2001; Luangtongkum et al., 2006; Van Overbeke et al., 2006) and a similarly high prevalence in other free-range chickens (Kazwala et al., 1993; Rivoal et al., 1999; El-Shibiny et al., 2005; Huneau-Salaün et al., 2007). Likely factors are thought to include the greater exposure of extensively-reared birds to environmental challenge, rearing for longer periods, which increases the risk of colonisation, and differences in susceptibility to *Campylobacter* infection among different breeds of chicken. However, recent work has questioned that the importance of environmental exposure (Colles et al., 2008).

Approximately 800 million chickens are slaughtered annually for meat production in the UK of which about 3% are extensively reared. Less than 1% of the total numbers are organically produced (Morris et al., 2009). However at present, there is increasing consumer interest in free-range poultry, mainly because of the perceived bird-welfare benefits (Fearnley-Whittingstall, 2008). It is important, however, that any public health risk associated with these birds is minimised. With regard to *Campylobacter*, such an aim will require a better understanding of factors involved in commercial bird-production systems and their influence on pathogen colonisation. In the present study, organic and free-range flocks belonging to major UK producers have been compared to each other and to conventionally reared birds in respect of *Campylobacter* colonisation. Particular attention has been given to the 'lag phase', the period following chick placement before *Campylobacter* colonisation becomes evident (Newell and Fearnley, 2003), which is often indicative of the rearing stage at which infection has occurred and may help to reveal deficiencies in biosecurity, leading to improvements in present practices.

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MATERIALS AND METHODS

Three separate studies were carried out to investigate *Campylobacter* colonisation in extensively-reared flocks. Twenty eight farms studied during 2004 and 2005 were associated with two (Company A and Company B) of the three major UK companies that specialise in the production of free-range and organic table chicken (16 and 12 farms from Company A and B respectively).

Study (i) and (ii) examined the time taken for *Campylobacter* colonisation to be first detected; 14 free-range flocks and 14 organic flocks from 28 farms were compared in study (i), while three organic farms from company A that had participated in study (i) were investigated over a further 7 crop cycles in study (ii), with environmental sampling being carried out on one of these farms. In addition, whole caeca were collected over a 4-month period (August to October) at a single processing plant in Company A to compare conventional and extensively-reared flocks for carriage of *Campylobacter* (study (iii)).

Rearing of the birds

The free-range flocks were reared in conventional-type broiler houses with access to an associated paddock and in accordance with European Union Poultrymeat Marketing Regulations (no. 1538/9). For organic flocks, the husbandry conditions were based on the requirements of Council Regulation (EEC) no. 2092/91, as amended. These flocks were brooded in static houses and moved into mobile houses at approximately 28 d of age except for one farm in Company A where they remained throughout in the same mobile house. In Company A, the mobile grower houses were situated on the same farm as the brooding houses. In contrast, the flocks in Company B were moved, by farm staff using their own crates and transport or by catching teams, to grower farms on different sites between 5 and 30 miles away.

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Three organic farms belonging to the same company were studied over seven crop cycles (study (ii)). Farm 1 brooded the birds in Modark modular houses (Creedy Carver Ltd, Crediton, Devon, UK). There were 4 such

houses, adjacent to each other in an open field, each containing about 1250 chicks. All these houses were filled at the same time. All houses had a concrete base and a gravel surround. Mains water was provided via a nipple drinking system and feed in pan feeders. The chicks were kept on wood shavings.

Farm 2 also had 4 Modark brooder houses, two of which were inside an old barn with a concrete floor and two outside in a concrete yard. Nipple drinkers supplied mains water to the birds and feed was provided in pan feeders. Wood shavings were used as litter. There was a weekly intake of approximately 1500 chicks with one of the 4 brooding houses being filled each week.

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At Farm 3, in a field, were two brooding houses constructed of wood on a steel frame, with a raised wooden floor. Each contained about 1000 chicks on wood shavings, with bell feeders and mains water provided in fountain-type drinkers. The brooding houses were filled alternately every two weeks and for the first week of each crop cycle an area was separated off by bales of straw at the front and rear of the house (designated ante area in this study). On this site, there were 12 separate growing houses, as well as some cattle, a free-range pig and two dogs.

