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A longitudinal study of environmental salmonella contamination in caged and free-range layer flocks

Andrew Wales, Mark Breslin, Ben Carter, Robin Sayers, Robert Davies*

*Department of Food and Environmental Safety, Veterinary Laboratories Agency,
Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom*

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Author for correspondence: Robert Davies

Telephone: 01932 357361

Fax: 01932 357595

E-mail: r.h.davies@vla.defra.gsi.gov.uk

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A longitudinal study of environmental salmonella contamination in caged and free-range layer flocks

Andrew Wales, Mark Breslin, Ben Carter, Robin Sayers, Robert Davies*

Abstract

The environmental contamination by salmonella was examined over a 12-month period in 74 commercial layer flocks from eight farms in the UK, which previously had been identified as being contaminated with salmonella. Samples of faeces, dust, litter, egg belt spillage and wildlife vectors were taken, plus swabs of cages, feeders, drinkers, floors, egg belts and boots. Some sampling was performed in each month of the year. Numerous serovars were detected but *Salmonella enterica* serotype Enteritidis was the only persistent serotype found among single-age flocks. There was a significant correlation between qualitative environmental samples and semi-quantitative faeces samples. The level of environmental contamination increased significantly over time. There were significant temperature and seasonal effects upon contamination. Wildlife vectors proved to be sensitive samples for the detection of salmonella. The efficacy of cleaning and disinfection upon residual salmonella contamination, and upon subsequent flock contamination, was highly variable between and within premises. The variability between detected prevalences over time and between flocks indicates a need for regular, sensitive monitoring of flocks for salmonella to permit targeting of control measures aimed at eliminating contamination of the layer environment by salmonella. There is substantial scope for improvement of cleaning and disinfection procedures.

1 Introduction

2 *Salmonella enterica* serotype Enteritidis is the most common identified cause of
3 human salmonellosis in the UK (Health Protection Agency, 2005) and is amongst the
4 most significant *Salmonella* serotypes in public health elsewhere, including other
5 parts of Europe (Fisher, 2004b) and North America (CDC, 2004). Undercooked and
6 raw eggs have been heavily implicated in human infection with *S. Enteritidis* (Coyle
7 *et al.*, 1988; St Louis *et al.*, 1988; Hogue *et al.*, 1997; Palmer *et al.*, 2000; CDC, 2004;
8 De Buck *et al.*, 2004; Crespo *et al.*, 2005). This serotype is able to cause long term
9 colonisation of the chicken reproductive tract and become deposited within egg
10 contents (Berchieri *et al.*, 2001; Okamura *et al.*, 2001; Amy *et al.*, 2004; Guard-
11 Bouldin *et al.*, 2004), and in addition will cause external contamination of the shell
12 (Messens *et al.*, 2005).

13 Improved biosecurity and hygiene in the UK poultry industry plus vaccination
14 of the majority of commercial laying birds and broiler breeders, introduced in the mid
15 to late 1990s, has been followed by a large reduction in reported incidents of
16 *S. Enteritidis* in poultry and in humans (Anonymous, 2005; Health Protection Agency,
17 2005). Similar improvements have also occurred in some other countries (Wegener *et*
18 *al.*, 2003; Marcus *et al.*, 2004; Mumma *et al.*, 2004) but there is still a significant
19 reservoir of infection in commercial laying flocks (Adak & Gillespie, 2004; Crespo *et*
20 *al.*, 2005). On commercial laying farms, persistent environmental contamination is
21 currently considered to be the predominant problem (van de Giessen *et al.*, 1994;
22 Davies & Breslin, 2003b; Gradel *et al.*, 2004). Vaccination and other interventions
23 such as competitive exclusion products do not reliably eliminate infection (Davies &
24 Breslin, 2003a; Davies *et al.*, 2003), and their effectiveness is reduced where there is a

1 heavy environmental challenge (Nakamura *et al.*, 1994; Davies & Breslin, 2003a,b;
2 Nakamura *et al.*, 2004).

3 Sampling the hen house environment, when coupled with suitable cultural
4 techniques, has proved to be a sensitive and cost-effective method of monitoring
5 salmonella carriage and excretion by layer hens (Kradel & Miller, 1991; Henzler *et*
6 *al.*, 1994), and there is good agreement between the level of environmental
7 contamination and the level of internal egg contamination and associated human
8 disease (Altekruse *et al.*, 1993; Henzler *et al.*, 1994; Schlosser *et al.*, 1995; Henzler *et*
9 *al.*, 1998; Mallinson *et al.*, 2000). The sensitivity of environmental sampling varies
10 between sample types (Davies & Wray, 1996), and in caged layers samples taken
11 from egg belts, from dust close to birds or cage stacks and from naturally accumulated
12 pooled chicken faeces on droppings belts or scrapers have proved especially useful
13 (Davies & Breslin, 2001).

14 The prevalence of eggs contaminated internally or externally by salmonella
15 from infected flocks appears to vary substantially, between less than 0.03% and 1%
16 overall, but with up to 35% of some batches positive (reviewed by Davies & Breslin,
17 2004; De Buck *et al.*, 2004). Although technical factors may account for some
18 variation between studies, it is unclear to what extent factors such as the time since
19 the introduction of salmonella into the flock, the stage of lay and stressors such as hot
20 weather can affect the level of infection in the flock and the production of
21 contaminated eggs. Longitudinal studies are an appropriate way to address these
22 questions, but the requirements of resource and cooperation by producers over months
23 or years tends to limit the number and scope of such investigations. Consequently,
24 there are few reports that have examined the levels of salmonella in laying houses and
25 hens in lay over an extended period of time (Davison *et al.*, 1999; Davies & Breslin,

1 2003b; Kinde *et al.*, 2005). The present report describes a longitudinal study
2 conducted on several layer premises over a 12-month period.

3

4 **Materials and methods**

5 **Sample collection.** A number of caged and free-range layer flocks were identified as
6 having *S. Enteritidis*, by reference to the Zoonoses Order Database, or by personal
7 contact or sampling. Approaches were made to the owners of these flocks and, when
8 permission for intensive sampling had been obtained, they were visited and
9 environmental samples were taken on successive occasions at differing stages of lay
10 and also following depopulation, cleaning and disinfection. Sampling was systematic
11 and targeted at sites and types of material that were likely to reveal salmonella
12 contamination if present, based upon previous experience. The number and types of
13 samples taken on each occasion was, in addition, determined by several factors. These
14 included: the need for reasonable coverage of hen houses of differing sizes, the
15 amount of certain sample types (e.g. dust, egg belt spillage and faeces) present
16 according to design and usage, and constraints on access to certain sample types
17 imposed by the hen house construction. On some visits an increased number of
18 samples was taken to better define the distribution of salmonella within the house.

