Colonization of internal organs by *Salmonella* Enteritidis in experimentally infected laying hens housed in enriched colony cages at different stocking densities

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ABSTRACT Epidemiologic analyses have linked the frequency of human infections with *Salmonella enterica* subspecies *enterica* serovar Enteritidis to the consumption of contaminated eggs and thus to the prevalence of this pathogen in commercial egg-laying flocks. Contamination of the edible contents of eggs by *Salmonella* Enteritidis is a consequence of the colonization of reproductive tissues in systemically infected hens. The animal welfare implications of laying hen housing systems have been widely debated, but no definitive consensus has yet emerged about the food safety significance of poultry housing options. The present study sought to determine the effects of two different bird stocking densities on the invasion of internal organs by *Salmonella* Enteritidis in groups of experimentally infected laying hens housed in colony cages enriched with perching and nesting areas. In two trials, groups of laying hens were distributed at two different stocking densities into colony cages and (along with a group housed in conventional cages) orally inoculated with doses of 1.0×10^7 cfu of *Salmonella* Enteritidis. At 5 to 6 d post-inoculation, hens were euthanized and samples of internal organs were removed for bacteriologic culturing. For both trials combined, *Salmonella* Enteritidis was recovered at a significantly ($P < 0.05$) greater frequency from hens in enriched colony cages at the higher stocking density than at the lower density from livers (75.0% vs. 51.4%) and ovaries (51.4% vs. 30.6%). However, spleens from hens in enriched colony cages at the higher stocking density were significantly less often positive for *Salmonella* Enteritidis than from hens in conventional cages at that same density (90.3% vs. 68.1%). These results suggest that stocking density can influence the susceptibility of hens to *Salmonella* Enteritidis, but other housing systems parameters may also contribute to the outcome of infections.

Key words: *Salmonella* Enteritidis, laying hens, conventional cages, enriched colony cages, stocking density

2016 Poultry Science 95:1363–1369
http://dx.doi.org/10.3382/ps/pew037

INTRODUCTION

Despite significant commitments of government and private industry resources to disease control efforts, the incidence of human *Salmonella* infections in the United States has remained nearly constant over time (Centers for Disease Control and Prevention, 2011; Chai et al., 2012). A recent epidemiological survey in 24 European nations identified laying hens as the principal reservoir for human salmonellosis, accounting for 42% of all cases, and 96% of these involved *Salmonella enterica* subspecies *enterica* serovar Enteritidis (De Knegt et al., 2015). Throughout the world, most human illnesses caused by *Salmonella* Enteritidis have been attributed to the consumption of contaminated eggs (Jackson et al., 2013; Pires et al., 2014). Active disease surveillance and retrospective epidemiologic analyses have directly linked the frequency of human infections with this pathogen to its prevalence in commercial egg-laying chickens (Havelaar et al., 2013; Arnold et al., 2014). However, sustained participation in comprehensive testing and risk reduction programs for poultry flocks has reportedly decreased both egg contamination and human illnesses in several countries (Esaki et al., 2013; O'Brien, 2013).

Contamination of the edible interior contents of eggs by *Salmonella* Enteritidis is a consequence of the ability of this pathogen to colonize reproductive tissues in laying hens (Gantois et al., 2009; Gast et al., 2011a). *Salmonella* Enteritidis can invade past the intestinal tract to reach internal organs such as the liver and spleen within a few hours after oral exposure (He et al., 2010), and subsequent systemic dissemination can involve both the ovary (site of egg yolk maturation and

Published by Oxford University Press on behalf of Poultry Science Association 2016. This work is written by (a) US Government employee(s) and is in the public domain in the US.

Received September 26, 2015.

Accepted January 8, 2016.

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release) and oviduct (site of albumen secretion around the descending yolk) of infected hens (Gast and Holt, 2000; De Buck et al., 2004). Internal organ colonization typically declines steadily during the first several weeks following the oral infection of mature hens (Gast et al., 2007, 2011b), but persistent infection in even a small percentage of birds can sometimes prolong the opportunities for infection to spread horizontally within flocks and thereby lead to the further production of contaminated eggs (Gast et al., 2009).

