# PROCESSING AND PRODUCTS

## Persistence of fecal shedding of *Salmonella* Enteritidis by experimentally infected laying hens housed in conventional or enriched cages

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**ABSTRACT** *Salmonella* Enteritidis can be deposited inside eggs laid by infected hens, so the prevalence of this pathogen in commercial egg-producing flocks is an important risk factor for human illness. Opportunities for the introduction, transmission, and persistence of salmonellae in poultry are potentially influenced by flock housing and management systems. Animal welfare concerns have spurred the development of alternatives to traditional cage-based housing. However, the consequences of poultry housing systems for food safety have not been fully resolved by prior research. The present study assessed the effects of two different housing systems (conventional cages and colony cages enriched with perching and nesting areas) on the persistence of fecal shedding of *Salmonella* Enteritidis by groups of experimentally infected laying hens. In each of two trials, 136 hens were distributed among cages of both housing systems and orally inoculated with doses of $10^8$ cfu of *Salmonella* Enteritidis (phage type 13a in one trial and phage type 4 in the other). At weekly intervals, samples of voided feces were collected from beneath each cage and cultured to detect *Salmonella* Enteritidis. Fecal shedding of *Salmonella* Enteritidis was detected for up to 8 wk post-inoculation by hens housed in enriched colony cages and 10 wk by hens housed in conventional cages. For both trials combined, the frequency of positive fecal cultures was significantly ($P < 0.05$) greater for conventional cages than for enriched colony cages at 1 wk (84.7 vs. 71.5%), 2 wk (54.2 vs. 31.3%), 3 wk (21.5 vs. 7.6%), and 4 wk (9.7 vs. 2.8%) post-inoculation. These results demonstrate that the susceptibility of hens to intestinal colonization by *Salmonella* Enteritidis can differ between conventional and enriched cage-based production systems, although this effect does not necessarily translate into a corresponding difference in the longer-term persistence of fecal shedding.

**Key words:** *Salmonella* Enteritidis, chickens, fecal shedding, conventional cages, enriched colony cages

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## INTRODUCTION

The contamination of eggs by *Salmonella enterica* subspecies *enterica* serovar Enteritidis continues to be an important international source of human illness (Jackson et al., 2013; Pires et al., 2014). A strong association between the prevalence of this pathogen in commercial egg-laying chickens and the incidence of human infections is indicated by both active disease surveillance and retrospective epidemiologic analyses (Havelaar et al., 2013; Arnold et al., 2014a). Sustained participation in comprehensive testing and risk reduction programs for *Salmonella* Enteritidis in poultry flocks has led to some reported declines in both egg contamination and human illnesses in individual countries (Esaki et al., 2013; O'Brien, 2013). Nevertheless, despite a considerable investment of public and private resources in disease control efforts, the incidence of *Salmonella* Enteritidis in the United States changed very little during the first decade of the present century (Centers for Disease Control and Prevention, 2011; Chai et al., 2012).

The deposition of *Salmonella* Enteritidis in the edible interior contents of eggs results from the colonization of reproductive tissues in systemically infected hens (Gantois et al., 2009; Gast et al., 2011a). Prolonged opportunities for hens to be exposed and infected are created by persistence of the pathogen in the environment of poultry facilities (Davies and Breslin, 2003). Because the presence of *Salmonella* Enteritidis in laying houses is epidemiologically correlated with egg contamination (but occurs at a far higher frequency), environmental samples are tested as an initial screening step in many protocols for identifying infected flocks (Gast, 2007). Fecal shedding of bacteria by infected hens can be a prominent source of environmental contamination with *Salmonella* Enteritidis (Trampel et al., 2014). The


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frequency of positive results from testing fecal samples has been reported to peak just before egg laying commences in commercial flocks and declines steadily thereafter (Li et al., 2007; Gole et al., 2014). Experimental infection of highly susceptible young chicks can establish *Salmonella* Enteritidis colonization of the intestinal tract that persists until maturity (Gast and Holt, 1998) and the inoculation of adult hens with large oral doses sometimes leads to fecal shedding for several months (Gast et al., 2011b).

The various poultry housing systems used by the contemporary commercial egg industry incorporate a wide range of complex facility characteristics and management practices that can potentially affect the persistence and transmission of *Salmonella* infections in laying flocks. Although the implications of these housing systems have been topics for widespread discussion and inquiry in recent years (from perspectives as diverse as animal welfare and economic viability), no definitive consensus about their consequences for food safety has yet emerged from the published scientific literature (Holt et al., 2011). Higher incidences of *Salmonella* detection have been attributed to either cage-based or floor-based (cage-free) poultry housing systems in different studies, but no consistent advantage has been convincingly shown for any one system regarding the persistence of *Salmonella* Enteritidis in either infected chickens or their environment (Holt et al., 2011). Enriched (furnished) colony cages and aviaries have been developed as intermediate alternatives between conventional battery cages and cage-free systems. The objective of the present study was to determine the effects of two different housing systems (conventional cages and colony cages enriched with perching and nesting areas) on the persistence of fecal shedding of *Salmonella* Enteritidis by groups of experimentally infected laying hens.