19 Samples were taken directly into 225 ml of buffered peptone water (BPW:
20 Merck) using gauze surgical swabs (Kleenex Readiwipes: Robinson Healthcare). For
21 qualitative detection of salmonellas, samples consisted of approximately 25 g faecal
22 material, spillage from egg belts and from floors under cages, litter from free-range
23 houses, dust from within and around cages and nest boxes (10 to 15 g), rodent faeces
24 (1 to 2 g) and flies from adhesive paper or contact insecticidal traps (1 to 2 g). In
25 addition, sterile swabs soaked in BPW were used to sample the surfaces (0.5 m²) of

egg belts, feeder troughs, cleaned droppings boards, floors beneath cages and the boots of free-range unit workers, The interiors of empty cages and drinker spillage cups or troughs were also swabbed, using composite samples of eight cages per swab. For semi-quantitative culture, bulked samples of chicken (approximately 30 g) and rodent (approximately 1 to 2 g) faeces were collected into dry pots.

All solid samples were returned to the laboratory at ambient temperature on the day of collection and processed immediately. Swab samples taken into BPW were, in addition, stored and transported in a cold box at below 10 °C. Mouse and rat carcasses were collected as available and 3 g of the liver, spleen and intestines were removed aseptically for culture at the laboratory.

Culture technique. For standard (qualitative) isolation, samples in BPW were pre-enriched at 37 °C for 18 h and then 0.1 ml of the pre-enriched mixture was inoculated onto modified semi-solid Rappaport-Vassiliadis agar with 0.01 % novobiocin (MSRV; Difco 218681) and incubated at 41.5 °C for 16 to 24 h. Where opaque growth was seen on MSRV, a 1 µl loop from the edge of the opaque growth zone was inoculated onto Rambach agar (Merck 107500). Rambach and associated MSRV plates were incubated at 37 °C and 41.5 °C respectively for 24 h. The plates were examined and any MSRV plates on which the growth had spread widely, but which were negative for salmonella on the Rambach plates, were subcultured again onto Rambach agar. Representative *Salmonella* isolates were confirmed by complete serotyping at the Salmonella Reference Laboratory at VLA - Weybridge according to the Kaufmann-White Scheme (Popoff, 2001).

For semi-quantitative culture (Wales *et al.*, 2006a), 90 ml BPW was thoroughly mixed with each 10 g bulked chicken faeces sample, and similarly 80 ml

BPW was mixed with 20 g samples. Ten or 20 ml BPW was added to pools of mouse and rat droppings respectively. From each of these initial tenfold or fivefold dilutions of chicken or rodent faeces, a 10% volume aliquot was dispensed into a universal container, to serve as the first in a decimal dilution series which was continued by taking 1 ml from the initial dilution, mixing it with 9 ml BPW and successively repeating this step five times, adding 1 ml of each consequent dilution to 9 ml BPW. A pre-enrichment incubation at 37 °C for 18 h was performed on all preparations, comprising the primary 1:10 or 1:5 mixture of faeces in BPW ('0'), the 10% volume aliquot of the same ('1'), and the decimal dilutions ('2' to '7'). After incubation, 0.1 ml of each of preparations '0' and '1' was cultured on selective (MSRV) and indicator (Rambach) media as described above. Preparations '2' to '7' were refrigerated, and were then similarly cultured if either the '0' or '1' preparations yielded growth. A semi-quantitative result was derived using the highest numbered preparation of each sample that yielded salmonellas to indicate the most probable range of salmonella colony-forming units (cfu) in the original sample. A semi-quantitative score was given to each bulked chicken faeces sample, depending on the highest dilution that yielded salmonellas upon culture (Table 1).

18

19 **Statistical analyses**

20 *Prevalence versus semi-quantitative data.* For each sampling visit from which semi-quantitative data was obtained, the overall house prevalence of positive samples, derived from the standard (qualitative) isolation procedure, was assigned to one of four bands: <20%, 20-40%, 40-60% and >60%. These were tabulated against the semi-quantitative score (zero to five) from faeces (caged) or litter (free-range)

24

samples. The exact probability for a linear by linear association test was calculated by StatXact software (Cytel, Cambridge, MA, USA).

Effects of season, temperature, stage of lay and vector activity. A non-linear mixed effects method was implemented to model the binary outcome: positive or negative for salmonella for each qualitatively-cultured sample. The data used for fitting the model was from caged single aged flocks, excluding the A&L farm that was visited only once. The model was fitted using a logit-link and the binomial distribution within Proc NLMIXED, in SAS (SAS Institute, Cary, NC, USA) (Gilmour *et al.*, 1985; Lindstrom & Bates, 1990; Pinheiro & Bates, 1995). The random effects matrix was estimated by a non-adaptive Gaussian quadrature method, with 201 points used. The linear predictor was:

$$\eta = \mu + \tau_i + \beta_0.MIH + \beta_1.Temp_i + Z$$

Where μ is the intercept, τ_i is the farm ($i = 1, 2, \dots, 5$), MIH is the age of the birds prior to entering the active experiment, and $Temp_i$ is the average monthly temperature recorded at the weather station closest to each farm i , as supplied by the Meteorological Office (2006). The random effect matrix Z includes the flock effect, over time. The model fitting process tested terms for their strength of evidence in affecting the odds of infection, including fly and wildlife infestation from semi-quantitative estimates of severity in each house, and the season of sample taken. Seasons were defined as: March-May (spring), June-August (summer), September-November (autumn) and December-February (winter). Since the temperature effect was completely nested within the season effect, nested models were compared and the

1 likelihood ratio test was used to show if there was any variation attributable to season
2 in addition to that accounted for by temperature. For each effect found to be
3 significant, confidence intervals were calculated for the odds ratio of a sample being
4 salmonella-positive when one state was compared to another, e.g. a particular number
5 of months in house compared to an additional increment of one month.

6

7 **Results**

8 Sampling was performed over a 12-month period from August 2004 to July 2005 and
9 a total of 74 flocks (59 caged, 15 free-range) were sampled from eight farms. All
10 flocks had been vaccinated against salmonella, the majority using a live *S. Enteritidis*
11 vaccine with or without a live *S. Typhimurium* component. In the remaining flocks an
12 inactivated *S. Enteritidis* plus *S. Typhimurium* (*Salenvac T*) or a live *S. Gallinarum*
13 vaccine providing cross-protection against *S. Enteritidis* (*Nobilis SG 9R*) had been
14 used. In addition, an oral competitive exclusion treatment had been given to
15 approximately half the flocks. All farms except two (Gra and Sut) operated an all-in-
16 all-out policy for stocking hen houses, potentially allowing thorough cleaning and
17 disinfection (C & D) of accommodation between flocks. Between one and four visits
18 were made per flock at intervals of two to six months (tables 2 and 3), with flock ages
19 varying between less than one and 19 months in house. Overall, and excluding rodent
20 and other vector samples, 19.4 % (736/3793) of cage house and 10.2 % (85/833) of
21 free-range house samples yielded salmonellas. However, there was wide variation in
22 the prevalence of positive samples between flocks and between farms (Tables 2 and
23 3).