The implications of different systems for housing commercial laying hens have been widely debated in a variety of contexts, including animal welfare, economic viability, and public health. Each housing system incorporates numerous complex facility characteristics and management practices which could potentially affect the persistence and transmission of infections with *Salmonella* Enteritidis and other pathogens. However, no definitive consensus has yet coalesced from the published scientific literature about the food safety consequences of using particular types of housing for egg-laying poultry (Holt et al., 2011; Whiley and Ross, 2015). Studies comparing conventional cage-based housing to cage-free housing, or to intermediate alternatives such as enriched (furnished) colony cages or aviaries, have produced variable results without demonstrating a consistent or convincing advantage for any one system in regard to *Salmonella* persistence in infected chickens or their environment (Holt et al, 2011). A recent multi-institutional field study reported that several different housing systems were associated with similar *Salmonella* prevalence in laying flocks, but each system posed unique inherent management challenges for sanitation and pathogen control (Jones et al., 2015). The stocking density of hens (expressed as the amount of available floor space per bird) is often identified as a characteristic of housing systems which might influence the likelihood of *Salmonella* infections. The objective of the present study was to determine the effects of two different bird stocking densities on the invasion of internal organs by *Salmonella* Enteritidis in groups of experimentally infected laying hens housed in colony cages enriched with perching and nesting areas.

MATERIALS AND METHODS

Experimental Housing of Laying Hens

In each of two similar trials, 142 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 35 wk old at the beginning of the first and second trials, respectively) were distributed into three separately housed groups in different rooms of a disease-containment facility containing cage systems designed to simulate commercial conditions. In one room, 42 hens were housed in conventional laying cages (6 hens per cage) which provided 648 cm^2 of floor space per bird. Hens in the other two rooms were housed in enriched colony laying cages, each of which included access to two perches and a single enclosed nesting area. In one enriched colony room, 40 hens were housed (20 per cage) at a stocking density of 973 cm^2 of floor space per bird. In the other enriched colony room, 60 hens were housed (30 per cage) at a stocking density of 648 cm^2 of floor space per bird. All hens were provided with water (via two automatic nipple-type drinkers in each conventional cage and six in each enriched colony 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 a phage type 4 *Salmonella* Enteritidis strain, originally isolated from the liver of an infected chicken by Dr. D. Munro at the Scottish *Salmonella* Reference Laboratory, Glasgow, UK. The inoculum strain was resuscitated for each trial by transfer into tryptic soy 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 10-fold dilutions in 0.85% saline produced a desired final cell concentration in each oral dose of approximately 1.2×10^7 cfu (confirmed by subsequent plate counts).

Fecal Samples

Immediately before 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 30 samples per room were collected, evenly distributed among all occupied cages. Each fecal sample was transferred into 9 mL of tetrathionate broth (Acumedia) and incubated for 24 h at 37°C. A 10-μL portion from each broth culture was then streaked onto brilliant green 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).

Internal Organ Samples

At 5 d post-inoculation in each trial, 18 hens were randomly selected from each of the three housing system treatment groups (drawn from three conventional cages and one enriched colony cage of each stocking

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Table 1. Recovery of *Salmonella* Enteritidis from internal organs of experimentally infected laying hens housed in conventional or enriched colony cages at different stocking densities.[1]

	Liver	Spleen	Ovary	Oviduct	Cecum
	S. Enteritidis-positive/total (%)				
Trial 1					
Conventional cages (648 cm^2/hen)	32/36 (88.9)[a]	33/36 (91.7)[a]	25/36 (69.4)[a]	14/36 (38.9)[a]	35/36 (97.2)[a]
Enriched colony cages (648 cm^2/hen)	26/36 (72.2)[a,b]	24/36 (66.7)[b]	18/36 (50.0)[a,b]	8/36 (22.2)[a,b]	32/36 (88.9)[a]
Enriched colony cages (973 cm^2/hen)	19/36 (52.8)[b]	18/36 (50.0)[b]	10/36 (27.8)[b]	4/36 (11.1)[b]	31/36 (86.1)[a]
Trial 2					
Conventional cages (648 cm^2/hen)	31/36 (86.1)[a]	32/36 (88.9)[a]	23/36 (63.9)[a]	12/36 (33.3)[a]	35/36 (97.2)[a]
Enriched colony cages (648 cm^2/hen)	28/36 (77.8)[a]	25/36 (69.4)[a,b]	19/36 (52.8)[a,b]	8/36 (22.2)[a,b]	33/36 (91.7)[a]
Enriched colony cages (973 cm^2/hen)	18/36 (50.0)[b]	20/36 (55.6)[b]	12/36 (33.3)[b]	4/36 (11.1)[b]	32/36 (88.9)[a]
Both trials					
Conventional cages (648 cm^2/hen)	63/72 (87.5)[a]	65/72 (90.3)[a]	48/72 (66.7)[a]	26/72 (36.1)[a]	70/72 (97.2)[a]
Enriched colony cages (648 cm^2/hen)	54/72 (75.0)[a]	49/72 (68.1)[b]	37/72 (51.4)[a]	16/72 (22.2)[a,b]	65/72 (90.3)[a]
Enriched colony cages (973 cm^2/hen)	37/72 (51.4)[b]	38/72 (52.8)[b]	22/72 (30.6)[b]	8/72 (11.1)[b]	63/72 (87.5)[a]

