Research Note: Contamination of eggs by *Salmonella* Enteritidis and *Salmonella* Typhimurium in experimentally infected laying hens in indoor cage-free housing

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ABSTRACT Contaminated eggs are a leading source of human *Salmonella* infections and this problem continues to challenge public health authorities and egg industries around the world. *Salmonella* invasion of the ovaries and oviducts of infected laying hens can result in bacterial deposition inside the edible portions of developing eggs. The introduction, persistence, and transmission of salmonellae in commercial egg-laying flocks are influenced by flock management practices, but the food safety ramifications of different types of laying hen housing remain unresolved. The present study assessed the frequency of internal contamination of eggs after experimental *Salmonella* Enteritidis and *S.* Typhimurium infection of laying hens in indoor cage-free housing. Groups of 72 hens were housed on wood shavings in isolation rooms simulating commercial cage-free barns with community kick-out nest boxes and perches and 1/3 of the hens in each room were orally inoculated with 8.0×10^7 cfu of 2-strain mixtures of either *S.* Enteritidis (2 rooms) or *S.* Typhimurium (2 rooms), and the entire internal contents of all eggs laid 5 to 30 d postinoculation in nest boxes or on the flooring substrate were cultured to detect *Salmonella.* Contaminated eggs were laid between 8 and 28 d postinoculation. The overall incidence of *S.* Enteritidis isolation from eggs (3.41%) was significantly ($P = 0.0005$) greater than *S.* Typhimurium (1.19%). The contamination frequencies associated with the 2 egg collection locations were not significantly different ($P > 0.05$). These results demonstrate that oral infection of a relatively small proportion of laying hens in indoor cage-free housing with invasive *Salmonella* serovars can result in the production of internally contaminated eggs at low frequencies over a period of nearly a month postinoculation.

Key words: *Salmonella* Enteritidis, *Salmonella* Typhimurium, laying hens, cage-free housing, eggs

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INTRODUCTION

Contaminated eggs can transmit *Salmonella* infections to humans and this problem has challenged public health authorities and egg industries around the world for more than 3 decades (Chousalkar et al., 2018). Eggs and egg products have been identified as the most common food vehicles for human salmonellosis in Europe, principally caused by *Salmonella enterica* serovar Enteritidis. The prevalence of *S.* Enteritidis in commercial egg-laying chickens has been directly correlated with the incidence of human infections, and human outbreak strains of this pathogen are often genetically identical to laying flock isolates. Contaminated eggs have also been implicated as the sources of human *S.* Typhimurium infections, particularly in Australia. Meaningful reductions in the occurrence of human *Salmonella* infections have been attributed to the widespread implementation of comprehensive risk reduction and flock testing programs for egg-producing flocks. Nevertheless, as the incidence of human *S.* Enteritidis infections in the United States has not declined significantly for more than a decade, refinements or improvements in pathogen control strategies for laying flocks continue to be topics of considerable interest.

Infections with invasive *Salmonella* serovars are typically initiated by oral ingestion of the pathogen, leading to intestinal colonization and rapid dissemination to internal organs such as the liver and spleen. In mature laying hens, subsequent involvement of the ovaries and

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oviducts can result in bacterial deposition inside the developing yolk and albumen prior to shell formation and oviposition (Gantois et al., 2009). Higher frequencies of egg contents contamination have often been observed with *S.* Enteritidis than with other serovars, associated with greater adherence to reproductive tract mucosa and invasion of ovarian granulosa cells. However, even after infection of hens with high oral doses, *S.* Enteritidis is generally found inside eggs at low frequencies and in low concentrations. Because hens in commercial laying flocks are most likely exposed to salmonellae from environmental reservoirs in relatively small doses, egg contamination occurs very infrequently.

