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Scratch area as an epidemiological risk factor for Spotty Liver Disease in cage-free layers in Australia

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ABSTRACT Spotty Liver Disease (**SLD**) is a serious problem in laying hens farmed in cage-free systems. The causative organism, *Campylobacter hepaticus*, is regarded as having a fecal-oral method of transmission and hence may build up and spread readily in housing systems which allow ease of direct contact of hens with the flock's fecal material. The epidemiology of SLD has not been thoroughly investigated. An initial cross-sectional analytical epidemiological survey of SLD in free range and barn layer systems was conducted in Australia over 2019 to 2021.The survey involved rearing flocks ($n = 32$) which were then followed through into laying flocks ($n = 24$) up to 40 wk of age. Cloacal swabs were collected during rearing and lay for *C. hepaticus* detection by PCR. Flocks were classified as "Cases" ($n = 18$) where clinical SLD according to the case definition was observed or "Controls" ($n = 6$) which were clinically unaffected. No *C. hepaticus* was detected in cloacal swabs from rearing houses whereas the organism was detected in 18 Case flocks in lay and from 2 Control flocks in lay. All layer houses that incorporated a scratch area ($n = 13$) were categorized as Cases. Thus, having a scratch area is a key determinant for SLD and no analyses of further contributory factors from these flocks were able to be made. Of the remaining 11 flocks which had floors fully covered by slats, 5 were Cases (45%). Further risk factor analysis was compromised by this small sample size and identification of other significant associations was not possible. A larger survey investigating flocks laying in houses with fully slatted floors was undertaken to further the understanding of SLD epidemiology and is reported in a companion paper.

Key words: Spotty Liver Disease, *Campylobacter hepaticus*, *Campylobacter bilis*, epidemiology, floor cover

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INTRODUCTION

The production of commercial eggs has increased worldwide substantially over the past few decades, providing an affordable and low cost source of animal protein (McMullin, 2022). The size of the Australian layer industry nearly doubled between 2005 and 2021, from 13 million birds to 22 million farmed per year (Australian Eggs Limited, 2023). The primary production systems for housing laying hens had undergone numerous changes, transitioning from predominantly cage-free systems (free-range and barn) in the 1960s to cage systems in the 1970 to 1980s (United Egg Producers, 2023). This was driven by more efficient production through the mechanization of animal feeding and drinking systems, as well as egg collection and packing, and better disease control by minimizing fecal-oral transmission (United Egg Producers, 2023). This was reflected in the exponential growth of cage systems in the United Kingdom, which increased from 19% in the early 1960s to 93% by the late 1970s, with a similar trend occurring in Australia (McMullin, 2022; Poultry Hub Australia, 2023). Several decades later, as public concern over the welfare of caged laying hens and the consumer perception that free-range housing systems resulted in 'happier and healthier' hens increased through the early 2000s (Matthews and Hemsworth, 2012), there was a steady expansion of cage-free systems in the layer industry, especially in Europe and Australia (Dikmen et al., 2016). In 2005, cage-free eggs accounted for 21% of Australian national sales by volume and by 2021 this had tripled to 64% of the country's retail sales (Australian Eggs Limited, 2023). Similar trends were observed in the United Kingdom, where cage-free systems accounted for 28% of national layer chicken production in 2000 and reached 60% by 2020 (McMullin, 2022).

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The return to cage-free production systems in recent years has been accompanied by the re-emergence of several infectious diseases, including internal parasites and bacterial diseases such as fowl cholera (Campbell et al., 2021). At the same time, in the early 2000s, there was an emergence of a clinical condition in both the United Kingdom and Australia, which challenged the frontiers of the cage-free systems, described as "Spotty Liver Disease" (**SLD**) (Jenner, 2001; Crawshaw, 2019). SLD was so called because of the characteristic hepatic lesions: white-grayish foci through the liver parenchyma. SLD was found to be readily responsive to antibiotic therapies (Grimes and Reece, 2011). Interestingly, this clinical condition closely resembled a condition termed Avian Vibrionic Hepatitis (**AVH**), which was reported in the United States in the mid-20th century, but subsequently disappeared (Moore 1958; Peckham, 1958). It was not until recently that Crawshaw et al. (2015) isolated a bacterial pathogen associated with SLD and that was subsequently named by Van et al. (2016), and was shown to fulfill Koch's postulates for SLD, as *Campylobacter hepaticus*. After comparing the clinical and bacterial characteristics of the 2 clinical conditions, it was concluded that these were in fact the same disease, both caused by *C. hepaticus* (Crawshaw, 2019). *C. hepaticus* has since been isolated from hens with SLD in the United States (Gregory et al., 2018), New Zealand (Crawshaw et al., 2021), and Jordan (Hananeh and Ababneh, 2021).