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Collection and handling of samples

Study (i): To compare the time taken for the onset of *Campylobacter* colonisation between organic and free-range flocks, 14 flocks of each management type were sampled from Company A and Company B (8 and 6 flocks of each type respectively). Farmers used Amies Charcoal Transport

swabs (Medical Wire, MW171) to sample 4 caecal or faecal droppings at each of 5 locations within a house (near each corner and at the centre). The 5 pooled samples were taken twice-weekly and sent by post overnight to the laboratory where they were processed individually.

For study (ii), three farms from Company A were sampled daily by taking ten samples of caecal or faecal droppings from the parts of the house described previously. These were transported to the laboratory under chill conditions and examined within two hours of receipt as pools of 5 samples from each house.

Samples were taken from the environment surrounding the two brooder flocks on one of these farms (Farm 3) during the first 5 d of the third crop cycle using a standardised sampling plan. Surfaces were sampled by swabbing an area of approximately 100 cm^2 from each site using a sterile Readiwipe (Robinson Healthcare Ltd, Chesterfield, UK) pre-moistened in a small amount of Maximum Recovery Diluent (MRD, CM733,Oxoid, Basingstoke, UK,). Boot swabs in the form of gauze overshoes (Mike Bowden Livestock Service, Attleborough, Norfolk, UK) were used for sampling large areas of grass or concrete. These were pre-moistened in MRD and worn over plastic overboots (A547, Arnold, Shrewsbury, UK). Both swabs and overshoes were aseptically transferred, on the farm, to labelled containers with 150 ml modified Exeter broth (Humphrey et al., 1995). Faecal droppings from other animals on the farm were collected by inverting sterile plastic bags using sterile disposable gloves and were immediately transferred into Exeter broth as above. Water present in drinkers or puddles

was collected with the aid of a sterile pipette and dispensed into a sterile container with an equal volume of double-strength Exeter broth.

To compare standard housed and extensively-reared flocks for carriage of *Campylobacter*, in study (iii) whole caeca from 40 standard housed flocks and 41 extensively-reared flocks were sampled randomly on 20 separate days. These were transported to the laboratory under chill conditions and examined within two hours of receipt as pools of 10 from each flock.

Isolation, enumeration and confirmation of Campylobacter

Whole caeca were opened aseptically, the contents removed and pooled as required. Each pooled sample was mixed well using a sterile, cotton-tipped swab and serial 10-fold dilutions prepared in MRD. Duplicate aliquots (50 µl) of each dilution were plated on Modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA, Oxoid CM739 and SR155) for microaerobic incubation at 37°C for 48 h. Presumptive colonies of *Campylobacter* were subjected to the confirmatory tests described below and counts expressed as $\log_{10}$ cfu/g. In addition, 2 g of pooled contents were used to prepare enrichment cultures in 20 ml amounts of modified Exeter broth. These were incubated aerobically, with a small headspace and tightly-closed caps, at 37°C for 48 h, and then used to inoculate mCCDA.

Caecal or faecal droppings and swab samples were pooled, where required, and used to inoculate plates of mCCDA for isolation purposes only. Enrichment cultures were also prepared and inoculated on to mCCDA after incubation as were the environmental samples.

Presumptive colonies of *Campylobacter* spp., up to three per sample, where available, were subcultured onto Oxoid Blood Agar Base No 2 (Oxoid CM0271) and incubated microaerobically at 42°C for 48 h. All isolates were tested for Gram-stain reaction, production of oxidase and absence of aerobic growth. A selection of isolates was also confirmed with the Oxoid Campylobacter Test Kit. (DR0150M).

Identification of isolates

Species identification of presumptive *Campylobacter* isolates was carried out by a real-time PCR assay according to the method of Best et al. (2003), using the MX3005p (Stratagene, Amsterdam, The Netherlands), as described previously (Ridley et al., 2008a).