Opportunities for the introduction, persistence, and transmission of salmonellae in commercial egg-laying flocks are strongly influenced by flock management strategies and practices as well as by some intrinsic design features of poultry housing facilities. In recent years, concerns about animal welfare have spurred the commercial egg industry to consider transitioning from conventional cage-based hen housing to less space-intensive alternatives such as indoor cage-free aviaries or outdoor free-range systems. However, the public health ramifications of laying hen housing are not entirely clear, as previous research regarding the effects of different poultry management systems on *Salmonella* and other food safety pathogens has yielded diverse and sometimes contradictory results. A number of confounding issues, including differences in bird stocking densities, levels of exposure to contaminated dust and feces, and populations of biological vectors, have often obscured meaningful comparisons between systems in these studies (Holt et al., 2011). Nevertheless, prior data has established that the unique inherent characteristics of each housing system present correspondingly unique challenges for pathogen control efforts (Jones et al., 2015). Understanding how housing and management factors affect the outcomes of *Salmonella* infections in poultry is a key issue for ensuring safe transition into cage-free systems. The objective of the present study was to assess the frequency of internal contamination of eggs after experimental *Salmonella* Enteritidis and *Salmonella* Typhimurium infection of laying hens in indoor cage-free housing.

MATERIALS AND METHODS

Experimental Housing of Laying Hens

In each of 2 similar trials, 144 female Tetra Brown chicks (a strain used by the commercial egg industry) were obtained from a breeding company and reared in cage-free barns at Purdue University (West Lafayette, IN) without vaccination against *Salmonella* infection. At 20 wk of age, the young hens were transferred to the US National Poultry Research Center (Athens, GA), distributed evenly between two separate rooms of a disease-containment facility, and allowed to acclimate to experimental housing conditions for 3 wk prior to *Salmonella* infection. Each isolation room simulated a commercial cage-free barn with community kick-out nest boxes and perches. Hens were housed on a wood shavings flooring substrate at a density of 1,710 cm^2 of horizontal space per bird. Water was supplied *ad libitum* via automatic nipple-type drinkers and feed (antibiotic-free, ground mash layer ration) was provided according to standards for commercial egg production. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the US National Poultry Research Laboratory.

Experimental Infection of Laying Hens With Salmonella

In each of 2 trials, 24 of the 72 hens in one isolation room were orally inoculated with a measured dose of a 2-strain mixture of *S.* Enteritidis and 24 of the 72 hens in the other room were similar infected with a 2-strain mixture of *S.* Typhimurium. One room in each trial was infected at 23 wk of age and the other room at 24 wk, with the order of administration of the 2 serovars reversed between trials. All *Salmonella* strains were originally isolated from internal organs of naturally infected chickens in commercial settings. Each inoculum strain was resuscitated by transfer into tryptic soy (**TS**) broth (Acumedia, Neogen Corp., Lansing, MI) for 2 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, equal numbers of the 2 inoculum component strains were combined, and further serial dilutions in 0.85% saline were performed to achieve the desired final cell concentration. Plate counts on brilliant green (**BG**) agar (Acumedia) confirmed that the final cell concentration in each 1.0-mL oral inoculum dose was 8.0×10^7 cfu. Orally infected hens were identified by colored leg bands.

Pre-inoculation Cloacal Swab Samples

Immediately before inoculation, sterile cotton swabs were used to collect cloacal swab samples from 56 randomly selected hens in each room. Each sample was transferred into 10 mL of buffered peptone water (Acumedia) and incubated for 24 h at 37°C. A 0.1 mL portion of each culture was then transferred into 10 mL of Rappaport-Vassiliadis broth (Acumedia) and incubated for 24 h at 41.5°C. A 10-μL portion from each of these broth cultures was then streaked onto BG agar supplemented with 0.02 mg/mL of novobiocin (Sigma Chemical Co., St. Louis, MO). These plates were incubated for 24 h at 37°C and then examined for the presence of typical *Salmonella* colonies.

