Comparison and assessment of necropsy lesions in end-of-lay laying hens from different housing systems in Denmark

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ABSTRACT Apperantly healthy laying hens at the end of production (60 to 91 wk) were investigated for the occurrence of pathology and bacterial infections. In total, 7,477 hens from 15 flocks representing the following production systems: Enriched cages, barn housed layers, and organic/free range layers were necropsied. Indications of bacterial infection were investigated by bacteriological cultivation. The overall prevalence of lesions was 16.60%, including lesions of both infectious and non-infectious origin. The most prevalent lesions were bursitis presternalis (6.65%), reproductive tract lesions (e.g., salpingitis and/or peritonitis and/or oophoritis) (3.50%), serosal scarification (e.g., fibrotic adhesive peritonitis) 1.55%, and neoplasm 1.73%. Significant differences were observed between different production systems and/or flocks in the prevalence of reproductive tract lesions, bursitis presternalis, serosal scarification, skin infections, juvenile hens, and traumas/fractures. No significant difference was observed between different production systems in the prevalence of neoplasia, infection of septicemic etiology, and pododermatitis. In total, 3.4% of the hens were out of lay, with significantly higher rate in organic flocks. Infections of the reproductive tract were the most prevalent lesions with bacterial etiology in all productions systems. In total, 40% of the hens with lesions associated to the oviduct were out of lay and significant difference between production systems were observed. *Escherichia coli* was the most commonly isolated bacteria and in 90% of the cases they were isolated from the reproductive tract lesions. The second most prevalent bacteria was *Gallibacteruim anatis*. Significant difference in the prevalence of *E. coli* positive hens was observed between production systems ($P < 0.05$). In conclusion, the prevalence of reproductive tract lesions in apparently healthy end-of-lay laying was higher than indicated in previous reports. These findings support the previous suggestions that *E. coli* and *G. anatis* are the major pathogens causing reproductive tract lesions.

Key words: laying hens, subclinical infections, pathology, bacteriology, reproductive tract

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INTRODUCTION

In the poultry industry, good health and management go hand- in-hand with animal welfare and production economy. Frequently observed causes of death of non-outbreak related mortality (i.e., normal mortality) in egg laying hens are chronic infections. The most frequent being salpingitis with large exudate impaction, chronic hock arthritis with fibrosis of the joint capsule, systemic amyloidosis, birds with pronounced emaciation/cachexia, and/or other sequelae like gouty nephropathy. These birds may have been diseased for a long period prior to death. Besides reduced animal welfare for the affected hens, the inflammation as such releases a whole range of both pro- and anti-inflammatory mediators. These mediators can suppress appetite, thus contributing to reduced availability of nutrients and induction of catabolic mechanisms in the host (Broom and Kogut, 2018). Some mediators (e.g., serum amyloid A, amyloidosis) may even deposit as insoluble proteins in various organs as a consequence of a chronic infection causing a prolonged inflammatory response in poultry (Landman et al., 1998; Bisgaard and Christensen, 2011; Breuer et al., 2014; Thøfner et al., 2015). Furthermore it has been suggested that both persistent asymptomatic parasitic and bacterial infections can have negative effects on the production results (Kogut and Arsenault, 2017; Sharma et al., 2018).

Infections in the reproductive tract are the most common production related disease in egg laying hens, which can be presented as salpingitis, peritonitis,

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salpingitis/peritonitis, and/or oophoritis (Lindgren, 1964; Bisgaard and Dam, 1981; Reid et al., 1984; Jordan et al., 2005; Nolan et al., 2017). Infection of the reproductive tract increase mortality, reduce egg production, and the eggshell quality (Bisgaard, 1995; Crespo et al., 2001; Landman et al., 2013). Poor quality of eggshells may result in a higher fraction of eggs infected by bacteria penetrating from the shell surface (De Reu et al., 2006). Bisgaard and Dam (1981) reported on the prevalence, etiology, and pathogenesis of salpingitis and peritonitis in laying hens in Denmark and found a prevalence of 1.26%, whereas a prevalence of 11% was demonstrated in Australia (Reid et al., 1984).

In laying hens, *Escherichia coli* is considered one of the most common bacterial pathogens associated with disease in the reproductive tract (Jordan et al., 2005; Stokholm et al., 2010; Nolan et al., 2017). The spread of *E. coli* into different parts of the reproductive tract via an ascending route of infection may cause salpingitis, and salpingitis/peritonitis (Jordan et al., 2005; Landman et al., 2013). The severity and outcome an *E. coli* infection related to the reproductive tract is highly dependent on the strains involved (Olsen et al., 2016b). Salpingitis, oophoritis, and/or peritonitis caused by *Gallibacterium anatis* may lead to egg drop and increased mortality (Bojesen et al., 2004; Jordan et al., 2005; Neubauer et al., 2009; Paudel et al., 2014; Persson and Bojesen, 2015).