Storage of isolates

Confirmed isolates were stored in cryovials containing porous beads and glycerol. After inoculation with a fresh culture suspension from an agar plate, the vial was closed and inverted four to five times to emulsify the suspension. Excess cryopreservative was then removed, leaving the inoculated beads as liquid-free as possible. Vials were stored at –80°C while awaiting typing.

Typing of strains

On farm 3, environmental isolates and isolates from two brooder flocks (n=58) and from two previous flocks (n=6) were characterised by pulsed-field gel electrophoresis (PFGE). Firstly, each isolate was plated on Blood Agar containing Skirrow's Supplement (Oxoid CM0271 and SR0069) plus actidione (100 µg/ml) and cefoperazone (30 µg/ml). Plates were incubated

microaerobically at 42°C for 24 h. To prepare chromosomal DNA, cells harvested in phosphate-buffered saline were lysed in 1.2% agarose blocks (Gibson et al., 1995). The DNA was digested overnight at 25°C using 20 U *Sma*I. PFGE was performed on a DRIII apparatus (BioRad, Hemel Hempstead, UK) at 6.6 V / cm for 22.5 h, with pulse times increasing from 0.5 to 40 s. Standardised parameters were as proposed by CAMPYNET (http://www.medvetnet.org/cms/templates/doc.php?id=99&searchstring=CAMPYNET). Gels were stained with ethidium bromide and the images captured using UV illumination with a video system (Alpha-Imager HP, Genetic Research Instrumentation Ltd., Braintree, UK). The macrorestriction profiles were analysed using Bionumerics Software (version 5.0, Applied Maths, Kortrijk, Belgium). Similarities between profiles were derived using the Dice coefficient, with position tolerance values set at 1.5%. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). Thirty isolates recovered from samples taken at days 1 and 2 as well as the 6 isolates from the previous flocks were also typed by PFGE using *Kpn*I at 6.0 V / cm for 24 h, with pulse times increasing from 0.2 to 20 s and analysed as above. To confirm flock and environmental matches, *fla*A SVR PCR and subsequent sequencing was performed on a total of 23 isolates with the primers FLA242FU and FLA625RU (Meinersmann et al., 1997).

A 321 bp sequence containing the *fla*A SVR nucleotide sequence was compared with the database at http://pubmlst.org/campylobacter/flaA/ (Dingle

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et al., 2005). To provide strain type designation the following nomenclature was adopted: *Sma*I PFGE type/ *Kpn*I type */fla* SVR type.

Statistical analysis

Analysis of variance (ANOVA) was undertaken where appropriate using 'Minitab' software.

RESULTS

Time to onset of *Campylobacter* colonisation and possible sources of infection

Campylobacter colonisation was studied by regular monitoring of 14 free-range and 14 organic flocks from Companies A and B, study (i). For each flock, the time at which *Campylobacter* colonisation was first detected was noted. The results for all flocks are given in Table 1. With organic flocks, the onset of colonisation was detected at a minimum of 4 d and a maximum of 32 d. The corresponding figures for free-range flocks were 7 and 57 d, indicating that organic flocks were usually colonised earlier than their free-range counterparts ($P<0.01$). The former were colonised mainly at the brooding stage (mean 14.1 d) in comparison with a mean of 31.6 d for the latter. Only one organic flock was colonised post-brooding. When Company A and B were compared, results for the two sets of organic flocks were similar, with means of 14.6 d for Company A and 13.5 d for Company B. There was a greater difference between the two companies with respect to free-range flocks. In this case, the onset of *Campylobacter* colonisation occurred at a mean of 35.4 d for Company A and 26.5 d for Company B. However these

were not statistically different since time to colonisation ranged from 7 to 48 d in Company B, with 4 of the flocks becoming positive at the brooding stage.

In study (ii), three organic farms from Company A were studied over 7 successive crop cycles (Table 2). The results confirmed the relative rapidity with which organic flocks become detectably colonised, with means of 6.7 d for Farm 1 and 5.9 d for Farm 3, although there was usually a longer delay in colonisation at Farm 2 (mean 14.6 d).