24

Salmonella serovars and phage types. Where *S. Enteritidis* was present on a farm, often there were two or three (in one case four) phage types detected (Table 4), although sometimes these were closely related types, such as PT4 and PT7. The same *S. Enteritidis* phage types were usually present in more than one flock on any one farm, and were present in both caged and free-range flocks where both were kept on the premises. Samples taken after C & D, and samples from successive flocks in the same house (Table 4), showed that endemic *S. Enteritidis* phage types frequently persisted after C & D and were present in contamination sampled from a subsequent flock. Other serovars, by contrast, typically were restricted to an individual flock and appeared only once in a series of sampling visits (Table 4).

Semi-quantitative versus qualitative samples. Semi-quantitative culture using 20 g faeces was adopted for later samples following evidence of a superior sensitivity compared with a 10 g sample. Semi-quantitative data is included in Tables 1 and 2, and no distinction is made between scores from the two sample sizes, as they were of the same order of magnitude. For values of prevalence and quantitative score from the same flock visit, the exact probability for a linear by linear association test is $p=0.0001$, indicating a highly significant association between results from the two culture techniques. The same data are plotted against each other in Figure 1, which shows an apparently higher sensitivity of the multiple qualitative samples compared with the single bulked faeces sample, manifested as many more non-zero prevalence values plotted against zero semi-quantitative scores than vice-versa. Nonetheless, there were many samples that were negative for both techniques: there are 13 superimposed data points at the origin in Figure 1.

1 **Stage of lay.** Figure 2 shows the prevalence of salmonella-positive samples against
2 time spent in-house by all single-age layer flocks. There is a substantial range of
3 prevalences observed, although the trend appears to be upwards over time for
4 sequentially-sampled flocks. Figure 3 shows the changes in salmonella prevalence
5 over time in caged flocks where there had been two or three visits to occupied houses,
6 for two sample sources: faeces/droppings boards and dust. Although the overall trends
7 are upwards, this is more pronounced for the dust than for the faeces and droppings
8 boards samples.

9 In the fitted non-linear mixed effects model, the time in house was significant
10 in respect of prevalence ($p < 0.0001$) with an average increase in odds ratio of 1.20
11 (95% CI: 1.13, 1.26) for each additional month in house.

12
13 **Effect of temperature and season.** The wide range and variation in the data is
14 illustrated in Figure 4 showing, month by month, the prevalence of salmonella-
15 positive samples from each flock visit as a deviation above or below the year-round
16 average prevalence for that particular farm.

17 The fitted non-linear mixed effects model revealed a significant ($p = 0.0014$)
18 effect between the odds of a positive sample and average monthly temperature. The
19 average increase in odds ratio for salmonella detection in a sample was 1.08
20 (95% CI: 1.03, 1.12) for each additional degree Celsius. Furthermore, after
21 additionally fitting the season effect it was found that the summer months
22 significantly increased the odds of a sample being positive, when compared to the
23 winter months ($p = 0.0486$), with an associated odds ratio of 3.41 (95% CI: 1.01,
24 11.55).

25

Wildlife vectors and free-range paddocks. Details of isolations from rodents (predominantly pooled mouse faeces, but also rat faeces and one set of mouse viscera), flies, litter beetles and free-range paddocks are given in Table 4. In flocks where no salmonellas were detected in samples from the house, any vector and paddock soil samples were also negative. The overall prevalence of salmonella-positive samples from wildlife vectors was 34/88 (38.6%), more than double the prevalence of positive samples from houses, which was 821/4626 (17.7%). Eleven positive samples were cultured by the quantitative method, yielding a range and mean of quantitative scores of 2 – 7 and 4.36 respectively. Where isolates from vectors were phage typed, the results generally correlated with those for samples from the same flock. In the statistical model, there was not enough evidence from the semi-quantitative wildlife data (not shown) to conclude that the severity of fly infestation caused a change in the odds of infection ($p=0.22$).

Cleaning and disinfection. On 17 occasions a hen house was sampled immediately after C & D, and in 10 of these cases the house had in addition been sampled shortly before depopulation. Figure 5 illustrates the degree of salmonella contamination before and after C & D in these 17 flocks, showing that the prevalence of positive samples fell after C & D in 9/10 cases, with no detectable salmonella following C & D in three of the 17 houses. The extent of contamination, as measured by the prevalence of positive samples, following C & D did not correlate with that detected before C & D. Two notable examples are flocks 53 and 55, both from the same farm, with similarly high pre-depopulation salmonella prevalences but widely differing post-C & D contamination. For all flocks except 13, 29 and 70, an aldehyde disinfectant was used, which should have been effective even in the presence of

residual organic matter. The breakdown of the data on contaminated sites seen post-C & D (Figure 5), illustrates that all areas of the hen houses were prone to residual contamination, including those sites (cages, drinkers and feeders) likely to pose an early challenge to a new flock.

Examination of the data from new flocks in cleaned houses (Tables 2, 3 and 4, plus data not shown) reveals that in three cases (flocks 26, 27 and 28, all on the same farm), high salmonella prevalences (40 to 60%) were found on first visits within five weeks of occupying houses that had had no detectable contamination after C & D. In the 14 other cases where the changeover of flocks was monitored, post-repopulation salmonella prevalences were 20% or lower, even in houses with detected residual contamination post-C & D.

Discussion

The present study examined the environmental contamination by salmonella in 74 flocks from eight farms over a 12-month period. Sampling was performed in every month of the year, although the data from any one farm tended to be clustered within a few months, and two farms (A&L and Gra) were visited only once. Sampling and detection was predominantly qualitative, yielding a positive or negative result for each sample and an overall percentage of positive samples (prevalence) figure for each site or flock. An abbreviated most probable number technique was used to estimate the number of viable salmonellas (cfu) in a single bulked faeces sample from many flock visits and in wildlife vector samples from a few. The detected prevalence of contamination at any particular visit varied widely, with many flocks (for example 30, 31, 32 and 65) having no detectable salmonella on any occasion and others (40 and 55) having a single-visit prevalence in excess of 80%. Those flocks that were sampled

1 more than once often showed substantial variations in prevalence from one visit to the
2 next, as illustrated in Figure 2. In most of the flocks examined, additional private
3 monitoring was being carried out by cloacal swabs and environmental stick swabs at
4 end of lay, and in only one case was contamination identified by these additional tests.