However little information on the prevalence of allegedly asymptomatic or subclinical disease, including infections in the reproductive tract, in egg-laying hens is available. End-of-lay laying hens (at the age between 60 and 90 wk) have been suspected to have a high incidence of chronic disease but no data has documented the prevalence for the old laying hens. The aim of the present study was to investigate subclinical disease and pathology not related to the gastrointestinal tract in order to gain more knowledge on the pathology and possible causes including bacterial etiology in apparently healthy flocks of laying hens at the end of production.

MATERIALS AND METHODS

Selection of Flocks

To investigate the prevalence of subclinical pathology and associated bacteriology in end-of-lay commercial layer hens, 15 flocks of laying hens were randomly selected from epidemiologically independent farms at different geographical locations in Denmark. The layer flocks were selected to represent the 3 most common production systems in Denmark including enriched cages, barn housed with either floor or aviary systems, and organic/free range layers (Table 1). From each flock 500 randomly selected hens were collected immediately after culling on farm at the end of the production period.

Table 1. Number of flocks and hens investigated in relation to production system.

	Number of flocks	Number of hens	Number of hens with lesions	Ratio of hens within lesion within production system (%)[1]
Enriched cages	5	2,497	434	13.74
Barn housed	5	2,500	374	14.96
Organic	5	2,480	433	17.46[a]
Total	15	7,477	1,241	16.60

[1]Chi square of the overall ratio of total lesions between production system; $P = 0.0262$.

[a]Denotes production system in which the ratio of hens with lesions is significantly different to the other.

Necropsy of Birds and Bacteriological Examination

In total, 7,477 collected freshly euthanized hens underwent full post mortem assessment as described in Bisgaard and Dam (1981) and Jordan et al. (2005). The hens were kept at 2°C for up to 48 h until necropsy. Bacteriological investigation was performed if macroscopic lesions typical of infection were present (e.g., fibrinous exudations, necrosis in liver or spleen, general vascular disturbances, pulmonary congestion, hyperemia, and generalized or localized organ swelling). In order to isolate bacteria, the organ surface was sterilized by searing with a flaming hot iron plate and the organ was subsequently swabbed using a sterile cotton swab which was streaked out on a blood agar (**BA**) plate (5% calf blood in BA base, Oxoid, Basingstoke, UK). In case of macroscopic reproductive tract lesions, samples were taken from the salpinx, peritoneum, ovary, and/or liver of these hens. The reproductive tract lesions observed included salpingitis, peritonitis, and/or oophoritis occurring as both active and chronic lesions. The agar plate was subsequently incubated aerobically overnight at 37°C.

From each plate showing abundant and pure bacterial growth with typical colonies, a single colony was sub-cultured in Brain Heart Infusion Broth (Difco, Brøndby, Denmark) and stored in 15% glycerol at –80°C. The colonies on the BA were tentatively identified based on colony morphology (Barrow and Feltham, 2003). Bacterial isolates were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Vitek MS, Biomérieux, Denmark; Kudirkiene et al., 2015) and a primary pathology/lesion category was assigned as cause of mortality for further analysis. All macroscopic lesions and bacteriological results were recorded.

Histological Examination

From selected hens with reproductive tract lesions or serosal scarification, tissue from affected organs and/or liver or spleen for histological evaluation was sampled. The tissue samples were fixed in 4% neutral buffered formaldehyde, dehydrated, embedded in paraffin wax,

Table 2. Overview on the distribution of lesions in end-of-lay hens in different production systems.

Lesion type	Production system number/count (% within housing system)[1]			Total
	Enriched cages	Barn housed	Organic/Free range	
Infection in the reproductive tract	71[a](2 84%)	78[a](3 12%)	113[b](4 56%)	262(3.50%)
Septicaemia	0(0%)	3(0.12%)	5(0.20%)	8(0.10%)
Focal necrosis liver/spleen	1(0.04%)	6(0.24%)	6(0.24%)	13(0.17%)
Endocarditis	1(0.04%)	2(0.08%)	0(0%)	3(0.04%)
Pododermatitis	6(0.24%)	8(0.32%)	9(0.36%)	23(0.31%)
Skin infection/abscess/cellulitis	1[a](0.04%)	12[b](0 48%)	7[a,b](0.28%)	20(0.27%)
Amyloidosis	0(0%)	3(0.12%)	2(0.08%)	5(0.07%)
Scarification/serosal fibrosis abdominal organs	31[a](1 24%)	61[b](2.44%)	24[a](0 97%)	116(1.55%)
Bursitis presternalis	212[a](8 49%)	131[b](5.24%)	154[b](6.21%)	497(6.65%)
Tumor	39(1.56%)	38(1.52%)	53(2.14%)	130(1.73%)
Fatty liver	1(0.04%)	0(0%)	3(0.12%)	4(0.05%)
Liver rupture/haemorrhage	1(0.04%)	0(0%)	0(0%)	1(0.01%)
Cardiac failure	3(0.12%)	1(0.04%)	3(0.12%)	7(0.09%)
Emaciation	6(0.24%)	5(0.20%)	2(0.08%)	13(0.17%)
Uraemia/nephropathia	4(0.16%)	5(0.20%)	5(0.20%)	14(0.19%)
Egg bound	0(0%)	1(0.04%)	0(0%)	1(0.01%)
Cannibalism/vent pecking	1(0.04%)	0(0%)	4(0.16%)	5(0.07%)
Juvenile	7[a](0 28%)	5[a](0.20%)	20[b](0.81%)	32(0.43%)
Trauma/fracture	22[a](0 88%)	7[b](0 28%)	12[ab](0 48%)	41(0.55%)
Miscellanous non-infectious	0(0%)	1(0.04%)	2(0.08%)	3(0.04%)
Unfit for necropsy/heavy autolysis	27[a](1.08%)	7[b](0.28%)	9[b](0.36%)	43(0.58%)
No lesions	2,063(82.62%)	2,126(85.04%)	2,047(82.54%)	6,236(83.40%)
Total	2,497	2,500	2,480	7,477