## MATERIALS AND METHODS

### Experimental Housing of Laying Hens

In each of two similar trials, 136 laying hens were obtained from the specific-pathogen-free flock of Single Comb White Leghorn chickens (negative for antibodies to *Salmonella* in periodic routine monitoring) at the Southeast Poultry Research Laboratory in Athens, GA. These hens (30 and 28 wk old at the beginning of the first and second trials, respectively) were distributed into four separately housed groups in different rooms of a disease-containment facility containing cage systems designed to simulate commercial conditions. Hens in two rooms (36 per room) were housed in conventional laying cages. Each of these cages housed six hens and provided 648 $cm^2$ of floor space per bird. Hens in two other rooms (32 per room) were housed in enriched colony laying cages. Each of these cages housed 16 hens and provided 1,216 $cm^2$ of floor space per bird, including access to two perches and a single enclosed nesting area. The stocking densities in both housing systems represented 2/3 of the maximum levels recommended by the cage manufacturer. All hens were provided with water (via two automatic nipple-type drinkers in each conventional cage and six in each enriched cage) and feed (a pelleted, antibiotic-free layer-breeder ration) ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Southeast Poultry Research Laboratory.

### Experimental Infection of Laying Hens with Salmonella Enteritidis

In each trial, all hens were orally inoculated with a measured dose of *Salmonella* Enteritidis. In Trial 1, hens were infected with a phage type 13a strain, originally isolated from a contaminated egg yolk by Dr. C. Benson at the University of Pennsylvania, Kennett Square, PA. In Trial 2, hens were infected with a phage type 4 strain, originally isolated from the liver of an infected chicken by Dr. D. Munro at the Scottish Salmonella Reference Laboratory, Glasgow, UK. Two different *Salmonella* Enteritidis phage types (both of which are epidemiologically important) were included in this study to minimize the strain-specificity of results. The inoculum strains were resuscitated by transfer into tryptic soy (TS) broth (Acumedia, Neogen Corp., Lansing, MI) for two successive cycles of 24-h incubation at 37°C. After cell numbers in each incubated culture were estimated by determining its optical density at 600 nm, further serial ten-fold dilutions in 0.85% saline produced a desired final cell concentration in each oral dose of approximately $1.25 \times 10^8$ cfu (confirmed by subsequent plate counts).

### Fecal Samples

Immediately before inoculation and at intervals of 1, 2, 3, 4, 5, 6, 8, 10, and 12 wk post-inoculation, sterile cotton swabs were used to collect samples of voided feces from polystyrene trays (food-grade but not sterile) placed under each cage. A total of 36 samples per room were collected on each sampling date, evenly distributed among all occupied cages (six samples per conventional cage and 18 samples per colony cage). Feces selected for sampling were visibly moist (recently voided) and dark in color (characteristic of cecal discharge). Each collected sample was transferred into 9 mL of Rappaport Vassiliadis broth (Acumedia) and incubated for 24 h at 41.5°C. A 10-$\mu$l portion from each broth culture was then streaked onto brilliant green (BG) agar (Acumedia) supplemented with 0.02 mg/mL of novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 h at 37°C. The identity of presumptive colonies of *Salmonella* was confirmed biochemically and serologically (Waltman and Gast, 2008).

**Table 1.** Recovery of *Salmonella* Enteritidis phage type 13a from voided fecal samples of experimentally infected laying hens in different housing systems.[1]

| Weeks post-inoculation | Conventional cages *Salmonella* Enteritidis-positive/total (%) | Enriched colony cages *Salmonella* Enteritidis-positive/total (%) |
|---|---|---|
| 1 | 60/72 (83.3)$^{a,A}$ | 52/72 (72.2)$^{a,A}$ |
| 2 | 36/72 (50.0)$^{a,B}$ | 26/72 (36.1)$^{a,B}$ |
| 3 | 16/72 (22.2)$^{a,C}$ | 5/72 (6.9)$^{b,C}$ |
| 4 | 9/72 (12.5)$^{a,C,D}$ | 3/72 (4.2)$^{a,C}$ |
| 5 | 6/72 (8.3)$^{a,D}$ | 2/72 (2.8)$^{a,C}$ |
| 6 | 4/72 (5.6)$^{a,D}$ | 1/72 (1.4)$^{a,C}$ |
| 8 | 1/72 (1.4)$^{a,D}$ | 1/72 (1.4)$^{a,C}$ |
| 10 | 1/72 (1.4)$^{a,D}$ | 0/72 (0)$^{a,C}$ |
| 12 | 0/72 (0)$^{a,D}$ | 0/72 (0)$^{a,C}$ |
| All | 133/648 (20.5)$^{a}$ | 95/648 (14.7)$^{b}$ |

[1]After oral inoculation of all hens with approximately $10^8$ cfu of *Salmonella* Enteritidis.