5 Typing of *Salmonella* isolates showed that *S. Enteritidis* was the only
6 persistent serotype on any farm with the exception, on evidence from the present and
7 previous studies, of *S. Livingstone* and *S. Infantis* on farm Sut, where houses with
8 individual cages were operated on a multi-age continuously occupied regimen. Whilst
9 the occurrence of hen house contamination by non-*S. Enteritidis* serovars appears
10 typically to be transient, these organisms are nonetheless found frequently and there is
11 evidence that certain serovars (e.g. *S. Infantis*) will contaminate both shells and
12 contents of eggs. Attention to biosecurity, particularly in respect of feed and wildlife,
13 should help reduce the chances of flock, and therefore egg, contamination by these
14 non-*Enteritidis* serovars.

15 Several different phage types of *S. Enteritidis* (4, 6, 7, 21b, 35) were detected
16 and each farm exhibited a particular, and persistent combination of these. Whilst PT4,
17 which has been strongly associated with infection of layers in the UK and Europe
18 (Cogan & Humphrey, 2003), was detected on six of the eight farms, it was present in
19 combination with other persistent *S. Enteritidis* phage types on five of these. This is
20 consistent with previous findings (Liebana *et al.*, 2001), whereby a variety of often
21 closely related phage types was seen in samples from poultry farms in geographically
22 varied UK locations. In addition, our findings show that these phage type mixes can
23 persist for an extended period of time on a farm. Where caged and free-range units
24 existed on the same premises, a very similar mix of *S. Enteritidis* phage types and/or
25 other serotypes was found in both production systems (Table 4). Egg surveys in the

1 last 15 years have shown evidence of an increasing diversity of *S. Enteritidis* phage
2 types isolated from eggs in the UK, with a waning dominance of PT4 (Food Standards
3 Agency, 2004). A long-term decline in the incidence of PT4 has also been observed in
4 isolates from humans in Europe (Fisher, 2004a; Health Protection Agency, 2005). The
5 present findings indicate that a diversity of *S. Enteritidis* phage types is also present at
6 the likely source of egg contamination.

7 Statistical investigations included a comparison of the qualitative and semi-
8 quantitative culture techniques, which showed significant correlation. The latter
9 technique shows promise as a research tool for the highlighting of areas and of vectors
10 where a high level of challenge may be encountered (Wales *et al.*, 2006a, b). In
11 addition, a non-linear mixed effects model was used to examine the effects of various
12 factors on the prevalence of contamination, incorporating the random effect of flock.
13 One limitation upon this statistical analysis is that, because many flocks were first
14 sampled in the cooler months towards the end of the calendar year, the environmental
15 temperature and the number of months in-house exhibited some confounding, i.e. the
16 'time in house' and the 'temperature' parameters both contributed to the same effect,
17 and there is little that can be done to isolate the effects of each of the two variables on
18 the response (Woodward, 1999). However, in most cases the overlap was not
19 complete and it is considered that the confounding is not severe enough to negate the
20 main statistical conclusions. The problem could have been ameliorated had a longer
21 study period and random sampling start dates been possible. Nonetheless,
22 confounding is difficult to avoid in epidemiological studies where (in contrast to
23 prospective experimental studies) different variables frequently cannot be controlled
24 separately. Temperature and season present another issue: as temperature is heavily
25 dependent upon season in the UK their effects cannot be considered independently.

1 The effect of temperature was nested within season and the impacts of temperature
2 and of season were assessed sequentially in the model. Therefore, the significance and
3 odds ratio of temperature was unadjusted for season, whereas the effect of season was
4 already adjusted for (i.e. in addition to) that of temperature.

5 When stage of lay (or the duration of house occupation) is considered, the data
6 shows that the longest continuously-occupied houses (multi-age flocks 39, 40, 41, 48,
7 49, 50) typically were heavily contaminated with salmonella. The findings amongst
8 the single-age flocks are more variable but there is a significant trend of an increase in
9 the prevalence of contamination of the environment with time. The pattern of
10 contamination appears to fluctuate more for faeces samples than for dust. As faeces
11 are periodically removed in most systems, this difference may reflect fluctuating
12 excretion by the hens (measured in faeces) against a background of a progressive
13 build-up of *Salmonella* organisms in the henhouse environment (seen in dust).

14 When considering the effects of season, it might be expected that higher
15 environmental temperatures in summer would increase bird stress and bacterial
16 multiplication rates, resulting in higher levels of henhouse contamination. The
17 statistical modelling does indeed show significant positive effects of temperature and,
18 additionally, of season upon the detected prevalence of contamination. This is in the
19 context of wide variation in detected contamination rates all year round. The season
20 effect in addition to temperature may be mediated by factors such as an increase in
21 vector numbers and activity. A more pronounced seasonal pattern is seen amongst the
22 free-range than the caged flocks in the present study (Figure 4), but there are too few
23 of the former to draw any firm conclusions about seasonal differences between the
24 two production systems. However, it may be that the control of bird stress and house
25 temperatures differs significantly between the two production systems.

1 It is noteworthy in this context that in another UK study a seasonal effect was
2 not seen for egg contamination (Davies & Breslin, 2004). In the British climate, well-
3 designed and well-insulated hen houses should not be subject to excessive
4 temperature fluctuations at any time of year, so a seasonal effect upon salmonella in
5 eggs may be more marked in accommodation which has serious deficiencies in
6 ventilation and insulation.

7 There is good evidence for the importance of wildlife vectors, especially
8 rodents and flies, in the introduction to hen houses of salmonella and its maintenance
9 thereafter (Davies & Breslin, 2001; Guard-Petter, 2001; Mian *et al.*, 2002; Garber *et*
10 *al.*, 2003). Wildlife vectors may also spread infection between flocks, by virtue of
11 their mobility. The observations that samples from vectors not only reflected the sero-
12 and phage types of the corresponding flock (as noted previously by Davies & Breslin,
13 2003b), but were negative when the flock samples were negative and were positive at
14 a high frequency in positive flocks, indicates the value of such samples for monitoring
15 flock infection. Furthermore, they highlight the importance of the control of vectors,
16 as the quantitative culture of rodent faeces showed that they frequently excrete high
17 concentrations of salmonella, and they have the potential to amplify residual
18 environmental contamination as well as to contaminate feeding and drinker systems
19 directly. A strong association has been found between the number of mice and the
20 detection of salmonella in layer houses (Garber *et al.*, 2003), but in the limited data of
21 the present study the association between wildlife score and prevalence was not found
22 to be significant.