[1]Different superscript letter denotes a subset of Production system categories whose column proportions differ significantly ($P < 0.05$).

and cut into 4 μm thick sections. Hematoxylin and eosin staining was performed according to a standard protocol (Stevens and Wilson, 1996). All histological changes were described and recorded.

Statistical Analysis

Data management and statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA) and SPSS Statistics 22 (IBM Corp., Armonk, NY). For determining differences in the prevalence of lesions, production status and bacteriology Bonferroni adjusted Chi-square or one-way ANOVA calculations were performed. The statistical level of significance was set to $P < 0.05$.

RESULTS

Post Mortem Examination

In total, 7,477 hens were necropsied (Table 1). Age at euthanasia ranged between 60 and 91 wk with a median age of 79 wk. The majority of the flocks (8) were white birds; 6 flocks were brown birds and 1 flock consisted of both white and brown birds. All of the flocks were commercial varieties from different hatcheries (e.g., Lohmann Brown, Lohmann LSL Lite, Lohmann LSL classic, Bovans Brown, and Hisex hybrids). None of the flocks received antibiotic treatment during the production period and the reported cumulative mortality ranged 2.1 to 9.6%. In 1,241 of all the birds (16.60%) gross pathology was observed. The majority of displayed lesions were located in the skin/subcutis, reproductive tract, or on serosal surfaces. The nature of the lesions could be assigned as inflammatory/infection, scarification and neoplastic. The inflammatory lesions were either in a chronic or acute/active state assessed on the presence/absence of edema, vascular disturbances, exudation, fibrosis, and fibrin (Table 2).

Inflammation of the sternal bursa, which is located as fluid-filled sac subcutaneously on the rim of the keel bone was the most prevalent finding among all productions types, with a significantly higher prevalence in hens from enriched cages (8.49%). The typical appearance was a significantly enlarged, edematous, hyperemic and thickened bursa with varying degrees fibrosis forming adhesions between the skin, bursa, and keel. Only in very few cases the bursa presented purulent/septic exudations (n = 7). In barn housed and organic flocks, 1 flock had a significantly higher prevalence of bursitis than other flocks from the same production system, whereas 3 of the caged flocks had higher prevalences than the rest of the caged flocks (Figure 1).

The second most prevalent lesion type was lesions associated to the reproductive tract (salpingitis and/or peritonitis and/or oophoritis) (Tables 2 and 3). These lesions varied both in terms of age of the lesion (acute-chronic), distribution (e.g., local infection in salpinx or more diffuse spread to ovary and/or peritoneum), as well as degree of inflammation and exudation. Furthermore, the exudate varied from being almost not present over watery and cell rich or fibrinous to large caseous impactions. Exudations were observed in the salpinx lumen and/or in the peritoneal cavity (serosal surfaces). The prevalence of infections related to the reproductive tract was significantly higher in the organic hens