Egg Contents Samples

In each trial, 60 eggs were collected (30 per room) prior to *Salmonella* inoculation. Beginning at 5 d postinoculation and continuing through 30 d postinoculation

in each trial, all eggs were collected daily from each room. Eggs were stored at room temperature for up to 3 d until transport to the laboratory for culturing to detect internal contamination with *Salmonella*. Eggs collected from nest boxes and from the flooring substrate were separately identified. Eggshell surfaces were disinfected by dipping for 5 s in 70% ethanol and the shells were then broken against a sharp edge covered by sterile foil strips. The entire liquid contents of each egg were transferred to 50 mL of TS broth, mixed by vigorous shaking for 15 s, and incubated for 24 h at 37°C. A 0.1-mL portion of each incubated TS broth culture was transferred to 10 mL of Rappaport Vassiliadis broth (Acumedia) and incubated for 24 h at 41.5°C. A 10-μL aliquot from each of these broth cultures was then streaked onto BG agar plus novobiocin and incubated for 24 h at 37°C. After incubation of these plates for 24 h at 37°C, typical *S.* Enteritidis colonies were subjected to biochemical and serological confirmation.

Statistical Analysis

For each replicate within the 2 serovar-specific trials (and for both replicates combined), significant differences ($P < 0.05$) between the 2 egg collection locations in the mean frequencies of *Salmonella* isolation from internal contents of eggs were determined by Fisher's exact test. Egg contamination frequencies associated with the 2 *Salmonella* serovars were similarly compared using the chi square statistic with Yates correction (because of the larger number of values involved). Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

None of the preinfection fecal samples were *Salmonella*-positive in either trial. Internal contamination with *S.* Typhimurium was detected in eggs laid from 10 to 28 d after oral inoculation of one-third of the hens (Table 1). The overall frequency of *S.* Typhimurium egg contamination was 1.19% (1.74% from eggs laid in nest boxes and 1.13% from eggs laid on the flooring substrate). Internal contamination with *S.* Enteritidis was detected from 8 to 26 d postinoculation (Table 1), at an overall frequency of 3.41% (3.37% for nest box eggs and 3.74% for flooring substrate eggs). For both *S.* Typhimurium and *S.* Enteritidis, no significant differences in egg contamination frequencies ($P > 0.05$) were found between the 2 experimental replicates or between the 2 egg collection locations. The frequencies of recovery of *S.* Enteritidis were greater than those of S. Typhimurium from eggs laid on the flooring substrate ($P = 0.0498$) and from all eggs ($P = 0.0005$).

Hens infected with both *Salmonella* challenge serovars in the present study laid internally contaminated eggs, but *S.* Enteritidis was recovered from the contents of significantly more total eggs (and also from more eggs laid on the flooring substrate) than was *S.* Typhimurium. *Salmonella* serovars Enteritidis and Typhimurium are both highly prevalent in commercial poultry. Both serovars are highly invasive and can colonize reproductive tissues (Gantois et al., 2008), although this has been shown to lead to deposition inside developing eggs more often for *S.* Enteritidis than *S.* Typhimurium. More intense tissue pathology and immune responses associated with *S.* Typhimurium infection have been postulated to diminish the likelihood of deposition inside eggs. Fecal contamination of shells appears to be the most likely source of *Salmonella* Typhimurium contamination of eggs in Australia (Pande et al., 2016). The ability of *S.* Enteritidis to persist in avian reproductive tracts and survive inside eggs has been attributed to genes which regulate cell surface lipopolysaccharides and flagella or are responsible for stress responses (Raspoet et al., 2014), often manifested as small changes in multiple genes.