$^{a,b}$Values in rows that share no common lower-case superscripts are significantly ($P < 0.05$) different.

$^{A–D}$Values in columns that share no common upper-case superscripts are significantly ($P < 0.05$) different.

**Table 2.** Recovery of *Salmonella* Enteritidis phage type 4 from voided fecal samples of experimentally infected laying hens in different housing systems.[1]

| Weeks post-inoculation | Conventional cages *Salmonella* Enteritidis-positive/total (%) | Enriched colony cages *Salmonella* Enteritidis-positive/total (%) |
|---|---|---|
| 1 | 62/72 (86.1)$^{a,A}$ | 51/72 (70.8)$^{a,A}$ |
| 2 | 42/72 (58.3)$^{a,B}$ | 19/72 (26.4)$^{b,B}$ |
| 3 | 15/72 (20.8)$^{a,C}$ | 6/72 (8.3)$^{a,C}$ |
| 4 | 5/72 (6.9)$^{a,D}$ | 1/72 (1.4)$^{a,C,D}$ |
| 5 | 2/72 (2.8)$^{a,D}$ | 1/72 (1.4)$^{a,C,D}$ |
| 6 | 1/72 (1.4)$^{a,D}$ | 0/72 (0)$^{a,D}$ |
| 8 | 0/72 (0)$^{a,D}$ | 0/72 (0)$^{a,D}$ |
| 10 | 0/72 (0)$^{a,D}$ | 0/72 (0)$^{a,D}$ |
| 12 | 0/72 (0)$^{a,D}$ | 0/72 (0)$^{a,D}$ |
| All | 127/648 (19.6)$^{a}$ | 78/648 (12.0)$^{b}$ |

[1]After oral inoculation of all hens with approximately $10^8$ cfu of *Salmonella* Enteritidis.

$^{a,b}$Values in rows that share no common lower-case superscripts are significantly ($P < 0.05$) different.

$^{A–D}$Values in columns that share no common upper-case superscripts are significantly ($P < 0.05$) different.

### Statistical Analysis

Within each trial, between the two trials, and for both trials combined, significant differences ($P < 0.05$) between housing systems, *Salmonella* Enteritidis strains, or sampling dates in the mean frequencies of *Salmonella* Enteritidis isolation from voided fecal samples were determined by Fisher's exact test. Because the two replicate groups of hens for each housing system did not differ significantly within either trial in the frequency of *Salmonella* Enteritidis recovery from fecal samples, their results were combined for analysis and presentation. Data were analyzed with Instated biostatistics software (GraphPad Software, San Diego, CA).

## RESULTS

In Trial 1, *Salmonella* Enteritidis (phage type 13a) was recovered from 83.3% of voided fecal samples collected at 1 wk post-inoculation from hens housed in conventional cages and from 72.2% of samples from hens in enriched colony cages (Table 1). The frequency of *Salmonella* Enteritidis isolation from feces in conventional cages decreased significantly ($P < 0.0001$) to 50.0% at 2 wk post-inoculation, further to 22.2% at 3 wk post-inoculation ($P = 0.0009$), and decreased again to 8.3% at 5 wk post-inoculation ($P = 0.0353$). In samples from enriched cages, *Salmonella* Enteritidis recovery declined significantly to 36.1% at 2 wk post-inoculation ($P < 0.0001$) and then to 6.9% at 3 wk post-inoculation ($P < 0.0001$). The last positive results for *Salmonella* Enteritidis detection from feces in this trial were at 8 wk post-inoculation in colony cages and at 10 wk in conventional cages. *Salmonella* Enteritidis was found in fecal samples significantly ($P = 0.0166$) more often from conventional cages than from enriched cages at 3 wk post-inoculation, but no differences between the two treatment groups were observed at any other sampling date. For all sampling dates combined, *Salmonella* Enteritidis was detected significantly ($P = 0.0069$) more frequently in feces from conventional cages (20.5%) than from enriched colony cages (14.7%).

In Trial 2, *Salmonella* Enteritidis (phage type 4) was isolated from 86.1% of fecal samples collected at 1 wk post-inoculation from hens housed in conventional cages and from 70.8% of samples from hens in enriched colony cages (Table 2). The frequency of *Salmonella* Enteritidis recovery from feces in conventional cages declined significantly ($P = 0.0003$) to 58.3% at 2 wk post-inoculation, to 20.8% at 3 wk post-inoculation ($P < 0.0001$), and then to 6.9% at 4 wk post-inoculation ($P = 0.0282$). In samples from colony cages, *Salmonella* Enteritidis isolation decreased significantly to 26.4% at 2 wk post-inoculation ($P < 0.0001$), to 8.3% at 3 wk post-inoculation ($P = 0.0074$), and again to 1.4% at 4 wk post-inoculation ($P = 0.0282$). The last positive detections of *Salmonella* Enteritidis from feces in this trial were at 5 wk post-inoculation in enriched cages and at 6 wk in conventional cages. The frequency of *Salmonella* Enteritidis recovery from fecal samples was significantly higher ($P = 0.0002$) from conventional cages than from colony cages at 2 wk post-inoculation, but no differences between the two treatment groups were observed on other sampling dates. For all sampling dates combined, *Salmonella* Enteritidis was found significantly ($P = 0.0002$) more often in feces from conventional cages (19.6%) than from enriched colony cages (12.0%).