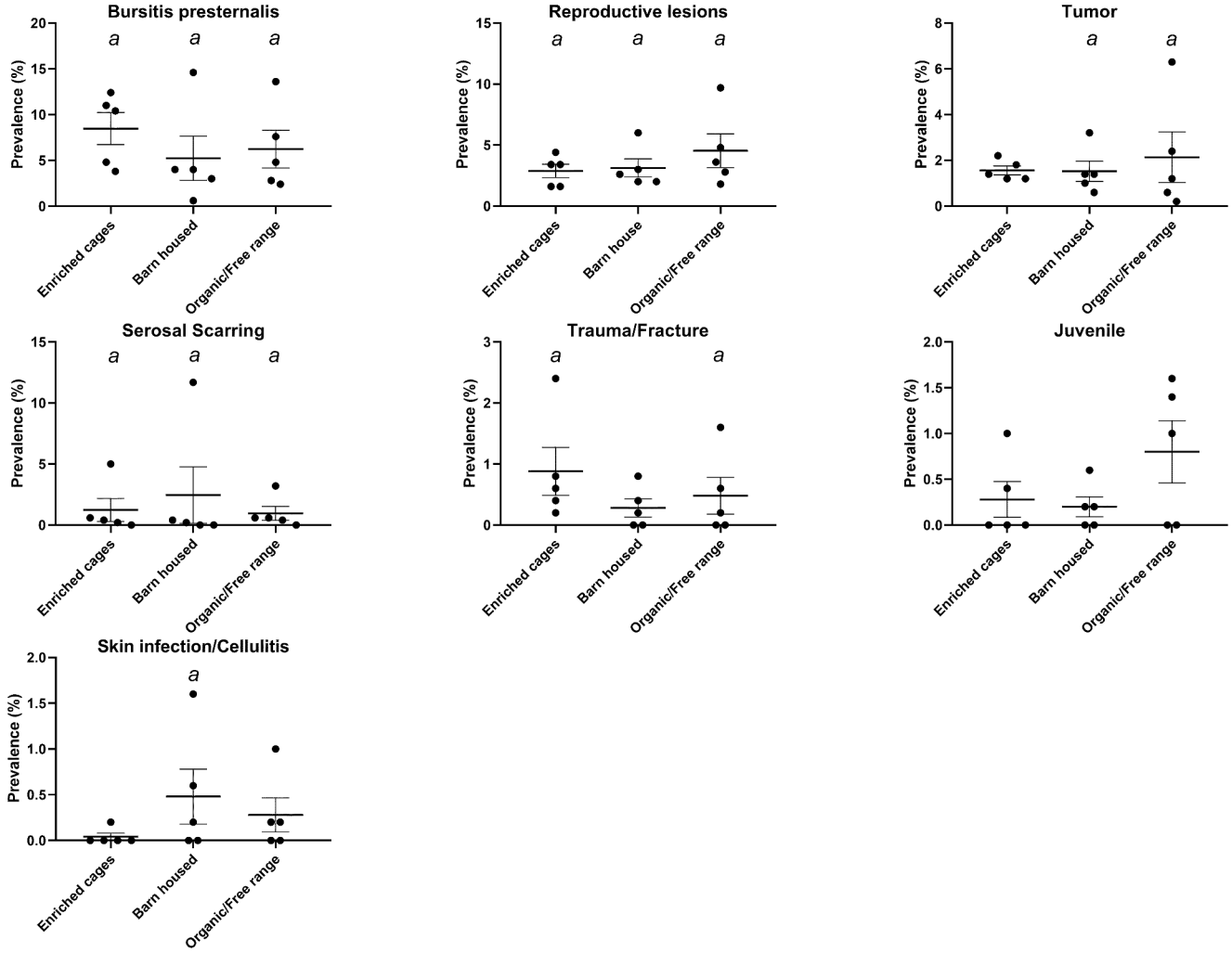

Figure 1. Scatter plot of flock prevalences (mean ± SEM) and variation for the most prevalent lesion types. Statistical difference ($P < 0.05$) within a production system is marked "a"

(4.56%) than in hens housed in enriched cages or in barns. However, considerable and significant variation between flocks within production systems was observed, most prominently among the organic flocks (Figure 1 and Table 3).

In total, 2 distinct macroscopic tumor manifestations were observed, at an overall prevalence of 1.73% with difference between the productions types, however among the barn housed and organic flocks 1 flock had a significantly higher prevalence. One manifestation was a single solitary tumor mass localized in the omentum of the oviduct. The size could vary from small to large (0.5 to >5 cm diameter) and they had a greyish and fibroid appearance. The second type of observed tumor was numerous small nodules (<1 to 5 mm in diameter) with a milliary distribution in the serosal surfaces and/or in the mucosal tissue of intestines and/or oviduct. These nodules were firm, solid and white in appearance, and only heavily affected hens had sequel lesions like large fluid accumulation in the body cavity (i.e., ascites). However, no significant difference in the prevalence between production types, significant flock difference were observed within both barn housed and organic flocks (see Table 2 and Figure 1). No systematic histological examination of the tumor tissue was included in the study.

The typical appearances of serosal scarification were completely fibrous adhesions of 1 internal organ to another (e.g., jejunal loops adhering to each other) or fibrous plaques and/or scars on the serosal surface of the liver capsule. A total of 1.55% of the hens displayed this lesion type. Despite a significantly higher prevalence in barn-housed hens, a significant flock variation was observed in all 3 production types, where 1 flock in each system had a higher prevalence than the rest (see Table 2 and Figure 1).

Substantial trauma lesions, primarily consisting of old but not always healed fractures of the limbs/wings or torn skin, were observed at an overall prevalence of 0.55%, with the prevalence in caged hens being significantly higher than in barn housed hens. Within the caged and organic flocks a significant flock variation

Table 3. Overview on the distribution of reproductive tract lesion in relation to production system and on flock level.