[1]In each trial, tissues were sampled 5 to 6 d after oral inoculation of groups of hens with approximately 10^7 cfu of a phage type 4 *Salmonella* Enteritidis strain. Hens were housed in conventional or enriched colony cages at the indicated levels of available floor space.

[a,b]Values in columns (within trials) that share no common superscripts are significantly ($P < 0.05$) different.

density) and humanely euthanized for bacteriologic culture of internal tissues. Portions (approximately 5 to 10 g) of the liver, spleen, ovary, oviduct (magnum-isthmus junction region), and ceca (including the ileocecal junction) from each hen were aseptically removed, transferred to 20 mL of buffered peptone water (Acumedia), and mixed by stomaching for 30 sec. After incubation for 24 h at 37°C, a 1-mL portion of each culture was transferred to 9 mL of tetrathionate broth and incubated for 24 h at 37°C. A 10-μL aliquot of each culture was then streaked onto brilliant green agar plus novobiocin. Following incubation of these plates for 24 h at 37°C, typical *Salmonella* Enteritidis colonies were subjected to biochemical and serological confirmation (Waltman and Gast, 2008). At 6 d post-inoculation, this necropsy procedure was repeated for 18 additional hens from each housing system treatment group.

Statistical Analysis

For each trial (and for both trials combined), significant differences ($P < 0.05$) between housing systems or between sampling dates in the mean frequencies of *Salmonella* Enteritidis isolation from internal organs were determined by Fisher's exact test. Because the two replicate sampling dates (5 and 6 d post-inoculation) did not differ significantly within either trial in *Salmonella* Enteritidis recovery from any of the five sampled tissues, their results were combined for analysis and presentation. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

RESULTS

None of the fecal samples collected prior to inoculation of the hens in either trial were *Salmonella*-positive. *Salmonella* Enteritidis was recovered from the ceca of 91.7% of all sampled hens in the two trials, with no significant differences observed between the three housing treatments in either trial or for both trials combined (Table 1). In Trial 1, the frequencies of *Salmonella* Enteritidis isolation (Table 1) were significantly greater among hens housed in conventional cages than among hens housed in enriched colony cages at the lower stocking density for sampled livers (88.9% vs. 52.8%; $P = 0.0015$), spleens (91.7% vs. 50.0%; $P = 0.0002$), ovaries (69.4% vs. 27.8%, $P = 0.0008$), and oviducts (38.9% vs. 11.1%; $P = 0.0130$). The recovery of *Salmonella* Enteritidis was significantly higher from hens in conventional cages than from enriched colony cages at the higher stocking density only from spleen samples (91.7% vs. 66.7%, $P = 0.0182$). The frequencies of *Salmonella* Enteritidis recovery did not differ significantly between the two enriched colony cage stocking density treatment groups for any of the sampled tissues in this trial.

In Trial 2 (Table 1), *Salmonella* Enteritidis was recovered significantly more often from hens housed in conventional cages than from enriched colony cages at the lower stocking density for samples of livers (86.1% vs. 50.0%; $P = 0.0002$), spleens (88.9% vs. 55.6%; $P = 0.0032$), ovaries (63.9% vs. 33.3%, $P = 0.0178$), and oviducts (33.3% vs. 11.1%; $P = 0.0451$). The frequency of *Salmonella* Enteritidis isolation was significantly higher among hens from the enriched colony cages at the higher stocking density than from the lower stocking density only from livers (77.8% vs. 50.0%, $P = 0.0263$). No significant differences were observed between the conventional cage and enriched colony cage (higher stocking density) treatment groups for any of the sampled tissues in this trial.