23 The variation in effectiveness of C & D between houses, even when in most
24 cases similar disinfection agents were being used, suggests that other factors, such as
25 the physical removal of organic matter and the mode of application of disinfectants,

are highly significant in the eventual reductions in contamination that may be achieved. Amongst the farms in the current study, disinfection was poorly applied, in that products were usually used at insufficient concentration and application rates. Also, key areas such as drinkers, dropping belts and boards (which normally form the ceilings of cages) were often poorly cleaned, usually by dry cleaning only, and inconsistently sprayed with disinfectant. The use of multi-age houses that cannot be effectively cleaned and disinfected between flocks appears, on the present evidence (flocks 39, 40, 41, 48, 49 and 50; Table 2), to afford very poor control of salmonella contamination. It is interesting that, in some of the present cases, apparently good C & D was followed by high prevalences of contamination within a month or so of repopulation. It seems likely that there was significant but undetected residual contamination. The evident difficulties in eliminating salmonella from any part of empty hen houses, plus the tendency of wildlife vectors to re-introduce the organisms (Garber *et al.*, 2003) implies that control measures such as vaccination and intestinal competitive exclusion will be important components of salmonella control for the foreseeable future, even with salmonella-free replacement stock. With free-range units there is an additional issue of the persistence of salmonella on paddocks even after the removal of detectable contamination in the house, as seen with flock 69 in the present study.

In conclusion, our investigation has shown a high degree of variation in the prevalence of salmonella contamination of hen houses, both between flocks on the same premises and within the same flocks over time. This has implications for monitoring programmes, when false negatives may occur. *S. Enteritidis* was predominant as the persistent serotype, and differing combinations of *S. Enteritidis* phage types proved stably persistent on various farms. The study reconfirmed the

value of sampling wildlife vectors and their faeces, if present. There was a significant tendency to increased contamination with increasing flock age, but the temporal patterns of salmonella contamination in the first months of lay can be highly variable. There may be significant differences between contamination patterns over time in faeces and non-faeces samples. There were significant effects of temperature and season upon salmonella contamination. In all cases there was a clear need to improve both rodent control and C & D, and in order to make further progress the egg industry must acknowledge the additional cost in terms of baits, traps, house maintenance, disinfectants, additional down-time between flocks and the labour to achieve this when salmonella is present. It is also vitally important that sensitive monitoring is introduced for laying flocks so that additional controls can be introduced, since routine and repeated use of the measures required to eliminate salmonella from infected premises would be prohibitively costly.

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Table 1: *Relationship between quantitative score and likely concentration of salmonella organisms in a sample*

Score	Range of salmonella colony-forming units	
	10 g sample (g ⁻¹)	20 g sample (g ⁻¹)
0	Not detected	Not detected
1	0.1 – 1	0.05 – 0.5
2	1 – 10	0.5 – 5
3	10 – 100	5 – 50
4	100 – 1000	50 – 500
5	1000 – 10 000	500 – 5000

Table 2: Details of overall salmonella prevalence (positive samples/total samples) from caged layer flock houses, and of quantitative scores of salmonella concentration from bulked faeces samples

Farm, visit	Flock number, positive samples/total samples taken; semi quantitative score ^a													
A&L	1	2												
Nov	0/25	0/28												
C&K	3	4	5	6	7	51	51 PCD ^c							
Sept	0/40; <u>0</u>	6/40; <u>2</u>	1/40; <u>0</u>			5/40; <u>1</u>	(9/60)							
Nov	1/40; <u>0</u>	12/40; <u>1</u>	0/40; <u>0</u>	0/40; <u>0</u>										
Apr	5/40; <u>0</u>	13/40; <u>2</u>		0/40; <u>0</u>	3/40; <u>0</u>									
Coc	8	9	10	11	12	13	14	15	16	17	18	70	73	74
Nov	2/19; <u>0</u>	3/19; <u>4</u>	3/18; <u>0</u>	12/20; <u>2</u>	0/20; <u>0</u>	12/19; <u>2</u>	10/18; <u>2</u>	8/20; <u>5</u>	2/19; <u>2</u>			(16/57)		
Feb	5/20; <u>2</u>	10/20; <u>0</u>	7/19; <u>5</u>	15/20; <u>0</u>	1/20; <u>0</u>	(9/63)			4/19	1/20; <u>0</u>	4/20			
Apr	9/20; <u>2</u>	7/20; <u>0</u>		4/20; <u>2</u>	1/20; <u>0</u>				2/20; <u>3</u>	0/20; <u>3</u>			1/20; <u>0</u>	1/20; <u>0</u>
Cots	19	20	21	22	23	24	25	26	27	28	52	53	54	55
Aug											22/40; <u>0</u>	28/40; <u>5</u>		
Sept											(4/60)	(19/60)	21/40	
Oct	1/40; <u>0</u>	2/40; <u>0</u>											(9/60)	
Nov			7/40; <u>0</u>	0/40; <u>0</u>	0/40; <u>0</u>	7/40; <u>2</u>								32/40; <u>4</u>
Jan	0/20; <u>0</u>	3/20; <u>0</u>												1/60
Mar						1/40; <u>2</u>	8/40; <u>2</u>							
May			10/40	10/40	8/40									
Jun			(0/60)	(0/60)	(1/60)									
Jul	7/40; <u>4</u>	25/40; <u>3</u>				29/40; <u>4</u>	4/40; <u>2</u>	16/40; <u>0</u>	21/40; <u>1</u>	24/40; <u>2</u>				
Fld	29	30	31	32	33	34	35	36	37	38	56			
Dec	0/13	0/13	0/14								12/40; <u>0</u>			
Jan	0/20	0/20	0/40	0/20	0/20	0/20	0/40				(5/60)			
Jul	7/20				1/20	0/20	1/20	7/20	1/20	3/20				
Aug	(10/60)													
Gra	39 ^b	40 ^b	41 ^b											
Apr	13/35; <u>3</u>	35/40; <u>5</u>	18/40; <u>0</u>											
Hum	42	43	44	45	46	47								
Oct	0/40; <u>0</u>	6/40; <u>1</u>	0/40; <u>0</u>	1/40; <u>0</u>										
Jan	11/40; <u>0</u>	(2/59)	0/40; <u>0</u>	4/40; <u>0</u>										
Jun	(7/58)		0/40; <u>0</u>		6/40; <u>0</u>	5/38; <u>0</u>								
Sut	48 ^b	49 ^b	50 ^b											
Mar	2/40	45/60; <u>3</u>	29/60; <u>0</u>											

Visits are listed in chronological order. ^a For key to numbers of salmonella per gram, see Table 1. ^b Multi-age flock. ^c Post cleaning and disinfection. Results in parentheses are from samples taken immediately after cleaning and disinfection following removal of the indicated flock.