Production system	Total no. hens	Infection in the reproductive tract[1]		Flock level			Salpingitis[1]		Flock level		Salpingitis-peritonitis-oophoritis (SPO)[1]		Flock level	
		n	%[2]	n	%	Chi square	n	%	n	%	n	%	n	%
Enriched cages	2,497	71	2.84 ± 0.55	17	3.4	$P = 0.0381$	29	1.16 ± 0.26	5	1.0	42	1.68 ± 0.34	12	2.4
				21	4.4				10	2.0			11	2.2
				8	1.6				2	0.4			6	1.2
				17	3.4				7	1.4			10	2.0
				8	1.6				5	1.0			3	0.6
Barn house/ aviary/floor	2,500	78	3.12 ± 0.74	30	6.0	$P = 0.0009$	25	1.0 ± 0.13	5	1.0	53	2.12 ± 0.74	25	5.0
				13	2.6				3	0.6			10	2.0
				15	3.0				7	1.4			8	1.6
				10	2.0				5	1.0			5	1.0
				10	2.0				5	1.0			5	1.0
Organic/free range	2,480	113[a]	4.54 ± 1.38	38	9.7	$P < 0.0001$	26	1.04 ± 0.29	9	1.8	87[a]	3.5 ± 1.21	39	7.9
				18	3.6				6	1.2			12	2.4
				14	2.8				7	1.4			7	1.4
				9	1.8				1	0.2			8	1.6
				24	4.8				3	0.6			21	4.2
Total	7,477	262	3.51 ± 0.55				80	1.06 ± 0.13			182	2.43 ± 0.50		

[1]Chi square of the overall ratio of reproductive lesion between production system; Total reproductive tract lesions: $P = 0.0020$, Salpingitis alone: $P = 0.8506$, Salpingitis-peritonitis-oophoritis $P = <0.0001$.

[2]Percentages expressed as mean ± SEM.

[a]Denotes production system in which the ratio of lesions is significantly different to other production systems.

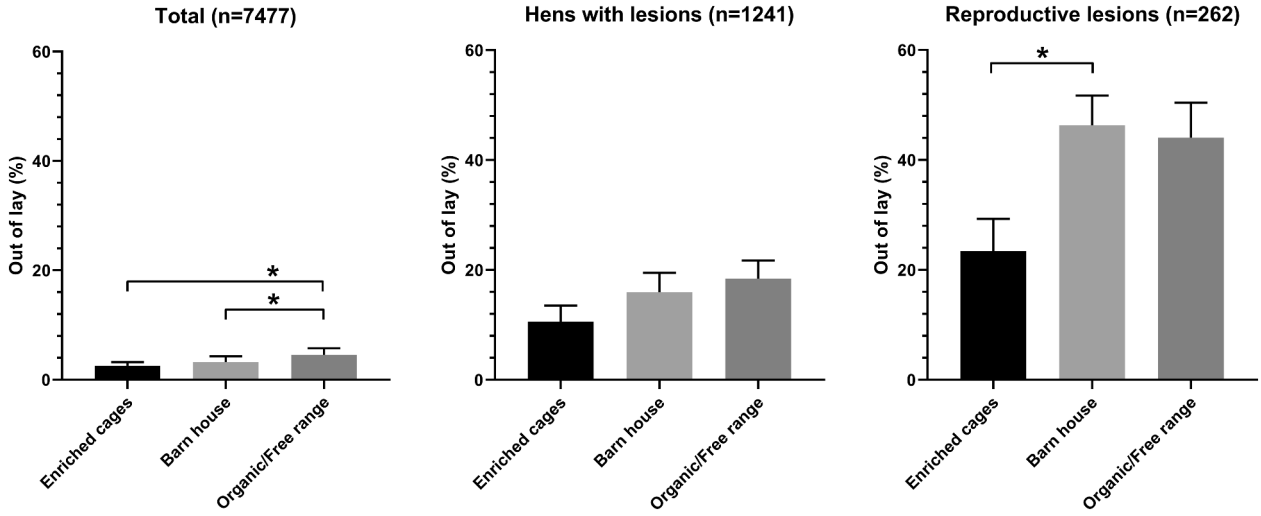

Figure 2. Percentages of birds out of lay in flocks from different production systems and in birds with lesions associated with the reproductive tract (mean ± SEM). Bars denote statistical difference ($P < 0.05$) between production systems.

was observed with 1 flock contributing significantly to the prevalence of the production system in question (see Table 2 and Figure 1).

In all production systems, a small number of hens had completely undeveloped or regressed reproductive organs, with follicles <1 mm and an oviduct diameter <2 mm. Common for these hens was that no other macroscopic lesions could be identified. Despite no exact time for the lack of development or total regression/atrophy of both the ovary and salpinx could be determined, and in combination with absence of other lesions, these birds were categorized as representing a juvenile state with respect to egg production status.The organic flocks had a significantly higher proportion (0.81%) of birds in a juvenile state at the age of "end-of-lay." Furtermore, a significant difference between flocks were observed within this production system (see Table 2 and Figure 1).