For both trials combined, significantly higher frequencies of *Salmonella* Enteritidis recovery were obtained from hens in conventional cages than from enriched colony cages (lower stocking density) for samples of livers (87.5% vs. 51.4%; $P < 0.0001$), spleens (90.3% vs. 52.8%; $P < 0.0001$), ovaries (66.7% vs. 30.6%, $P < 0.0001$), and oviducts (36.1% vs. 11.1%;

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$P = 0.0007$). A significantly higher proportion of samples was positive from hens in conventional cages than from enriched colony cages (higher stocking density) only for spleens (90.3% vs. 68.1%, $P = 0.0018$). The frequency of *Salmonella* Enteritidis isolation from hens in enriched colony cages was greater for the higher stocking density than the lower density for samples of livers (75.0% vs. 51.4%, $P = 0.0055$) and ovaries (51.4% vs. 30.6%, $P = 0.0173$). No significant differences were observed between the two trials in the frequencies of *Salmonella* Enteritidis recovery from any of the five sampled tissues.

DISCUSSION

Salmonella Enteritidis is deposited inside eggs when systemic infection involves reproductive tissues. Experimental infection studies have typically produced relatively low incidences of egg contamination, even after very large oral doses of *Salmonella* Enteritidis are administered to hens (Gast et al., 2011b, 2013a). Commercial laying flocks, exposed more sporadically to lower pathogen doses from environmental sources, produce contaminated eggs very infrequently (DeWinter et al., 2011; Esaki et al., 2013). The ability of *Salmonella* Enteritidis to reach the contents of developing eggs may be associated with enhanced adherence to reproductive tract mucosa (Wales and Davies, 2011). The in vivo persistence of *Salmonella* Enteritidis in the reproductive tract of chickens and bacterial survival in forming eggs have been associated with genes found in the major pathogenicity islands, involved in cell wall or lipopolysaccharide structure, or related to stress responses (Guard-Bouldin et al., 2004; Coward et al., 2013; Raspoet et al., 2014). For example, genes of the *Salmonella* pathogenicity island 1 type III secretion system have been found to play important roles in colonization of the gastrointestinal tract and invasion of the host epithelial cell layer (Jones et al., 2007). Reduced expression of these genes at the body temperature of poultry may restrict systemic bacterial proliferation, thereby leading more often to asymptomatic carriage than to clinical illness (Troxell et al., 2015). Small changes in multiple genes may be responsible for the sequential expression of diverse bacterial characteristics which mediate systemic infection and egg contamination (Guard et al., 2010, 2011). If environmentally mediated influences (such as those exerted by housing systems in the present study) affect the expression of these *Salmonella* virulence factors or their interactions with host cells, they could have a significant impact on the outcome of flock infections.

Environmental conditions in egg production facilities can create opportunities for the introduction and dissemination of pathogens in laying flocks (Trampel et al., 2014). Persistence in the production environment creates a reservoir for *Salmonella* Enteritidis introduction into successive flocks and from which strains with heightened abilities to cause systemic infection and egg contamination might periodically emerge (Dewaele et al., 2012a,b). *Salmonella* Enteritidis isolates with reduced resistance to environmental stressors have reportedly also exhibited reduced pathogenicity for chickens (Shah, 2014). Contaminated feces and dust are common sources for environmental persistence of salmonellae (Im et al., 2015), and rodent or insect vectors can sustain and amplify contamination (Lapuz et al., 2012; Wallner-Pendleton et al., 2014). The environmental prevalence of *Salmonella* in laying flocks has been attributed to diverse risk factors, including larger flock size, greater flock age, housing in older facilities, and multiple-age stocking (Van Hoorebeke et al., 2010a; Pitesky et al., 2013; Denagamage et al., 2015). After introduction from environmental sources, *Salmonella* Enteritidis can spread rapidly and extensively throughout poultry flocks (Thomas et al., 2011; Gast et al., 2014b). Stress caused by feed deprivation, water deprivation, or environmental heat can increase the susceptibility of chickens to horizontally transmitted infection (Humphrey, 2006; Okamura et al., 2010). Infected hens can shed *Salmonella* Enteritidis into the laying house environment via contaminated feces for several months (Gast et al., 2011c).