Until relatively recently, nearly all egg-laying hens were housed in conventional cage systems. Considerable data are available that characterize the progress, outcomes, and control of *Salmonella* infections in caged poultry. High stocking densities in conventional cages have been associated with an increased frequency of

Table 1. Recovery of *Salmonella* Typhimurium and *Salmonella* Enteritidis from the internal contents of eggs laid by experimentally infected laying hens in indoor cage-free housing.[1]

	Replicate 1	Replicate 2	All replicates	First and last contaminated eggs (d postinfection)
	Salmonella-positive/total (%)			
S. Typhimurium				
Eggs laid in nest boxes	1/27 (3.70)[a]	1/88 (1.14)[a]	2/115 (1.74)[abcd]	13–22
Eggs laid on flooring substrate	7/572 (1.22)[a]	6/577 (1.04)[a]	13/1149 (1.13)[ac]	10–28
All eggs	8/599 (1.34)	7/675 (1.08)	15/1264 (1.19)[ab]	10–28
S. Enteritidis				
Eggs laid in nest boxes	16/493 (3.25)[a]	15/426 (3.52)[a]	31/919 (3.37)[d]	8–26
Eggs laid on flooring substrate	3/71 (4.23)[a]	1/36 (2.78)[a]	4/107 (3.74)[bd]	10–21
All eggs	19/564 (3.37)	16/462 (3.46)	35/1026 (3.41)[cd]	8–26

[1]Eggs were collected for sampling between the 5th and 30th d after 24 of 72 hens in each cage-free housing room were orally inoculated with approximately 8.0×10^7 cfu of two-strain mixtures of either *S.* Typhimurium or *S.* Enteritidis. The remaining hens were exposed to infection by horizontal contact.

[a,b,c,d]Values in columns within individual replicate trials or in the column for all replicates combined that share no common superscripts are significantly ($P < 0.05$) different.

internal organ invasion by *S.* Enteritidis in experimentally infected hens (Gast et al., 2016), perhaps due to immunosuppressive stress. Less information is available about *Salmonella* in laying flocks housed in noncage systems. Comparative studies have produced variable results, as some studies have found higher frequencies of *Salmonella* in environmental, tissue, and egg samples from conventional cage systems and others have reported greater *Salmonella* prevalence and horizontal transmission in cage-free systems. In an experiment that examined conventional cage, enriched colony, and aviary housing systems managed under commercial conditions at the same location, no differences were observed in *Salmonella* contamination of environmental and egg shell samples (Jones et al., 2015). Dust, feces, rodents, and insects contribute to perpetuating *Salmonella* contamination in poultry facilities and supporting horizontal dissemination of infection among hens. Higher risks of *Salmonella* infection are reported among laying flocks that are larger, consist of older birds, or are housed in older facilities.

Despite the fact that all hens in the current experiment were of identical genetic stock and had been raised in the same location under identical conditions as chicks and pullets, the groups infected with *S.* Typhimurium laid the majority of their eggs on the flooring substrate and the groups infected with *S.* Enteritidis laid the majority of their eggs in the nest boxes. This illustrates some of the difficulties producers face in effectively managing consistent egg production with these types of housing systems. However, although exposure to *Salmonella* from feces would presumably increase the likelihood of shell contamination among eggs laid on the flooring substrate, no differences in egg contents contamination were observed between the 2 egg collection sites for either serovar. In a prior study using the same cage-free system and a similar experimental infection model as the current investigation, extensive horizontal spread among co-housed hens occurred within two weeks after the introduction of *S.* Enteritidis infection (Gast et al., 2020). In the present study, internal egg contamination was detected at relatively low overall frequencies between 8 and 28 d after one-third of the hens were orally inoculated with *S.* Enteritdis or *S.* Typhimurium. Earlier data have suggested that individual hens seldom lay contaminated eggs for longer than 3 wk after oral infection (Gast et al., 2019). The present study demonstrated that infection of a relatively small proportion of laying hens in indoor cage-free housing with invasive *Salmonella* serovars could result in the production of internally contaminated eggs at low frequencies over a period of nearly a month postinoculation. Horizontal transmission of salmonellae in cage-free systems, perhaps enhanced by extensive access of hens to each other and to feces-contaminated flooring substrate, may prolong opportunities for recently infected individuals to lay contaminated eggs.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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