However, low in overall prevalence (0.27%) a significantly higher prevalence of skin infections/cellulitis was observed in barn housed hens (0.48%) compared to caged hens (0.04%). Most of the hens with this lesion type in the barn housed flocks originated from 1 flock, where the prevalence reached about 1.5% (see Table 2 and Figure 1).

Regarding the production status, a total of 3.44 ± 0.58% (mean ± SEM) of all the hens had stopped laying eggs (range 1.0 to 8.7%). Within the group of hens with any lesions the overall prevalence was 14.97 ± 1.95% (range: 3.6 to 26.5%) and among the hens with reproductive tract lesions, 37.93 ± 4.18% (range: (12.5 to 61.5%) showed no signs of egg production (see Figure 2). The overall prevalence of barren hens was significantly higher in organic/free range flocks (4.56 ± 1.18%), whereas in the groups of hens with any lesion no difference in the level in hens out of lay was observed between production systems. Among the hens with reproductive tract lesions the prevalence of barren hens was significantly lower in caged flocks compared to barn housed flocks (Figure 2).

Table 4. Overview on the bacteriology on hen level. Hens may have been sampled at more than one site (lesion or organ) and more than one bacterial species could be identified for a single hen, thus numbers of identified bacterial species may add up to more than number of sampled hens.

	Enriched cages	Barn housed	Organic	Total
Total hens sampled for bacteriological investigation[1]	**87**[a]	**103**[a,b]	**132**[b]	**322**
E. coli (Salpingitis/Peritonitis/oophoritis)	**14**[a]	**23**[a,b]	**35**[b]	**72**
E. coli (other lesions)	2	4	2	8
G. anatis	6	4	6	16
E. faecalis	1	5	4	10
S. aureus	3	2	1	6
Other Gram +[2]	0	4	6	10
Unknown spp./Unidentifiable	2	0	3	5
Mixed, unspecified	3[a]	26[b]	9[a]	36
No growth/sterile	57[a,b]	40[b]	69[a]	166

[1]Different superscript letter denotes a subset of Housing System categories whose column proportions differ significantly ($P < 0.05$).
[2]Other Gram positive bacteria identified were *Enterococcus avium* (3), *Staphylococcus lentus* (2), *Staphylococcus equorum* (2), *Enterococcus faecium* (1), *Aerococcus viridans* (1), *and Listeria monocytogenes* (1).

Bacteriological Examination

Of the 1,241 hens with lesions, 322 underwent bacteriological examination of one or more organs (e.g., salpinx, ovary, peritoneum, skin, footpad, joint, spleen, and/or liver) determined by the location of the lesion with tentative/suspected infectious origin. Of the 322 hens sampled, 87, 103, and 132 represented caged hens, barn housed hens, and organic hens, respectively. The proportion of organic hens sampled for bacteriological evaluation was significantly higher compared to caged hens ($P = 0.0052$) (see Table 4). As observed for the

lesion prevalence significant flock variation in the proportion of hens sampled for bacteriology was observed. An overview of the prevalence and distribution of the isolated bacteria is presented in Table 4. Despite observed pathological changes in the birds, 166 of the samples were sterile. The overall ratio of samples with no growth was significantly higher in samples from organic hens (n = 69, 2.8% of total organic hens) when compared to barn housed flocks (n = 40, 1.6% of barn housed hens) ($P < 0.05$). The remaining 156 sampled hens were culture-positive, of these 115 had abundant growth of single species. A total of 15 hens were culture-positive for either 2 bacterial species in the same organ or had 2 organs/sampling sites culture positive for 1 bacterial species. *E. coli* was isolated from 80 hens, hereof which 72 had lesions associated with the reproductive tract (27.48%, 72/262). On production system level *E. coli* was isolated significantly more often from organic flocks than from caged flocks (Table 4), whereas no difference was observed between production system when only looking at the hens sampled for bacteriology. Furthermore, it was observed that 33.8% of the *E. coli* positive hens originated from 2 flocks (20% and 13.8% of all *E. coli* positive hens, respectively), both flocks were organic flocks and had significantly higher prevalence of lesions related to the reproductive tract than the other organic flocks, 9.72% and 4.80%, respectively ($P < 0.05$). A subset of *E. coli* isolates from the reproductive tract (n = 42) were whole genome sequenced for other purposes (data not shown). A brief overview on the whole genome MLST sequence types revealed following STs: ST10, ST58, ST69, ST95/ST140, ST117, ST131, ST141, ST155, ST349, ST371, with ST95/ST140 (21.3%), ST117 (11.9%), ST141 (7.3%), ST10 (4.8%), and ST155 (4.8%) as the most prevalent STs. The phylogroups of the isolates had the following distribution: phylogroup A (14.3%), B1 (19.0%), B2 (40.5%), and D (26.2%).