Table 3: *Details of overall salmonella prevalence (positive samples/total samples) from free-range layer flock houses, and of quantitative scores of salmonella concentration from bulked faeces samples*

Farm/visit	Flock number, positive/total samples taken; <u>semi quantitative score</u>				
A&L	57	58	59		
Nov	0/40	0/37	0/37		
Fld	60	61	62	63	71
Jan	0/20	0/18	0/40		(5/50)
Jul		25/39	23/39	8/40	
Aug		(9/50)			
Gra	64				
Apr	3/8				
Hum	65	66	67		
Oct	0/44	0/50			
Jan	0/44		0/40		
Jun	0/40		0/40; <u>0</u>		
Sut	68	69	72		
Sept	0/51	7/41; <u>0</u>			
Oct		(0/25)			
Mar	1/40; <u>0</u>		1/40; <u>0</u>		

Footnotes as for Table 2.

Table 4: Details of *Salmonella* types isolated from layer houses and vectors

First flock in house				Second flock in house		
Farm / house	Flock	Serovars / PTs ^a (visits 1: 2: 3: 4)	Rodent [Insect / soil] samples ^b	Flock	Serovars / PTs ^a (visits 1: 2: 3)	Rodent [Insect] samples ^b
C&K/ 2	3	0: e21B: e21B	no samples			
C&K/ 3	51	e21B: e21B ^c	no samples			
C&K/ 4	4	e21B: e21B: e21B	2/2			
C&K/ 5	5	e21B: 0	no samples	7	t, o	0/1
Coc/ 1	8	a, bi: e4/7, n: e4/7, l, k	1/1			
Coc/ 2	9	e4/7, br: e4/7: e4/7, br, l	0/2			
Coc/ 3	10	a: e4/7	0/2			
Coc/ 6	11	e4/7: e4/7, k: e4/7	no samples			
Coc/ 7	12	0: e4/7: e4/7	no samples			
Coc/ 8	13	e4/7: e4/7 ^c	0/3	73	e4/7	
Coc/ 9	14	e4/7	0/1	17	e4/7: 0	no samples
Coc/ 10	70	e4/7 ^c	1/1	18	e4/7	0/1
Coc/ 11	15	e4/7, a	no samples	74	e4/7	0/1
Coc/ 12	16	e4/7, a: e4/7: e4/7	1/1			
Cots/ 1	21	e4&6: e6: 0 ^c	1/2	26	vx, ou	5/6 (e6&35, vx)
Cots/ 2	52	e: e ^c	0/2	19	e6: 0: e6/35	2/2
Cots/ 3	22	0: e6&7: 0 ^c	1/2 (e6)	27	e6/35, vx	6/6 (e6&35, vx)
Cots/ 4	23	0: e-, z: e- ^c	0/2	28	e6/35, co, m, te, y	3/6 (e6&35, a)
Cots/ 5	53	e6&35: e ^c	2/2	20	e6: e4&6: e6/35	1/2
Cots/ 6	54	e: e6 ^c	2/2 (e35)	24	e4&6: r: e6/35	0/1
Cots/ 8	55	e4/6: e4	1/2 (e7)	25	e4, co, y: e4, a, cu	0/3
Fld/ 1R	29	0: 0: e4/7: e4/7/35 ^c	} 2/5 [1/3 insect]			
Fld/ 1L	36	e4/7				
Fld/ 2R	37	e4/7	} [0/1 insect]			
Fld/ 2L	38	e4/7, s				
Fld/ 3R	33	0: e4/7	[0/1 insect]			
Fld/ 4R	56	e: e4 ^c	0/2	35	0: e4/7	1/3
Fld/ 4L (FR)	63	e4/7	1/3			
Fld/ 5R (FR)	61	0: e4/7: e4/7/35 ^c	[0/10 soil]			
Fld/ 5L (FR)	71	e4 ^c	no samples	62	0: e4/7	no samples
Gra/ 1	39	e6	0/1			
Gra/ 2	40	e6	1/1			
Gra/ 3	41	e4	no samples			
Gra/ P (FR)	64	e4	[3/10 soil]			
Hum/ 2	42	0: e4, a: e4 ^c	1/2			
Hum/ 3	43	a, ty: e4 ^c	0/1	46	e4	no samples
Hum/ 6	45	e4: e4	[0/10 insect]	47	e4	[1/2]
Sut/ A	48	l	no samples			
Sut/ 9	49	l	0/1			
Sut/ 10	50	l, i	0/1			
Sut/ frA (FR)	69	i: 0 ^c	[4/10 soil: 2/5 soil ^c]	72	w	[0/10]
Sut/ frB (FR)	68	0: l	no samples			

^a Lower-case letters indicate serovars as detailed below; numbers and upper-case letters indicate phage types (PTs) for *S. Enteritidis*. '0' indicates no isolates at that visit; '/' indicates 'and/or'. Where no *S. Enteritidis* PT is given, the isolate was untypable (-), was not typed (Fld) or a phage infection prevented typing (Cots).

^b Results are number of positive samples/total samples taken at all visits, or at separate visits for flock 69. Not all presumptive *Salmonella* isolates were typed. ^c Visit after cleaning and disinfection. (FR) indicates free-range flock. e – *Salmonella* Enteritidis; a – *S. Agona*; bi – *S. Binza*; br – *S. Braenderup*; co – *S. Corvallis*; cu – *S. Cubana*; i – *S. Infantis*; k – *S. Kedougou*; l – *S. Livingstone*; m – *S. Mbandaka*; n – *S. Newport*; o – *S. Oranienburg*; ou – *S. Ouakam*; r – *S. Rissen*; s – *S. Seftenburg*; te – *S. Tennessee*; ty – *S. Typhimurium*; vx – *S. Enteritidis* vaccine strain; w – *S. 4,12:i:-* incomplete; y – *S. 3,19* incomplete; z – *S. 6,4:D:-*

FIGURE LEGENDS

Figure 1: *House salmonella prevalence versus quantitative culture of bulked faeces.*