The overall frequency of *Salmonella* Enteritidis recovery from samples of voided feces did not differ significantly ($P > 0.05$) between hens inoculated with the phage type 13a strain and hens inoculated with the phage type 4 strain in either conventional cages (20.5% vs. 19.6%) or in enriched colony cages (14.7% vs. 12.0%), nor did these values differ significantly at any individual sampling interval. For both trials (and thus for both *Salmonella* Enteritidis strains) combined,

the frequency of positive results from fecal samples was significantly greater for conventional cages than from enriched colony cages at 1 wk (84.7% vs. 71.5%; $P = 0.0099$), 2 wk (54.2% vs. 31.3%; $P = 0.0001$), 3 wk (21.5% vs. 7.6%; $P = 0.0013$), 4 wk (9.7% vs. 2.8%; $P = 0.0259$), and for the total of all sampling dates (20.1% vs. 13.3%, $P < 0.0001$). None of the fecal samples collected before inoculation in either trial were positive for *Salmonella*.

## DISCUSSION

The persistence of environmental contamination in egg production facilities creates an important potential reservoir for introducing *Salmonella* Enteritidis infection into successive laying flocks (Dewaele et al., 2012a, b; Lapuz et al., 2012). Distributed widely throughout laying houses in association with dust and feces (Garber et al., 2003; Kinde et al., 2005), *Salmonella* Enteritidis contamination can be magnified by severe rodent or insect infestations to levels that will survive standard cleaning and disinfection procedures (Carrique-Mas et al., 2009b; Snow et al., 2010). A variety of risk factors have been linked to elevated environmental *Salmonella* prevalence, including larger flock size, greater flock age, and housing in older facilities (Huneau-Salaün et al., 2009; Van Hoorebeke et al., 2010a; Pitesky et al., 2013). Following introduction from environmental sources, horizontal transmission of infection can disseminate *Salmonella* Enteritidis rapidly and extensively throughout flocks (Gast et al., 1998; Thomas et al., 2011). Stress associated with feed or water deprivation or with environmental heat can increase the susceptibility of hens to horizontally transmitted *Salmonella* Enteritidis infection (Humphrey, 2006; Okamura et al., 2010). Fecal shedding of *Salmonella* by infected hens can be a major contributor to overall environmental contamination levels, but the magnitude of fecal shedding does not always correlate strongly with the probability of pathogen detection by environmental sampling. (Wales et al., 2006). Dust samples have been reported to provide both a higher frequency of *Salmonella* Enteritidis isolation (Arnold et al., 2014b) and a longer duration of positive results (Gole et al., 2014) than fecal samples. The sensitivity of *Salmonella* detection from chicken feces (70 cfu) has been found to be lower than the comparable value for poultry house dust (Martelli et al., 2014). Combinations of feces and dust are sometimes recommended for optimal detection of environmental contamination (Carrique-Mas and Davies, 2008; Arnold et al., 2010).

Fecal shedding of salmonellae is a product of their ability to adhere to cells of the avian intestinal tract. Intestinal colonization by *Salmonella* usually declines steadily following experimental infection of mature chickens (Gast et al., 2005, 2011b), but can sometimes persist for an extended period (Li et al., 2007; Gast et al., 2009). However, persistent fecal shedding has been an inconsistent predictor of the likelihood of systemic infection and egg contamination by *Salmonella* Enteritidis (Gast and Holt, 2000; Gast et al., 2005). The prevalence of fecal shedding by infected hens in commercial laying flocks can fluctuate over time (Wales et al., 2007; Schulz et al., 2011). Nevertheless, persistent shedding of *Salmonella* Enteritidis by even a small proportion of hens in a flock could prolong opportunities for horizontal transmission to other birds and ultimately the production of contaminated eggs. In the present study, oral infection with doses of $10^8$ cfu of *Salmonella* Enteritidis led to detectable fecal shedding for up to 8 wk by hens housed in enriched colony cages and 10 wk by hens housed in conventional cages. These results paralleled those of a prior study in which inoculation with $\geq 10^6$ cfu led to fecal shedding for at least 8 wk (Gast et al., 2013b). The frequency and duration of fecal shedding of *Salmonella* Enteritidis are directly correlated with the oral exposure dose (Gast and Holt, 2000; Gast et al., 2011b; Gast et al., 2013a). However, horizontal contact transmission of naturally occurring infections in commercial flocks typically involves relatively low exposure doses and accordingly results in correspondingly infrequent infection and egg contamination (Gast and Holt, 1999; DeWinter et al., 2011; Esaki et al., 2013). The two *Salmonella* Enteritidis phage types used to infect hens in the present study were recovered from fecal samples at similar overall frequencies, although the phage type 13a strain was shed in feces for a longer post-inoculation interval (10 wk) than the phage type 4 strain (6 wk). The phage typing scheme for *Salmonella* Enteritidis has been epidemiologically useful for establishing relationships between strains from different sources, but it does not delineate consistent differences in the potential of individual strains to cause egg contamination (Gast and Holt, 2000; Gantois et al., 2009; Guard et al., 2011).