G. anatis was cultivated and isolated from 16 hens in total, with no observed difference between production systems. All of the isolated *G. anatis* originated from lesions associated to the reproductive tract (6.11%, 16/262). A total of 5 of the hens with *G. anatis* had a dual infection with *E. coli* or *E. faecalis.* Out of the 10 hens where *E. faecalis* was isolated 6 was in dual infections with *E. coli* or *G. anatis.* A total of 4 of the hens with *E. faecalis* infection were diagnosed with pododermatitis; the rest was associated with lesions of the reproductive tract. All 6 hens with *S. aureus* infection had pododermatitis or skin infection/cellulitis.

Finally, bacterial culture from 36 hens resulted in mixed bacterial growth of more than 5 distinct colony types on the BA, with no observed dominating colony morphology type and in most cases covered by swarming *Proteus* spp. This was considered as contamination by efflux through the intestinal wall. Almost half of these samples (15/36) originated from 1 flock (barn housed).

Histological Examination

A total of 82 hens were sampled for further histological examination of lesions related to salpinx, ovary and peritoneum. The lesions included varying degrees of necrosis of spleen, liver, and necrosis of epithelium in the salpinx. Furthermore, fibrinous exudation, edema, mononuclear cell infiltration, and congestion were also detected in spleen, liver, and salpinx mucosa. In hens with adhesive peritonitis/scarification no to very mild inflammatory or degenerative lesions were observed.

DISCUSSION

The aim of the present investigation was to determine the prevalence of subclinical lesions and pathology in layer hens at the end of lay. The 3 most common production types in Denmark, enriched cages, barn-housed and organic, was investigated. The production system was considered as a factor that could affect the prevalence of lesions and bacterial infections (Bojesen et al., 2003).

In the present study, inflammation of the sternal bursa was by far the most prevalent lesion among all productions systems. The observed large farm variation may be related to a difference in the design of the house interior including perches. A recent systematic review by the European Food Safety Authority, concluded that perch design and material is correlated to both plumage damage and keel bone deformities (EFSA, 2015). As the sternal bursa is an anatomical feature for cushioning the sternal crest against excessive wear (e.g., pressure from perches when resting). If excessive wear arises the bursa will compensate by increasing the amount of fluid in the bursa, this process will initiate a local inflammatory response and result in bursitis presternalis (breast blister). Although inflamed bursas were highly prevalent, only 7 out 497 hens with bursitis had progressed to become infected by bacteria, thus the risk of secondary infection is considered to be low as long the integument stays intact.

Reproductive tract lesions have previously been reported as a major cause of increased mortality, drop in egg production and decreased welfare in chickens, ducks, and geese (Bisgaard, 1995; Crespo et al., 2001; Stokholm et al., 2010; Landman et al., 2013). Bisgaard and Dam (1981) reported that the prevalence of salpingitis and peritonitis in laying hens was 1.26% in hens slaughtered at the end of lay. The reason for the increase in prevalence found in this study (3.50%) could be because of an increased number of eggs produced per hen per year, which has increased by 31% from approximately 256 eggs/364 D in 1980 to 335 eggs/364 D in 2013 (Yding Sørensen, 2013). Additionally a change in production systems, where there has been a shift from predominantly caged birds to alternative systems where the hens are free to move indoor and outdoor, thus leading to an increased risk of transmitting

infection between flock mates, may also contribute to the increased prevalence. The reproductive tract associated lesions were divided into 2 groups reflecting the macroscopic manifestation. When the salpinx was the only visibly affected organ, the hen was categorized as having only salpingitis. In these cases the left salpinx and/or the residual right salpinx was distended and filled with fluid to caseous exudate consisting of fibrin, albumen, bacteria, cellular debris, and/or pus. In some cases the size/weight of an affected salpinx could contract up to 50% of the total body weight (1 case where the salpinx weighed >1,200 g and total body weight including salpinx was just over 2,300 g). In this study, 30.5% (80/262) of the hens with reproductive tract lesions had infections in the salpinx alone. This is somewhat higher than reported in a previous study by Jordan et al., (2005) where the proportion of hens with salpingitis alone was 25.9% (21/81). The second group of lesions associated with infections in the reproductive tract involves spread of the infection to other organs, most commonly the ovary and/or the peritoneum. In these cases all combinations of inflammation in the salpinx, peritoneum and ovary may occur. In this study 182/262 (69.5%) hens had lesions in the peritoneum and/or the ovary and/or the salpinx. Jordan and colleagues reported that almost 75% of the hens dying from infections in the reproductive tract had salpingitis and/or peritonitis (Jordan et al., 2005). Both manifestations may be caused by ascending bacteria from the cloacal region, which has been proposed as a major route of infection (Cumming, 2001; Ozaki and Murase, 2009). Recently it has been demonstrated, by the use of an experimental model, that infection with different *E. coli* strains via the salpinx give rise to lesions in salpinx, ovary, and/or the peritoneum (Olsen et al., 2016b; Pors et al., 2014). However significant flock variation was observed on the prevalence of reproductive tract lesions, the overall prevalence of reproductive tract lesions in organic end-of-lay hens was significantly higher than both barn-housed and caged layers. This is in line with the causes of mortality of layers as documented by Stokholm et al. (2010) where the lower levels of biosecurity, hygieine and persistence of pathogens in the environment that comes with the access to outdoor areas, contributed significantly to the level of infections as opposed to the confined production systems.