In order to investigate the possible role of environmental contamination in the infection of brooder chicks, samples were collected from Farm 3 following placement of a single flock in each brooding house (Houses A and B) on the third crop cycle. One hundred and eighty one samples were taken from in and around the designated target flock in house A and the flock in the adjacent house (B) over the first 5 d following placement to identify possible sources of infection that might explain such early colonisation (Table 3). A total of 52 isolates from 45 samples recovered from the surrounding environment and an additional 6 isolates (6 samples) from the previous flocks, sampled during the second crop cycle, were typed by PFGE and compared with the strains recovered from the chicks. *fla*A SVR was performed on 23 of the isolates covering the present flocks, surroundings and the previous flocks for confirmation of genotypic identity. Faecal samples from both flocks were positive by day 2 (Table 3). The 6 isolates recovered from the 4 different faecal samples in the target flock and the adjacent flock (House B) belonged to two different genotypes (*Sma*I PFGE type 3-1 / *Kpn*I type I */fla* SVR type 497 (3-1/I/497) and 3-8/I/208), (PFGE types are shown in Figure 1). Isolates

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belonging to 3-1/I/497 were predominant (4/6) in faeces and were identified as *C. coli;* those of 3-8/I/208 were *C. jejuni*. Multilocus sequence typing (MLST), carried out using the method of Dingle et al. (2001) by the Dept of Zoology at the University of Oxford, demonstrated that all 9 isolates of type 3-1/I/497 examined belonged to MLST sequence type (ST) 829, part of clonal complex 828, while both isolates of type 3-8/I/208 were identified as *C. jejuni* MLST type 877. Chick isolates belonging to the predominant genotype, 3-1/I/497, were indistinguishable by *Sma*I PFGE and *fla*A SVR from 4 isolates recovered from 3 different samples collected from the adjoining field, around the brooding houses and from the ante area of the house on day 1. However, using the additional enzyme *Kpn*I, only 3 of these environmental isolates (from between the two houses and those from the ante-area of House A) matched by all genotypic methods investigated (Table 3, Figure). One of 6 isolates from the two previous brooding flocks in the same house also matched by *Sma*I PFGE (a mix of 3-1 and 3-3) and *fla*A SVR (497), but not by *Kpn*I, the colonising strain in the target flock. The relationship between these genotypes with identical *Sma*I PFGE and *fla*A SVR types but not *Kpn*I is unclear and requires further investigation. Nevertheless, the combined PFGE/SVR typing indicated that the ante-area and ground between the two tested houses were the most likely sources of flock contamination.

The second chick strain, (3-8/I/208) was only detected in House B flock samples but was also identified in an in-house overshoe sample from House A prior to placement (Figure 1). Thus, it appears as though the strains first colonising the chicks were present in and around the brooder house

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environment at the time of chick placement. In this flock the chicks were colonised by day 2, thereafter strains that genotypically matched the predominant flock type (3-1/I/497) were isolated from the main drive and cow faeces and from faeces from older poultry, which had been let out on to pasture in an adjoining field (Table 3, Figure 1) as well as from drinking water within the house, a foot-dip, water collected from old fountain drinkers stored outside the house, puddles on the main drive, and from a stile located between the area of the brooding houses and the farmhouse. Thus, once colonisation was detectable in the chicks, rapid dissemination by this strain occurred throughout the surrounding environment. For some environmental isolates, only minor changes in the size of a single band of the *Sma*I and/or *Kpn*I PFGE profiles were observed, indicating the presence of subtypes. Such minor variations in band size are well recognised in *Campylobacter* and are indicative of genomic instability (Wassenaar et al., 1988), possibly as a consequence of exposure to environmental stresses.

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In contrast, the second campylobacter strain found in the flock, 3-8/I/208, while detected in two environmental sites at day 2 (anteroom of house A and heater of house B), was not recovered from any of the 19 positive environmental samples investigated on day 5 suggesting poor environmental dissemination.

Species present, levels of colonisation and strain diversity

A total of 86 *Campylobacter* isolates obtained from the 28 organic and free-range flocks, study (i), was identified to species level. Of these, 47 (55%)

were identified as *C. jejuni* and 39% as *C. coli*, while 5 (6%) belonged to neither species and were not studied further.