The management systems and facilities used to house laying hens can exert powerful influences on the sources, transmission, and persistence of pathogens such as *Salmonella* Enteritidis within flocks (Carrique-Mas et al., 2009a). However, diverse and sometimes contradictory results have emerged from prior research comparing the effects of the various housing systems on the prevalence of *Salmonella* infection and environmental contamination. For example, cage-free housing systems have been associated with higher levels of Enterobacteriaceae on egg shells (Jones and Anderson, 2013), more frequent *Salmonella* isolation from environmental samples, and a greater likelihood of horizontal transmission of *Salmonella* infection within flocks (De Vylder et al., 2011; Hannah et al., 2011; Watanabe et al., 2012). Access of poultry to outdoor areas vulnerable to pathogen introduction from external sources can be a particularly significant *Salmonella* risk factor (Mollenhorst et al., 2005). In other studies, cage-based housing systems were associated with a higher probability of *Salmonella* infection in flocks, especially in the presence of large populations of rodents

(Huneau-Salaün et al., 2009; Snow et al., 2010; Van Hoorebeke et al., 2010b). A third group of studies identified no significant differences between cage-based and cage-free flocks in either *Salmonella* fecal shedding or environmental contamination (Siemon et al., 2007; Jones et al., 2012). Likewise, the reported frequencies of *Salmonella* isolation from conventional cage and enriched colony cage systems have been similar in several previous investigations (De Vylder et al., 2009; Nordentoft et al., 2011; Van Hoorebeke et al., 2011). In the present study, strains of two different phage types of *Salmonella* Enteritidis were recovered from fecal samples collected from experimentally infected groups of laying hens at significantly higher overall frequencies from conventional cages than from enriched colony cages. For both strains combined, a significant difference between housing types was observed at each of the first four weekly post-inoculation sampling intervals. These results suggest that parameters such as stocking density and behavioral restriction (which are the most readily discernible distinctions between conventional and enriched cage systems) may influence the susceptibility of hens to the establishment of intestinal colonization by *Salmonella* Enteritidis. A corresponding effect on systemic invasion to internal organs was observed in a similarly designed prior study (Gast et al., 2013b). Stress associated with housing poultry at high stocking densities has been linked to the suppression of both humoral and cellular immunity and to increased organ invasion by *Salmonella* Enteritidis (Gomes et al., 2014). Impaired lymphocyte function within intestinal lymphoid tissues such as Peyer's patches and cecal tonsils could compromise the hen's ability to effectively clear infection from the gut (Holt et al., 2010). Nevertheless, groups of chickens housed in conventional and enriched cages did not differ significantly in the observed persistence of fecal shedding in the present study or in the frequencies of either horizontal transmission of infection or the production of internally contaminated eggs in earlier investigations (Gast et al., 2014a, b). Therefore, although housing systems may directly influence some specific aspects of the susceptibility of chickens to *Salmonella* Enteritidis, other secondary manifestations or consequences of infection may not be similarly affected. Carefully controlled experimental infection studies provide a unique opportunity to evaluate the effects of narrowly defined treatments, but they cannot fully encompass the complexity of the numerous management and environmental parameters that are present under commercial production conditions. Several multi-institutional field studies, which are currently in progress, should help provide an improved context for assessing the diverse experimentally generated data regarding *Salmonella* in different housing systems.

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## REFERENCES

Arnold, M. E., J. J. Carrique-Mas, and R. H. Davies. 2010. Sensitivity of environmental sampling methods for detecting *Salmonella* Enteritidis in commercial laying flocks relative to the within-flock prevalence. Epidemiol. Infect. 138:330–339.

Arnold, M. E., F. Martelli, I. McLaren, and R. H. Davies. 2014a. Estimation of the rate of egg contamination from *Salmonella*-infected chickens. Zoonoses Pub. Health 61:18–27.

Arnold, M. E., F. Martelli, I. McLaren, and R. H. Davies. 2014b. Estimation of the sensitivity of environmental sampling for detection of *Salmonella* in commercial layer flocks post-introduction of national control programmes. Epidemiol. Infection. 142:1061–1069.

Carrique-Mas, J. J., M. Breslin, L. Snow, I. McLaren, A. R. Sayers, and R. H. Davies. 2009a. Persistence and clearance of different *Salmonella* serovars in buildings housing laying hens. Epidemiol. Infect. 137:837–846.

Carrique-Mas, J. J., and R. H. Davies. 2008. Sampling and bacteriological detection of *Salmonella* in poultry and poultry premises: a review. Rev. Sci. Tech. Off. Int. Epiz. 27:665–677.