Spotty Liver Disease is now considered to be one of the most significant infectious diseases in Australian commercial layer flocks, directly impacting bird welfare, productivity, and extending the use of therapeutic antibiotics (Courtice et al., 2018; Noormohammadi, 2021). The disease is most commonly seen in cage-free systems, which include laying hens housed in barn and free-range environments (Crawshaw, 2019). The clinical condition was first reported in the United Kingdom and Australia in the 1980s (Jenner, 2001; Swarbrick, 2003). Cage-free production systems continue to grow as components of commercial chicken egg production generating ongoing concern for managing SLD within these systems.

Susceptible layer flocks experiencing SLD may show a drop in production by 10-35% and an increase in mortality by 10-15% (Courtice and Jenner, 2022). Based on a cost model presented by Courtice and Jenner (2022), in a flock of laying hens experiencing an outbreak of SLD with a mortality of 6% and a 7.2% drop in egg production at peak lay, a net cost of lost eggs, dead birds (and the associated loss of egg production due to lost hens), cost of treatment ameliorated by savings on feed, due to less hens, could result in a loss of AUD $4.24 per hen (Courtice and Jenner, 2022).

The epidemiology of SLD is poorly understood and, to our knowledge, analytical epidemiological studies have not been reported. Descriptive epidemiology seeks to describe the pattern and occurrence of disease in terms of the incidence and prevalence rates due to the host, the causative organism and the environment in space and time (the who, what, when, and where of disease) while analytical epidemiology searches for factors that may modify the risk of a disease occurring (the "why and how" of disease) (Martin et al., 1987; Thrusfield, 2005). A limited number of publications have attempted to address the descriptive epidemiology of SLD (Jenner, 2001; Grimes and Reece, 2011; Scott et al., 2016; Kotiw et al., 2018; Phung et al., 2020). These reports describe SLD as mostly occurring in early lay, causing mortality (10%−15%) and a drop in egg production of 10% to 35%; when *C. hepaticus* is introduced into a susceptible flock and SLD may result. Further, SLD can occur in a flock at any age after sexual maturity, but once endemic on a property, it is typically observed during early lay (Phung et al., 2020). The causative organism has also been detected in cloacal swabs from birds at least 8 wk prior to clinical disease and from rearing layers from 12 wk of age (Phung et al., 2020). The organism has also been detected from environmental sources including soil, dust, mud and water sources and from feces of wild birds and rats (Phung et al., 2020) and also from flies and feces of fauna (including kangaroos) (Kotiw et al., 2018).

To our knowledge, this is the first detailed analytical epidemiological study of SLD attempted in commercial poultry. The aim of this study was to search for and identify risk factors which may be statistically associated with the occurrence of SLD, particularly those factors that could be considered to be "key determinants" (i.e., risk factors that are amenable to management procedures; Martin et al., 1987). The overarching objective was to provide guidance to cage-free egg producers to assist in reducing the impact of SLD on their operations.

MATERIALS AND METHODS

Ethics Statement

The cross-sectional survey of the Australian cage-free layer industry was conducted under the supervision of the Animal Ethics Committee of the University of Sydney (protocol number 2019/1589) and the Human Ethics Committee of the same institute (protocol number 2019/662).

Cross-Sectional Analytical Epidemiological Survey

The hypothesis of the study was that there will be identifiable management factors that may modify the occurrence of SLD in cage-free flocks and that some of these may be manipulated to reduce the deleterious effects of SLD.

Selection of Flocks

All flocks enrolled in the survey were commercial cage-free laying flocks housed in either barn or free-range production systems in Australia. To capture flocks experiencing different climate conditions, flocks were recruited from 5 Australian States: Queensland, New

South Wales (**NSW**), Victoria, South Australia, and Western Australia. The sampling frame was established based on veterinarians' acquaintance with cage-free flocks and from farmers who volunteered to participate following their attendance at several workshops conducted by an industry body (Australian Eggs Limited) across Australia.