Comparison of standard housed and extensively reared flocks for carriage of *Campylobacter*

From within a single poultry company, (Company A), organic (n=21), free-range (n=20) and conventionally reared (n=40) flocks were sampled by randomly collecting 10 caeca per flock from the common processing plant. The sampling of flocks from each type of rearing regime took place on 20 occasions equally spread over a 4-month period from August to October. The caecal contents were analysed for *Campylobacter* in positive birds as described. The data given in Table 4 show that there were no significant differences in carriage levels (mean $\log_{10}$/g 6.2 - 6.7) between the three production systems, despite the differences in flock age at slaughter. However, whereas 95 – 100% of the 41 organic and free-range flocks tested were *Campylobacter*-positive, the organism was found in only 55% of 40 flocks that had been reared conventionally ($P < 0.001$).

DISCUSSION

In this study, attention has been largely focussed on the lag phase, the period that precedes the onset of *Campylobacter* colonisation in poultry. This phenomenon has been partly attributed to the protective effect of maternal antibodies that are present initially in chicks obtained from infected parent stock (Cawthraw et al., 1994; Sahin et al., 2001, 2003), but other host factors, such as changes in gut-flora composition during development, could play a

part in resistance to early colonisation. Coliform bacteria and enterococci with antagonistic activity against *Campylobacter* have been isolated from chickens (Mead et al., 1996) within the lag phase and these microbial groups are among those known to be abundant in the young bird (Mead, 1989). Also, with conventional broiler flocks, it has been shown that the length of the lag phase can be usefully extended by improving flock biosecurity (Gibbens et al., 2001).

For extensive flocks in particular, it appears that the lag phase can be a useful indicator of the stage in rearing where a flock has become colonised by *Campylobacter*. It is clear from the results obtained that some free-range flocks remained uninfected for relatively long periods, up to 57 d, but organic flocks were colonised much earlier and this occurred mainly at the housed brooding stage that has a duration of 24 – 28 d.

At Farm 3, an organic farm that was studied in detail, the predominant colonising strain had matching *Sma*I PFGE and *fla*A SVR types to that associated with the previous brooding flock, suggesting either a poor standard of biosecurity or inadequate cleaning and disinfection of the house after the previous birds were transferred to growing houses. The farm had no concrete area on which to contain the chicks and used fountain drinkers that are more prone to microbial contamination than the nipple drinkers used on the other two farms studied. Also, Farm 3 contained a mixture of other animals, which may have been reservoirs of *Campylobacter*, thus increasing the risk of flock infection. Supportive evidence for this was obtained from PFGE typing, which showed that a strain isolated from a cow on the site on day 2 was

indistinguishable from those found in the study flock. Genotypic evidence to support the role of cattle as a possible source of infection of broiler flocks has recently been reported (Ridley et al, 2008a; Zweifel et al, 2008). Although the proximity of pig holdings has been associated with an increased risk of broiler flocks testing positive for *Campylobacter* (Lyngstad et al, 2008), *Campylobacter* was not recovered from the pig faecal samples collected during this study. Once the brooding flock had been colonised, strain 3-1/I/497 spread rapidly in the vicinity of the house, again an indication of poor biosecurity.

Among the organic farms, the lag phase was longest at Farm 2, where chicks were held initially within an enclosed concrete area that would have facilitated post-crop cleaning and disinfection, and there was a clear separation between the brooding area and the growing houses that were situated some distance away. Although the potential for practising appropriate biosecurity was seen to vary between the farms being studied, it is evident that more attention to this aspect of bird husbandry could be beneficial in reducing the risk of *Campylobacter* colonisation, at least during the stage at which the birds are housed. In a Belgian study (Van Overbeke et al., 2006), organic flocks were detectably colonised after a much longer period, between weeks 7 and 10, suggesting that farm biosecurity measures were more effective in this case.