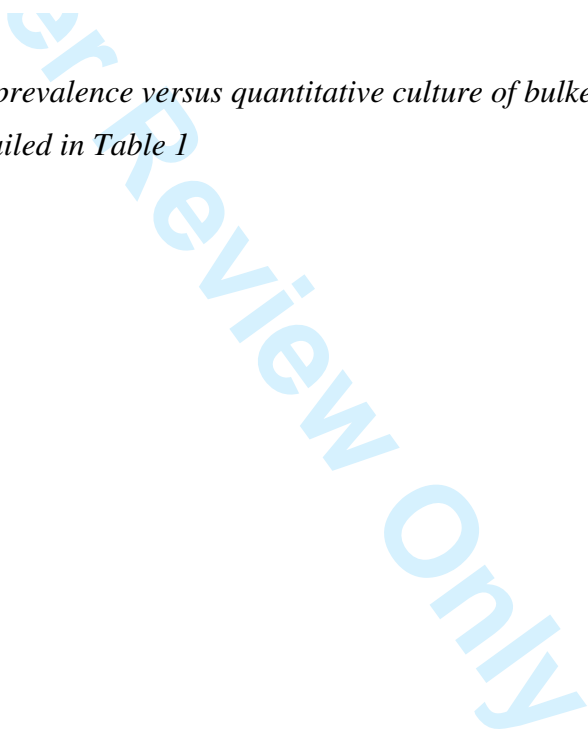
**Quantitative score is as detailed in Table 1.*

Figure 2: *Overall prevalence of salmonella-positive samples over time in 66 single-age layer flocks. Prevalence values from consecutive visits to the same flock are joined by straight lines. Values from flocks sampled only once are indicated by triangles.*

Figure 3: *Changes in the prevalence of salmonella-positive samples over time. Prevalence values from initial visits are marked by solid circles and are connected to prevalence values from subsequent visits to the same flock by straight lines.*

Figure 4: *Month-by-month deviation from farm/housing system averages of salmonella prevalence for all flocks.*

Figure 5: *Results of cleaning and disinfection in 17 hen houses. *Free-range flock.*



*Quantitative score is as detailed in Table 1

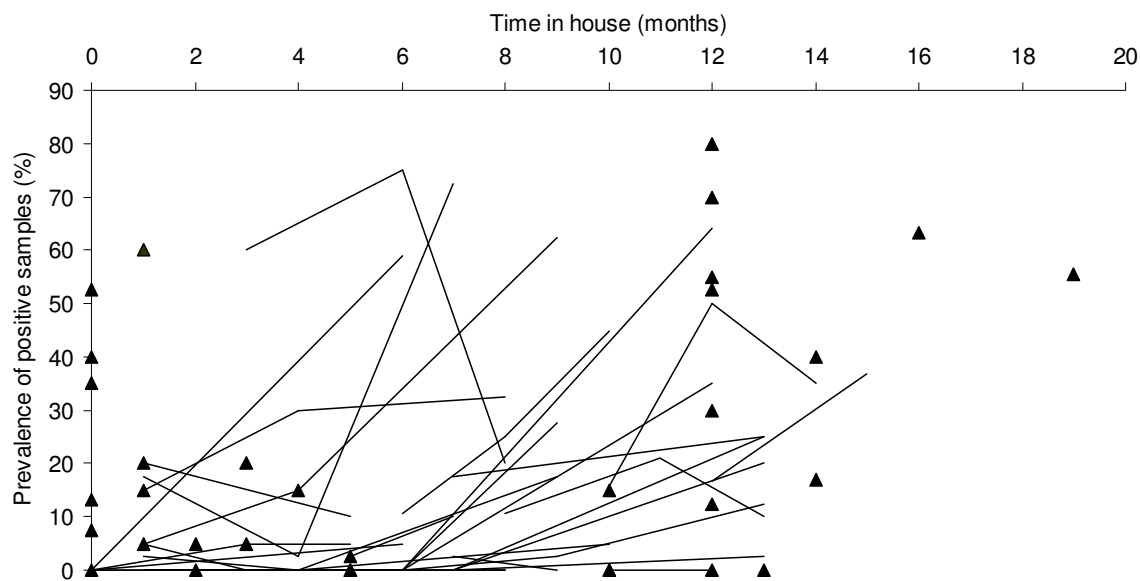
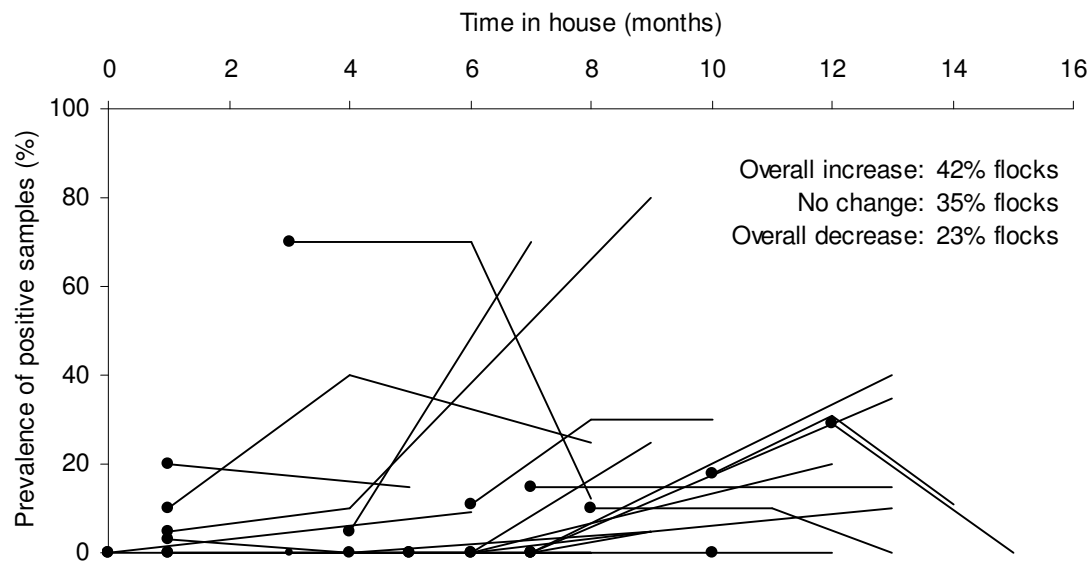


Figure 2: Overall prevalence of *salmonella*-positive samples over time in 66 single-age layer flocks. Prevalence values from consecutive visits to the same flock are joined by straight lines. Values from flocks sampled only once are indicated by triangles.