Carrique-Mas, J. J., C. Marín, M. Breslin, I. McLaren, and R. H. Davies. 2009b. A comparison of the efficacy of cleaning and disinfection methods in eliminating *Salmonella* spp. from commercial egg laying houses. Avian Pathol. 38:419–424.

Centers for Disease Control and Prevention. 2011. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food — foodborne diseases active surveillance network, 10 U.S. sites, 1996–2010. Morbid. Mortal. Weekly Rep. 60:749–755.

Chai, S. J., P. L. White, S. L. Lathrop, S. M. Solghan, C. Medus, B. M. McGlinchey, M. Tobin-D'Angelo, R. Marcus, and B. E. Mahon. 2012. *Salmonella enterica* serotype Enteritidis: increasing incidence of domestically acquired infections. Clin. Infect. Dis. 54:S488–S497.

Davies, R. H., and M. Breslin. 2003. Persistence of *Salmonella* Enteritidis phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ. Microbiol. 5:79–84.

De Vylder, J., J. Dewulf, S. Van Hoorebeke, F. Pasmans, F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Horizontal transmission of *Salmonella* Enteritidis in groups of experimentally infected hens housed in different housing systems. Poult. Sci. 90:1391–1396.

De Vylder, J., S. Van Hoorebeke, R. Ducatelle, F. Pasmans, F. Haesebrouck, J. Dewulf, and F. Van Immerseel. 2009. Effect of the housing system on shedding and colonization of gut and internal organs of laying hens with *Salmonella* Enteritidis. Poult. Sci. 88:2491–2495.

Dewaele, I., G. Rasschaert, C. Wildemauwe, H. Van Meirhaeghe, M. Vanrobaeys, E. De Graef, L. Herman, R. Ducatelle, M. Heyndrickx, and K. De Reu. 2012a. Polyphasic characterization of *Salmonella* Enteritidis isolates on persistently contaminated layer farms during the implementation of a national control program with obligatory vaccination: a longitudinal study. Poultry Sci. 91:2727–2735.

Dewaele, I., H. Van Meirhaeghe, G. Rasschaert, M. Vanrobaeys, E. De Graef, L. Herman, R. Ducatelle, M. Heyndrickx, and K. De Reu. 2012b. Persistent *Salmonella* Enteritidis environmental contamination on layer farms in the context of an implemented national control program with obligatory vaccination. Poult. Sci. 91:282–291.

DeWinter, L. M., W. H. Ross, H. Couture, and J. F. Farber. 2011. Risk assessment of shell eggs internally contaminated with *Salmonella* Enteritidis. Int. Food Risk Anal. J. 1:40–81.

Esaki, H., K. Shimura, Y. Yamazaki, M. Eguchi, and M. Nakamura. 2013. National surveillance of *Salmonella* Enteritidis in commercial eggs in Japan. Epidemiol. Infect. 141:941–943.

Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, R. Gast, T. J. Humphrey, and F. Van Immerseel. 2009. Mechanisms of egg contamination by *Salmonella* Enteritidis. FEMS Microbiol. Rev. 33:718–738.

Garber, L., M. Smeltzer, P. Fedorka-Cray, S. Ladely, and K. Ferris. 2003. Salmonella enterica serotype enteritidis in table egg layer house environments and in mice in U.S. layer houses and associated risk factors. Avian Dis. 47:134–142.

Gast, R. K. 2007. Serotype-specific and serotype-independent strategies for preharvest control of food-borne *Salmonella* in poultry. Avian Dis. 51:817–828.

Gast, R. K., J. Guard-Bouldin, R. Guraya, and P. S. Holt. 2009. Effect of prior passage through laying hens on invasion of reproductive organs by *Salmonella enteritidis*. Int. J. Poult. Sci. 8:116–212.

Gast, R. K., J. Guard-Bouldin, and P. S. Holt. 2005. The relationship between the duration of fecal shedding and the production of contaminated eggs by laying hens infected with strains of *Salmonella* Enteritidis and *Salmonella* Heidelberg. Avian Dis. 49:382–386.

Gast, R. K., R. Guraya, and J. Guard. 2013a. *Salmonella* Enteritidis deposition in eggs after experimental infection of laying hens with different oral doses. J. Food Prot. 76:108–113.

Gast, R. K., R. Guraya, J. Guard, and P. S. Holt. 2011a. The relationship between the numbers of *Salmonella* Enteritidis, *Salmonella* Heidelberg, or *Salmonella* Hadar colonizing reproductive tissues of experimentally infected laying hens and deposition inside eggs. Avian Dis. 55:243–247.

Gast, R. K., R. Guraya, and P. S. Holt. 2011b. Frequency and persistence of fecal shedding following exposure of laying hens to different oral doses of *Salmonella enteritidis*. Int. J. Poult. Sci. 10:750–756.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2013b. Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 92:468–473.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2014a. Contamination of eggs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 93:728–733.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2014b. Horizontal transmission of *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 93:3145–3151.