In contrast to the organic flocks studied here, those designated free-range were mostly colonised after the brooding stage and therefore showed a significantly longer lag phase. Several factors may have affected the extent

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of this lag phase. Firstly, the birds were kept in the same houses and adjoining paddocks throughout the rearing period, thus limiting exposure to environmental organisms. Secondly, the management system allowed a longer cleaning period between crops, with the surrounding area being free from poultry for at least 5 weeks at a time perhaps enabling a significant reduction in *Campylobacter* contamination of the environment. Although both free-range and organic birds were of the same slower-growing breed (Hubbard), the free-range farms were more like those used for conventional flocks, with comparable standards of cleaning and disinfection, and the birds were subject to the same degree of biosecurity while they were housed. These flocks were of a single age, whereas the organic flocks of Company A were kept on "multi-age" sites and, in the case of Company B, the intake of chicks was staggered over a 6-d period, thereby increasing the risk of exposure to *Campylobacter*. The importance of biosecurity during the initial stages of rearing was evident from a French study (Huneau-Salaün et al., 2007) where 71% of free-range flocks studied were colonised by *Campylobacter* before being released on to pasture.

Once the birds are released from the house, standard biosecurity measures are thought to be largely irrelevant, because of the unavoidable exposure to environmental campylobacters (Rivoal et al., 2005). More recently, however, this view has been challenged and Colles et al. (2008), in a study of free-range chickens, found no evidence of flock-to-flock transmission, no association of ranging behaviour with the likelihood that the birds would shed *Campylobacter*, and a clear distinction between the

population of *C. jejuni* carried by certain wild birds and the chickens. Nevertheless, it seems most likely that transmission into all broiler flocks is horizontal from the environment and this is supported by convincing evidence using highly discriminatory typing methods reporting the presence of identical strains in the farm environment prior to colonisation in the flock (Bull et al., 2006; Johnsen et al., 2006).

Given the opportunity for challenge of extensively-reared flocks by environmental strains a wide diversity of colonising strains would be anticipated. Strain tracking indicated, like those reared conventionally in the UK (Ayling et al., 1996), the two target flocks on Farm 3 carried a restricted number of predominant strain types. However, these belonged to different species (*C. jejuni* and *C. coli*).

The presence of both species in extensively–reared flocks has been described elsewhere (Rivoal et al., 2005), but significant biodiversity among strains of *Campylobacter* was observed. In the present study almost 40% of caecal isolates examined from 28 extensively reared flocks were identified as *C. coli*, which was consistent with data from another study of organic flocks in the UK by El-Shibiny et al. (2005). One suggestion is that *C. coli* has a colonisation advantage over *C. jejuni*, over time, thus accounting for its frequent presence in extensively-reared birds at the time of slaughter. One interesting observation was diversity in strains recovered from flock and environmental samples, as detected by minor changes in PFGE patterns, as a result of apparent genetic rearrangements. Similar observations were reported by Rivoal et al., (2005). Such rearrangements may be a consequence of

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environmental stresses during colonisation (Ridley et al., 2008b), which over the longer period of chicken rearing might become more evident.

Whatever the risk of flock infection from exposure to pasture, the present study has confirmed previous work from other countries showing that chicken flocks reared under extensive conditions are particularly susceptible to *Campylobacter* colonisation and often have a higher prevalence of colonisation at slaughter than, conventionally-reared birds (Kazwala et al., 1993; Heuer et al., 2001; Luangtongkum et al., 2006; Van Overbeke et al., 2006). However, this could not be explained entirely by environmental exposure or the longer rearing period, and appeared to be more to do with in-house husbandry conditions than any other single factor. With the growing popularity for the consumption of organic and free-range chickens in the UK, it appears that any associated public health risk from *Campylobacter* would be related primarily to the high prevalence of flock infection rather than increased levels of intestinal carriage, which were found to be similar in samples from organic, free-range and conventionally-reared flocks. Work is now being done to establish the most effective biosecurity measures for use in extensive poultry production.

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Table 1. Comparison of free-range and organic farms: time taken for *Campylobacter* colonisation to be first detected in each flock studied.