In total, 2 types of tumor manifestations, single tumors in the salpinx mesentery or multiple nodules of leucocytic origin dispersed in the serosal lining and/or in the mucosa of the visceral organs (e.g., gastrointestinal tract, reproductive tract) were observed in the present study. Despite most of the observed tumors were related to the reproductive tract almost none of them influenced the production status of the affected hens. In birds, tumors in the reproductive tract is not uncommon (Scott Echols, 2002), however ovarian neoplasia is uncommon in hens younger than 2 yr old (Fredrickson, 1987). Very little information on the incidence of neoplasms in commercial poultry is available. Regarding tumors related to the reproductive tract, the prevalence increase with increasing age (almost absent <2 yr of age reaching 30 to 60% at 6 to 7 yr of age) and considerable flock variation occurs (Fredrickson, 1987). In hens aged 2 to 7 yr, ovarian neoplasm was the most prevalent and it was found in 32% in a study population of 466 hens, oviductal tumors 8% and 5% benign leiomyomas in the suspending ligament of the oviduct (Fredrickson, 1987). Although, the solitary tumors in the present study exclusively were located in the mesentery of the oviduct, their tissue origin can only be speculated and further characterization would require systematic histological evaluation.

The observed multicentric nodular type can be of lymphoproliferative origin, which is often associated with viruses like Marek's Disease virus (Nair, 2014; Reddy et al., 2017); avian leucosis viruses (Nair and Fadly, 2017) and reticuloendotheliosis virus (Nair et al., 2017). However, distinction of the cells involved, require histological examination before any conclusion on the origin of the tumor can be established.

Scarification and fibrous adhesion between the organs in the body cavity is regarded as an end-point of inflammatory process from fibrinous adhesive peritonitis were fibrinous exudation undergo remodeling into fibrous connective tissue. Therefore, the presence of serosal scars and adhesions are an indication of previous inflammation of peritoneum. This is not surprising as peritonitis is a common manifestation when inflammation in the reproductive tract occurs.

A total of 80 hens were culture positive for *E. coli,* of which 72 of the isolates were associated with reproductive tract lesions. About half of these originated from organic flocks. This significantly higher proportion is expected as data on the *E. coli* associated normal mortality in layer flocks demonstrated high association in organic flocks vs. indoor flocks (Stokholm et al., 2010) by bacteriological examination. A total of 6 samples were identified as both *E. coli* and *G. anatis* positive.

In 1981, Bisgaard and Dam (1981) reported the prevalence of *E. coli* and "*Pasteurella haemolytica*" (now *G. anatis*) from hens with salpingitis to be 42.7% and 9.3%, respectively. In the present study, the prevalence of *E. coli* and *G. anatis* from the 262 hens with reproductive tract lesions was 27.48% and 6.11%, respectively. Significantly, fewer hens in the enriched cages had *E. coli* infections, but no significant difference of the prevalence of *G. anatis* was observed between the production systems. It was expected that the prevalence of *G. anatis* would be higher in the production systems where the hens can move freely and thus have a higher risk of getting infected in contrast to hens from enriched cages where only restricted contact to flock mates is possible (Stokholm et al., 2010). However, considerable flock variation within each production system was observed.

Despite infections in the reproductive tract, 62% of the hens were still laying eggs. These birds may represent a risk of transmission of bacteria to the eggs. A previous study has shown that egg contents (yolk and albumen) from commercial laying hens had a prevalence of *E. coli* approximately 5% (Kilonzo-Nthenge et al., 2016). Furthermore, horizontal spread to flock mates while infected could pose a risk for continuous infection in the flock. Colonization or infection with *E. coli* or *G. anatis* in reproductive tract of laying hens opens for the possibility of vertical transmission to the developing embryo as is a well-known route for *E. coli* transmission (Poulsen et al., 2017). The most frequently observed STs (ST95/140 and ST117) have the potential to cause massive losses in poultry (Olsen et al., 2016a; Ronco et al., 2017). The infectious potential of the present *E. coli* strain is also reflected in the distribution of the phylogroups, with B2 and D comprising 2/3 of the isolates. This observation is in line with previous reports (Rodriguez-Siek et al., 2005)

In conclusion, this is the first investigation on the prevalence of subclinical disease other than parasitic infestations/gastrointestinal disease in poultry/layers. The results revealed that of apparently healthy end-of-lay hens a significant proportion presented lesions that can be attributed to the housing and management system (e.g., inflamed sternal bursa and perches, higher frequency of bacterial infections, and associated pathology in organic layers). Of the lesions with a bacterial etiology, infection related to the reproductive tract was of importance as they pose a considerable risk of transmission of the bacteria to eggs and flock mates, with *E. coli* and *G. anatis* as the major pathogens involved. Other findings like tumors were at similar levels regardless of production system, thus more related to the age of the birds.

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