Gast, R. K., and P. S. Holt. 1998. Persistence of *Salmonella enteritidis* from one day of age until maturity in experimentally infected layer chickens. Poult. Sci. 77:1759–1762.

Gast, R. K., and P. S. Holt. 1999. Experimental horizontal transmission of Salmonella enteritidis strains (phage types 4, 8, and 13a) in chicks. Avian Dis. 43:774–778.

Gast, R. K., and P. S. Holt. 2000. Deposition of phage type 4 and 13a Salmonella enteritidis strains in the yolk and albumen of eggs laid by experimentally infected hens. Avian Dis. 44:706–710.

Gast, R. K., B. W. Mitchell, and P. S. Holt. 1998. Airborne transmission of Salmonella enteritidis infection between groups of chicks in controlled-environment isolation cabinets. Avian Dis. 42:315–320.

Gole, V. C., C. G. B. Caraguel, M. Sexton, C. Fowler, and K. K. Chousalkar. 2014. Shedding of Salmonella in single age caged commercial layer flock at an early stage of lay. Int. J. Food Microbiol. 189:61–66.

Gomes, A. V. S., W. M. Quinteiro-Filho, A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, E. Baskeville, A. T. Akamine, C. S. Astolfi-Ferreira, A. J. P. Perreira, and J. Palermo-Neto. 2014. Overcrowding stress decreases macrophage activity and increases *Salmonella* Enteritidis invasion in broiler chickens. Avian Pathol. 43:82–90.

Guard, J., C. A. Morales, P. Fedorka-Cray, and R. K. Gast. 2011. Single nucleotide polymorphisms that differentiate two subpopulations of *Salmonella* enteritidis within phage type. BMC Res. Notes 4:369.

Hannah, J. F., J. L. Wilson, N. A. Cox, L. J. Richardson, J. A. Cason, D. V. Bourassa, and R. J. Buhr. 2011. Horizontal transmission of *Salmonella* and *Campylobacter* among caged and cage-free laying hens. Avian Dis. 55:580–587.

Havelaar, A. H., S. Ivarsson, M. Löfdahl, and M. J. Nauta. 2013. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. Epidemiol. Infect. 141:293–302.

Holt, P. S., R. H. Davies, J. Dewulf, R. K. Gast, J. K. Huwe, D. R. Jones, D. Waltman, and K. R. Willian. 2011. The impact of different housing systems on egg safety and quality. Poult. Sci. 90:251–262.

Holt, P. S., L. E. Vaughn, and R. K. Gast. 2010. Flow cytometric characterization of Peyer's patch and cecal tonsil T lymphocytes in laying hens following challenge with *Salmonella enterica* serovar Enteritidis. Vet. Immunol. Immunopathol. 133:276–281.

Humphrey, T. J. 2006. Are happy chickens safer chickens? Poultry welfare and disease susceptibility. Brit. Poult. Sci. 47:379–391.

Huneau-Salaün, A., C. Marianne, S. Le Bouquin, F. Lalande, I. Petetin, S. Rouxel, M. Virginie, P. Fravalo, and N. Rose. 2009. Risk factors for *Salmonella enterica* subsp. *Enterica* contamination in 519 French laying hen flocks at the end of the laying period. Prev. Vet. Med. 89:51–58.

Jackson, B. R., P. M. Griffin, D. Cole, K. A. Walsh, and S. J. Chai. 2013. Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998–2008. Emerg. Infect. Dis. 19:1239–1244.

Jones, D. R., and K. E. Anderson. 2013. Housing system and laying hen strain impacts on egg microbiology. Poult. Sci. 92:2221–2225.

Jones, D. R., K. E. Anderson, and J. Y. Guard. 2012. Prevalence of coliforms, *Salmonella*, *Listeria*, and *Campylobacter* associated with eggs and the environment of conventional cage and free-range egg production. Poult. Sci. 91:1195–1202.

Kinde, H., D. M. Castellan, D. Kerr, J. Campbell, R. Breitmeyer, and A. Ardans. 2005. Longitudinal monitoring of two commercial layer flocks and their environments for *Salmonella enterica* serovar Enteritidis and other Salmonellae. Avian Dis. 49:189–194.

Lapuz, R. R. S. P., D. V. Umali, T. Suzuki, K. Shirota, and H. Katoh. 2012. Comparison of the prevalence of *Salmonella* infection in layer hens from commercial layer farms with high and low rodent densities. Avian Dis. 56:29–34.

Li, X., J. B. Payne, F.B. Santos, J. F. Levine, K. E. Anderson, and B. W. Sheldon. 2007. *Salmonella* populations and prevalence in layer feces from commercial high-rise houses and characterization of the *Salmonella* isolates by serotyping, antibiotic resistance analysis, and pulsed field gel electrophoresis. Poult. Sci. 86: 591–597.