Free-range farm/Flock No	Time to detectable colonisation (d)	Organic farm/Flock No	Time to detectable colonisation (d)
Company A			
A / FR 1	57	A / OR 1	32
A / FR 2	37	A / OR 2	16
A / FR 3	46	A / OR 3	20
A / FR 4	30	A / OR 4	20
A / FR 5	27	A / OR 5	6
A / FR 6	37	A / OR 6	4
A / FR 7	26	A / OR 7	8
A / FR 8	23	A / OR 8	11
Company B			
B / FR 1	18	B / OR 1	14
B / FR 2	7	B / OR 2	7
B / FR 3	42	B / OR 3	14
B / FR 4	28	B / OR 4	18
B / FR 5	48	B / OR 5	7
B / FR 6	16	B / OR 6	21
Mean (d)	31.6 ± SD 13.8		14.1 ± SD 7.7 (p<0.001)

Table 2. First detection of *Campylobacter* colonisation: comparison of data from seven successive crop cycles on each of three organic farms belonging to Company A.

	Time to detectable colonisation (d)							
Farm	Crop no.							
	1	2	3	4	5	6	7	Mean ± SD
1	4	9	10	5	5	6	8	6.7 ± 2.3^{a}
2	10	20	18	11	15	11	17	14.6 ± 3.9^{ab}
3	9	6	2	3	12	2	7	5.9 ± 3.8^{b}

[a & b] significant difference p=0.001

Table 3. Samples positive for *Campylobacter* spp. on Farm 3.

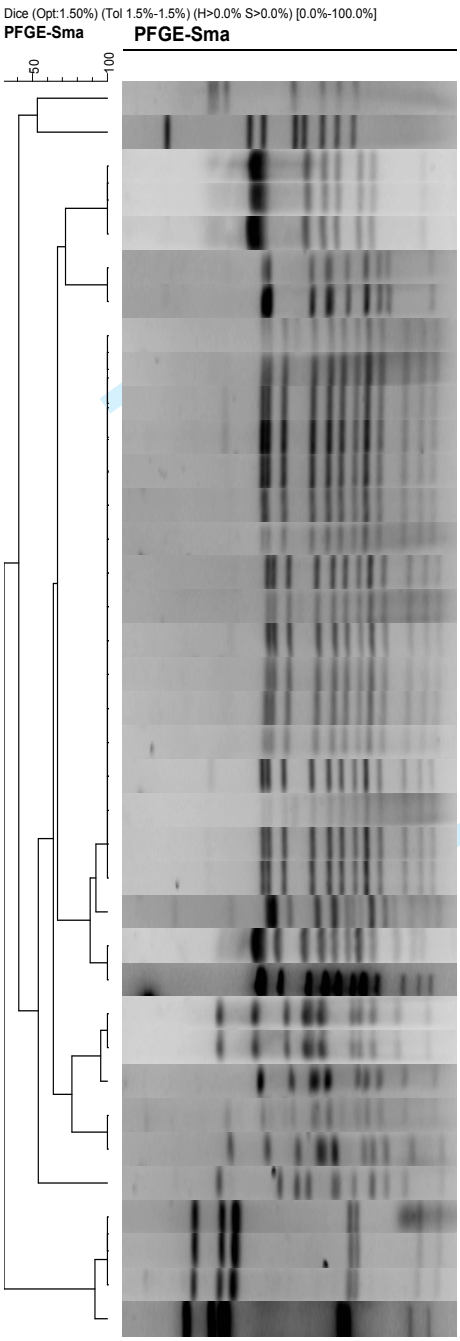
Bird age (days)	No. of positive samples	Total no. of samples	Description of culture-positive environmental samples	combined PFGE matches to flock[d]
1	7	53	Overshoes in adjoining field	1/2
			Overshoes around houses	1/2
			Overshoes in ante area	1/1
			Overshoes in house A [b]	0/1
			Rat faeces outside house A	0/1
2[a]	23	70	Overshoes around houses	1/3
			Overshoes front drive	1/1
			Cow faeces in adjoining field	1/1
			Swab from stile	1/1
			Puddle water on front drive	0/1
			Old water from drinkers	1/1
			Overshoes and faeces from 'older' chickens	2/3
			Ante area house A	2/3
			Litter from house A	1/1
			Litter from house B[c]	2/3
			Swab from under roof outside house A	0/1
			Foot-dip water house A	1/1
			Drinkers in house B	2/2
			Heater in house B	1/1
5	19	58	Faeces from cow in adjoining field	1/1
			Faeces from 'older' chickens in adjoining field	1/1
			Overshoes between house B and field	2/2
			Ante areas in house A	3/3
			Feed house A	1/1
			Swabs from drinkers in house A	1/1
			Swabs from drinkers in house B	2/2
			Litter from house A	2/2
			Overshoes from house A	2/2
			Litter from house B	4/4
Previous flocks	6	10	Previous crop in house A	0/3
			Previous crop in house B	0/3