The various types of facilities and management practices used for commercial laying hens have diverse and complex influences on environmental sources of *Salmonella* and other pathogens (Carrique-Mas et al., 2009a), but the wide range of results obtained in previous research has made it difficult to reach a consensus about the food safety consequences of poultry housing options (Holt et al., 2011; Whiley and Ross, 2015). In some studies, conventional cage-based housing systems were associated with a higher prevalence of *Salmonella* infection or environmental contamination in egg-laying flocks, particularly if large rodent populations were present (Snow et al., 2010; Van Hoorebeke et al., 2010b; Denagamage et al., 2015). However, other published reports have linked cage-free housing systems to more frequent *Salmonella* isolation from egg shells or environmental samples, and more extensive horizontal dissemination of *Salmonella* infection within flocks (De Vylder et al., 2011; Hannah et al., 2011; Parisi et al., 2015). Poultry housing which provides access to outdoor areas can be particularly vulnerable to the introduction of salmonellae from external sources (Mollenhorst et al., 2005). In many instances, research has not identified any meaningful differences between cage-based and cage-free housing (Siemon et al., 2007; Jones et al., 2012) or between conventional cage and enriched colony cage systems (De Vylder et al., 2009; Nordentoft et al., 2011; Van Hoorebeke et al., 2011) in either flock infection or environmental contamination with *Salmonella*. In one recent investigation, no significant overall differences in the prevalence of *Salmonella* in environmental and egg shell samples were observed between conventional cage, enriched colony cage, or aviary housing under commercial conditions (Jones et al., 2015).

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Reservoirs for the persistence of *Salmonella* contamination which are specific to the facilities and practices of each poultry housing system may create correspondingly unique food safety risk factors (Carrique-Mas et al., 2009b; Jones et al., 2015).

In a series of prior studies, *Salmonella* Enteritidis was recovered from internal organs and voided feces at significantly higher overall frequencies from experimentally infected hens in conventional cages than in enriched colony cages, but no similar effects were observed in the persistence of fecal shedding, the frequency of horizontal transmission of infection, or the production of internally contaminated eggs (Gast et al., 2013b, 2014a,b, 2015). These results suggested that parameters which differed between the two housing systems under consideration, notably stocking density, can influence the susceptibility of hens to *Salmonella* Enteritidis infection. However, some secondary manifestations of these infections (including egg contamination, the most critical food safety parameter) may not always be correspondingly affected. Systemic infection is a necessary precursor to the deposition of *Salmonella* Enteritidis inside eggs, but the frequency or magnitude of reproductive organ invasion is an inconsistent predictor of the likelihood of egg contamination (Gast et al., 2004, 2007, 2011b).

The present study focused on the potential effects of stocking density by comparing larger and smaller groups of hens housed in similar enriched colony cage units. As in earlier studies, the frequency of *Salmonella* Enteritidis invasion to a diversity of internal organs (liver, spleen, ovary, and oviduct) was significantly greater in conventional housing than in enriched colony cage housing when the latter system employed a lower stocking density. However, when compared at a similar hen density, conventional cages yielded a higher frequency of *Salmonella* Enteritidis isolation than enriched colony cages only for spleen samples. When two different stocking densities were used in enriched colony cages, the higher bird density resulted in significantly higher *Salmonella* Enteritidis isolation from both livers and ovaries. These results suggest that stocking density is an important contributor to the susceptibility of hens to *Salmonella* Enteritidis infection, but some other characteristic of conventional cage housing may also play a role.

Stocking density could exert an effect on the susceptibility of poultry to pathogens by either diminishing immune responses or increasing the likelihood and magnitude of exposure by horizontal contact. Housing chickens in crowded, unsanitary conditions decreases their resistance to infection (Asakura et al., 2001). Stress associated with high poultry stocking densities has been shown to suppress both humoral and cellular immunity and to increase *Salmonella* Enteritidis invasion of internal organs (Gomes et al., 2014). Significant effects on colonization of the spleen (an important secondary lymphatic organ) in both the present and prior studies (Gast et al., 2013b) are consistent with this stress-related explanation. A stress-mediated reduction in the ability of B cells to secrete mucosal IgA might facilitate gastrointestinal colonization by salmonellae (Vaughn et al., 2008). Alternatively, impaired lymphocyte function in lymphoid tissues could compromise the clearance of infection from the hen's tissues (Holt et al., 2010). Alterations in the complex regulatory circuitry which coordinates immune responses could also damage the efficacy of host defenses against pathogens (Babu et al., 2012; Shanmugasundaram et al., 2015).

Experimental infection models provide an assessment of the effects of narrowly defined treatments under carefully controlled conditions, but they cannot account for all of the complex management and environmental parameters found under commercial production conditions. A complete perspective on the food safety consequences of poultry housing systems requires the integration of experimental information about susceptibility to infection with field data regarding pathogen persistence in the environment. Neither of these lines of inquiry have demonstrated a consistent food safety advantage for any one type of housing, but the emerging broader perspective is that effective pathogen control may result from addressing the challenges posed by each system's unique risk factors.

ACKNOWLEDGMENTS

We gratefully express appreciation for excellent technical assistance from Robin Woodroof and laboratory support services from Garrett Ward (U. S. National Poultry Research Center, Athens, GA).

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