a. Cage layers faeces / droppings board samples



b. Cage layers dust and floor samples

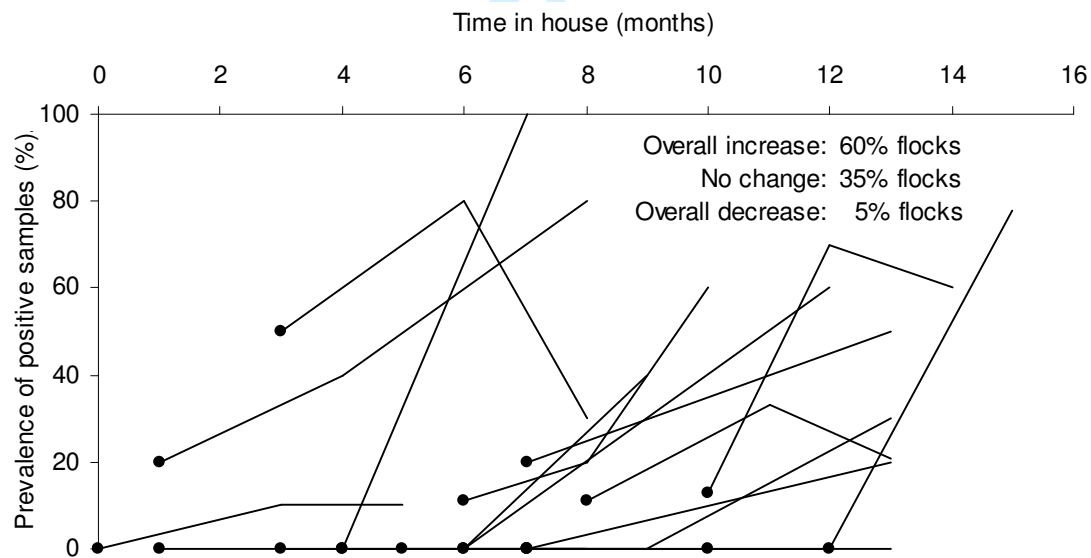


Figure 3: Changes in the prevalence of *salmonella*-positive samples over time. Prevalence values from initial visits are marked by solid circles and are connected to prevalence values from subsequent visits to the same flock by straight lines.

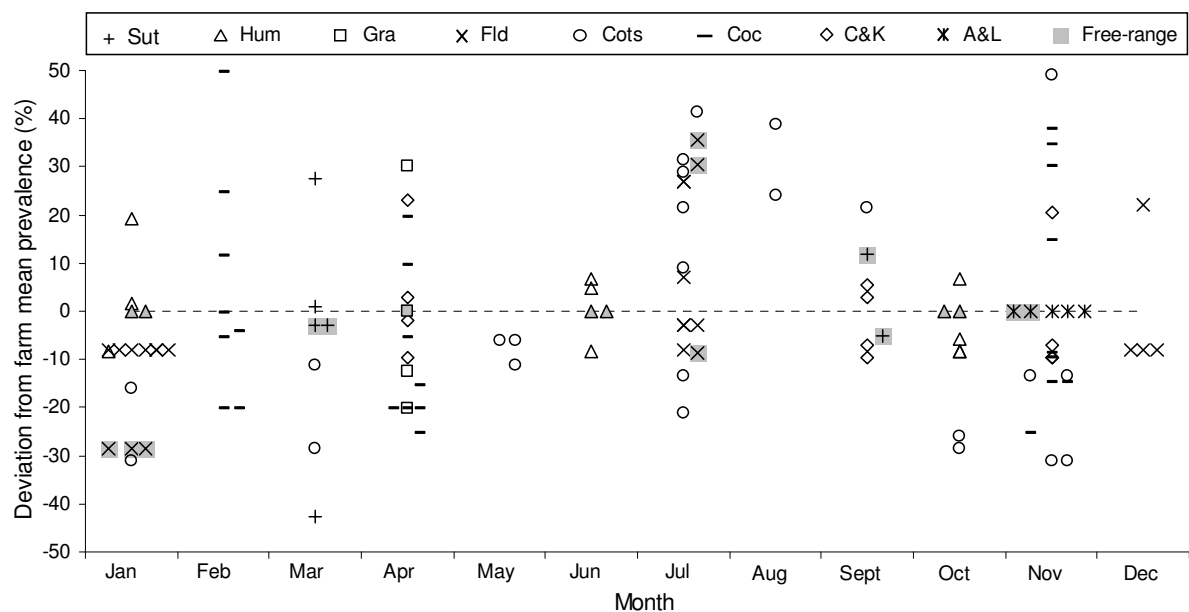


Figure 4: Month-by-month deviation from farm/housing system averages of *salmonella* prevalence for all flocks.

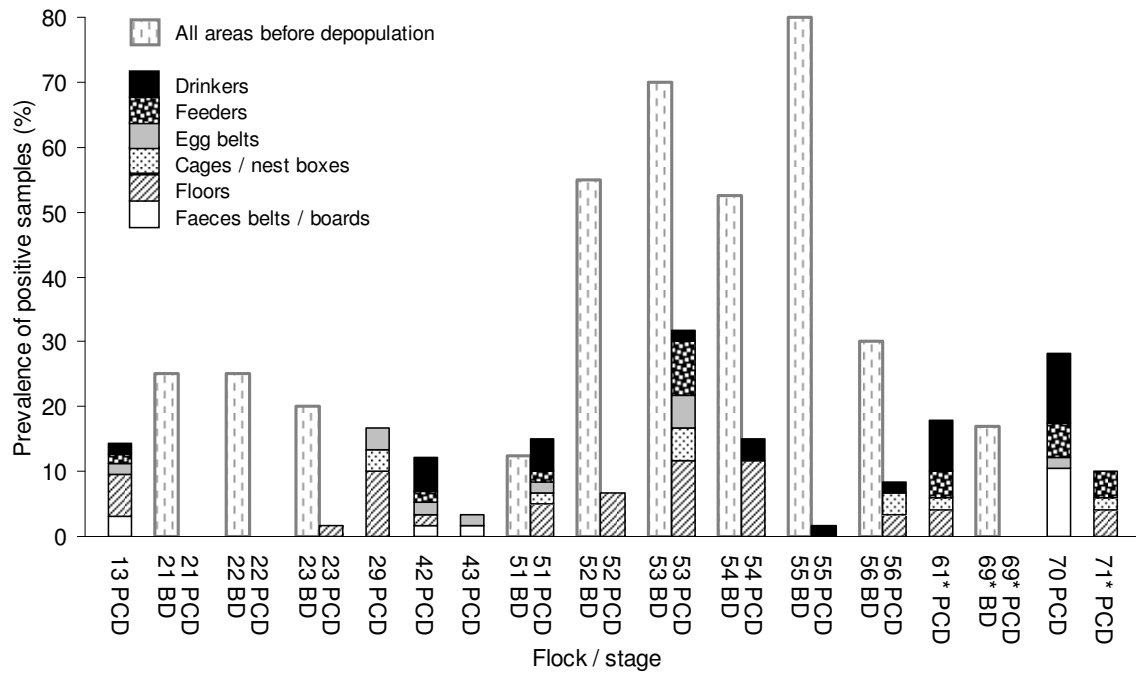


Figure 5: Results of cleaning and disinfection in 17 hen houses. *Free-range flock.