Martelli, F., R. Gosling, I. McLaren, A. Wales, and R. Davies. 2014. Development and testing of external quality assessment samples for *Salmonella* detection in poultry samples. Lett. Appl. Microbiol. 59:443–448.

Mollenhorst, H., C. J. van Woudenbergh, E. G. M. Bokkers, and I. J. M. de Boer. 2005. Risk factors for *Salmonella enteritidis* infections in laying hens. Poult. Sci. 84:1308–1313.

Nordentoft, S., L. Mølbak, L. Bjerrum, J. De Vylder, F. Van Immerseel, and K. Pedersen. 2011. The influence of the cage system and colonisation of *Salmonella* Enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. BMC Microbiology 11:187.

O'Brien, S. J. 2013. The "decline and fall" of nontyphoidal *Salmonella* in the United Kingdom. Clin. Infect. Dis. 56: 705–710.

Okamura, M., M. Sonobe, S. Obara, T. Kubo, T. Nagai, M. Noguchi, K. Takehara, and M. Nakamura. 2010. Potential egg contamination by *Salmonella enterica* serovar Typhimurium definitive type 104 following experimental infection of pullets at the onset of lay. Poult. Sci. 89:1629–1634.

Pires, S. M., A. R. Vieira, T. Hald, and D. Cole. 2014. Source attribution of human salmonellosis: an overview of methods and estimates. Foodborne Pathogens Dis. 11:667–676.

Pitesky, M., B. Charlton, M. Bland, and D. Rolfe. 2013. Surveillance of *Salmonella* Enteritidis in layer houses: a retrospective comparison of the Food and Drug Administration's Egg Safety Rule (2010–0211) and the California Egg Quality Assurance Program (2007–2011). Avian Dis. 57:51–56.

Schulz, J., S. Van Hoorebeke, B. Hald, J. Hartung, F. Van Immerseel, I. Radtke, S. Kabell, and J. Dewulf. 2011. The dynamics of *Salmonella* occurrence in commercial laying hen flocks throughout a laying period. Avian Pathol. 40 243–248.

Siemon, C. E., P. B. Bahnson, and W. A. Gebreyes. 2007. Comparative investigation of prevalence and antimicrobial resistance of *Salmonella* between pasture and conventionally reared poultry. Avian Dis. 51:112–117.

Snow, L. C., R. H. Davies, K. H. Christiansen, J. J. Carrique-Mas, A. J. C. Cook, and S. J. Evans. 2010. Investigation of risk factors for *Salmonella* on commercial egg-laying farms in Great Britain, 2004–2005. Vet. Rec. 166:579–586.

Thomas, E., A. Bouma, and D. Klinkenberg. 2011. A comparison of transmission characteristics of *Salmonella enterica* serovar Enteritidis between pair-housed and group-housed laying hens. Vet. Res. 42:40.

Trampel, D. W., T. G. Holder, and R. K. Gast. 2014. Integrated farm management to prevent *Salmonella* Enteritidis contamination of eggs. J. Appl. Poult. Res. 23:353–365.

Van Hoorebeke, S., F. Van Immerseel, F. Haesebrouck, R. Ducatelle, and J. Dewulf. 2011. The influence of the housing system on *Salmonella* infections in laying hens. Zoonoses Public Hlth. 58:304–311.

Van Hoorebeke, S., F. Van Immerseel, J. De Vylder, R. Ducatelle, F. Haesebrouck, F. Pasmans, A. de Kruif, and J. Dewulf. 2010a. The age of production system and previous *Salmonella* infections in laying hen flocks. Poult. Sci. 89:1315–1319.

Van Hoorebeke, S., F. Van Immerseel, J. Schulz, J. Hartung, M. Harisberger, L. Barco, A. Ricci, G. Theodoropoulos, E. Xylouri, J. De Vylder, R. Ducatelle, F. Haesebrouck, F. Pasmans, A. de Kruif, and J. Dewulf. 2010b. Determination of the within and between flock prevalence and identification of risk factors for *Salmonella* infections in laying hen flocks housed in conventional and alternative systems. Prev. Vet. Med. 94:94–100.

Wales, A., M. Breslin, B. Carter, R. Sayers, and R. Davies. 2007. A longitudinal study of environmental salmonella contamination in caged and free-range layer flocks. Avian Pathol. 36:187–197.

Wales, A., M. Breslin, and R. Davies. 2006. Semiquantitative assessment of the distribution of *Salmonella* in the environment of caged layer flocks. J. Appl. Microbiol. 101:309–318.

Waltman, W. D., and R. K. Gast. 2008. Salmonellosis. Pages 3–9 in A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 5th ed. L. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. Pearson, W. M. Reed, M. W. Jackwood, and P. R. Woolcock, eds. American Association of Avian Pathologists, Athens, GA.

Watanabe, O., M. Ogino, A. Iwamoto, T. Akiyama, and T. Miki. 2012. Henhouse feeding style and *Salmonella* Enteritidis contamination in unvaccinated flocks of egg farms, April 1994 -March 2001. J. Vet. Med. Sci. 74:575–582.