Rearing Survey and Questionnaire

Thirty-two flocks of rearing birds destined for cage-free laying facilities were enrolled for the study. Each flock was visited once between the age of 12 wk until the end of rearing (usually 16 wk of age). An extensive questionnaire designed using an online questionnaire recording system, REDCap (Harris et al., 2009) was completed on each visit. Cloacal swabs were collected from 12 randomly selected birds in each rearing flock during each visit. The number of rearing flocks ($n = 32$) surveyed exceeded that of laying flocks ($n = 24$) as more than one of the smaller rearing flocks can contribute to a single layer flock. Note however that in some instances, birds reared in one flock were distributed to more than one layer facility. During each visit a comprehensive management and facility description questionnaire was completed, and 12 cloacal swabs were collected from random pullets in each flock, for subsequent PCR analysis for the presence of *C. hepaticus*. All rearing flocks enrolled in the study were hatched between July and November 2019. The rearing survey was completed before March 2020, after which the COVID-19 pandemic restricted travel and face to face farm visits in Australia.

Laying Survey

Cage-free layer flocks that were supplied with point of lay birds from the surveyed rearing flocks were enrolled in the layer survey. A total of 24 flocks were surveyed, COVID-19 pandemic travel restrictions which were imposed in New South Wales (**NSW)** and across Australia during 2020 prohibited interstate travel in 2020 and also restricted local movements within NSW. As a result, flocks could only be visited by the research team where these restrictions allowed. Some flocks in other states (Queensland, Victoria, South Australia, and Western Australia) were interviewed remotely. The questionnaire was sent to participants to complete and local responding veterinarians assisted with cloacal swab sampling and submission. As a result of the travel restrictions and concerns over COVID-19, some farmers withdrew their flocks from the survey resulting in some data being incomplete. All rearing flocks were transferred to their laying quarters between October 2019 and March 2020. Survey visits occurred between January and September 2020 coinciding with when the flocks were between 35 and 40 wk of age. This age range was targeted to allow for any likely incursion of SLD to have become evident. A detailed questionnaire was completed and cloacal swabs were collected from 12 random birds or detection of *C. hepaticus* by PCR. Small samples of fresh feces were randomly collected by gloved hand throughout the house from the slatted floor surface and pooled.

Case Definition

Participating flocks were categorized as "Cases" or "Controls." The case definition used was that a "Case" flock experienced a rise in mortality and a decline in egg production associated with the occurrence of typical gross pathology of SLD: that is, multiple focal necrotic lesions (spots) in the liver, and a fibrinous perihepatitis possibly with icterus.

"Control" flocks consisted of flocks which had no identified clinical SLD nor reported increased mortality nor unexpected egg production declines by the age of 40 wk.

Campylobacter Hepaticus

PCR: A PCR developed by Van et al. (2017) was used to detect *C. hepaticus* from fecal material on cloacal swabs and was used as described.

DNA from cloacal swabs was prepared using ISOLATE II Genomic DNA Kit (Meridian Bioscience, Cincinnati, Ohio), according to the manufacturer's instructions. PCR amplification was performed using Applied Biosystems 7500 FAST Real-Time PCR System (Thermo Fisher Scientific, Macquarie Park, NSW, Australia), by detecting the unique *C. hepaticus* glycerol kinase gene (**GK**) with the primers G2F3 and G2R2 as described by Van et al. (2017). Each reaction was carried out in a final volume 20 μL, which includes 5 μL of template DNA, 400 nM of primers, by using SensifFAST HRM Kit (Meridian Bioscience). The following temperature cycling conditions were used: 95°C for 5 min, followed by 40 cycles of 5 s at 95°C, 20 s of 57°C annealing temperature, and final extension for 30 s at 72°C. For each set of PCR reactions, DNA template of a known *C. hepaticus* isolate and distilled water were used as positive and negative controls, respectively. A melt curve analysis was performed on the completion of PCR to confirm that the expected fragment had been amplified. The PCR products were subjected to 1°C/s increments between 60°C and 95°C. The melting profiles were analyzed using Applied Biosystems software High Resolution Melt (**HRM**) Software v2.0. Normalization regions of 77°C to 78°C were applied for detection of *C. hepaticus*.

In 2021, another *Campylobacter* species capable of causing SLD, designated as *Campylobacter bilis,* due to its ability to be isolated from bile was identified (Phung et al., 2022; Van et al., 2023). It is understood that the PCR method used in this study would detect the presence of both *C. hepaticus* and *C. bilis* (R. Moore, personal communication). Hence, the identification of *C. bilis* after the completion of this survey does not compromise these findings, as both organisms would have been detected from samples collected.