[a] Flocks found to be colonised at this visit.
[b] Target brooder house.
[c] Adjacent brooder house.
[d] Number matching by both *Sma*I and *Kpn*I types/Total number tested

Table 4. Prevalence and levels of *Campylobacter* spp. in pooled caecal samples* from colonised flocks reared under different systems within a single company (company A).

Flock type	Free-range	Organic	Conventional
Slaughter age	54-56days	72-74days	41-43 days
No. examined	20	21	40
No. positive (%)	19 (95)	21 (100)	22 (55)
Geometric mean count (log_{10} cfu / g)	6.70 ± SD 1.27	6.52 ± SD 1.21	6.23 ± SD1.85
Median	7.15	6.35	6.74

* Ten samples taken from each flock.



Isolate ref	age of birds	source	*Sma*I type	*Kpn*I type
3/1/A/c	1 day	in between A and B	3-4	VII
3/2/A/21a	2 days	under roof of house	3-6	VIII
caeca a 15/10	N/A	previous flock	3-3	III
caeca b 15/10	N/A	previous flock	3-3	III
caeca c 15/10	N/A	previous flock	3-3	III
3/2/A/7c	2 days	anteroom house A	3-5	V
3/2/A/68a	2 days	puddle on drive	3-5	V
3/1/A/3a	1 day	in between A and B	3-1	I
3/1/A/3b	1 day	in between A and B	3-1	I
3/1/A/5c	1 day	anteroom house A	3-1	I
3/2/A/17a	2 days	faeces house A	3-1	I-a
3/1/B/1a	1 day	field	3-1	II
3/2/A/70a	2 days	old water from drinkers	3-1	I
3/2/A/7a	2 days	anteroom house A	3-1	I
3/2/A/27a	2 days	footdip house A	3-1	I
3/2/A/28a	2 days	anteroom door hs A	3-1	II
3/2/B/36a	2 days	faeces in house B	3-1	I
3/2/B/37a	2 days	faeces house B	3-1	I
3/2/B/46a	2 days	between house B and field	3-1	I
3/2/B/50a	2 days	older bird houses	3-1	I
3/2/B/69a	2 days	drinkers	3-1	I
3/2/B/53a	2 days	stile	3-1a	I
3/2/B/51	2 days	older bird houses	3-1	I
3/2/B/55	2 days	cows	3-1	I
3/2/A/59a	2 days	front drive	3-1b	I-a
caeca a 1/10	N/A	previous flock	3-1/3-3	II
3/2/A/7a	2 days	anteroom	3-1	I
caeca b 1/10	N/A	previous flock	3-2	IV
caeca c 1/10	N/A	previous flock	3-2	IV
3/1/A/42a	1 day	rat faeces in field	3-2a	IV
3/1/B/2a	1 day	field	3-2	IV
3/2/A/63a	2 days	front of houses	3-2	IV
3/2/A/66a	2 days	older bird houses	3-9	VI
3/2/A/29a	2 days	front of anteroom	3-8	IX
3/2/B/35b	2 days	faeces house B	3-8	IX
3/2/B/41a	2 days	heater in house B	3-8	IX
3/1/A/20a	1 day	in-house pre-placement	3-8	IX

Fig. 1. Relationships of *Sma*I and *Kpn*I PFGE types for *Campylobacter* isolates from brooder houses and surrounding environment at Farm. 3, initially (day 1) and at the time the flock was found to be positive (day 2)

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