Statistical Analysis

Data was transferred from REDCap to MS-Excel and uploaded in STATSTICA v6 (Statsoft Inc, 2003) for analysis. All survey variables in both rearing and laying compartments were assessed in a univariate analysis using a contingency table analysis for categorical variables or Student's t test for continuous variables with the Case or Control definition as the dependent variable. Any variable displaying a probability of an association being due to chance value of <0.20 (as suggested by Hosmer et al., 2013) was selected for further inclusion in any multivariate model building approach. This less robust probability value was used as a screening level for selection of potentially important factors the significance of which may be hidden within the complexities of the data. Pearson χ^2 tests were used to assess probability due to chance. Given the restricted sample size, Fisher's exact test (2-tailed) was used when an expected value was <5. The Mantel−Haenszel stratified analysis technique (Martin et al., 1987; Thrusfield, 2013) was used to control for confounding through stratified analyses where appropriate. Continuous variables expressing interest (selection level of $P < 0.20$) were divided into ordinal categories, using the median value as the break point and these were further analyzed as categorical variables. The selected variables were then combined and analyzed in a multiple logistic regression to control for confounding, examine interactions and statistically develop a parsimonious model for the outcome variable (SLD case). The multiple logistic regression model was analyzed using JMP v16 statistical software (SAS, 2021).

RESULTS

The association between observed factors in the rearing and laying section of the survey were cross tabulated against the occurrence ("Case") or nonoccurrence ("Control") of clinical SLD outbreaks in the surveyed flocks. In some situations, more than one rearing flock supplied a single layer flock and in others, a single rearing flock contributed birds to more than one layer flock. Twenty-three flocks contributed cloacal swabs (a total of 276 swabs were examined). No cloacal swabs from rearing flocks returned a positive PCR result for *C. hepaticus*, although it was detected in pooled feces from one rearing flock. Table 1 shows the proportion of cloacal swabs which gave a positive detection of *C. hepaticus* by PCR from the laying flocks ($n = 23$ as samples from one flock were not submitted due to difficulties with COVID-19 restrictions).

Case flocks had a significantly higher proportion of cloacal swabs giving positive *C. hepaticus* detection than did control flocks ($P = 0.0004$, Mann−Whitney U test) (Table 1). All of the Case flocks had positive cloacal swabs (4 or more positive cloacal swabs /12 for each case flock). A zero detection from a sample size of only 12 samples provides a 95% confidence that the actual level was below 25% of the population, hence declaring a flock to be "negative" on this sample size is not valid. Obtaining a zero positive swab detection result is within a 95% confidence interval of between 0 and 3 positive swabs per 12 birds sampled. Noting this condition, out of the 6 Control flocks, 4 gave zero positive swabs (95% confidence interval 0−3) while 2 Control flocks had 2 positive swabs (95% confidence interval 0.5−5.4).

Tables 2 and 3 show the observed associations between SLD occurrence and categorical variables for rearing and laying flocks respectively (assessed by contingency table analysis) and Tables 4 and 5 show continuous variables for rearing and laying flocks respectively (assessed using Student's t test for independent samples). The surveyed flocks were located predominantly in NSW (13 flocks), and there were limited numbers participating from Victoria, Queensland, Western Australia, and South Australia (Table 3). The total number of birds in lay represented in the surveyed flocks was 468,420, with a mean flock size of 19,518 (Table 4). It became immediately obvious that one particular variable, the presence of partially slatted flooring (Figure 1A), showed a highly significant association with the occurrence of clinical SLD in the layer flocks ($P = 0.003$, Table 3). All flocks (100%) with partial slatted flooring (i.e., having houses that allowed some bird contact with the solid floor, described as a "scratch area", $n = 13$) were Cases of SLD, while flocks in houses with fully slatted flooring (Figure 1B) exhibited only 45% Case flocks. The cross tabulation for the partial slats variable reveals a zero-cell value (for Control flocks where partial slats were present) and hence the odds ratio for this association is undefined (infinite). Because of the nonoccurrence of Control flocks, no further analyses within this category of house were possible.

The presence of a zero-cell value in a contingency table causes major difficulties for any multiple factor analysis as it seriously inflates standard error values. Hence the scratch area presence factor strongly confounded the putative effect of any other associated factor in houses with a scratch area. Hence, even though several factors met the further selection criterion of an association with SLD occurrence with $P < 0.20$ in the univariate analyses (other animals on rearing farm

Table 1. Number cloacal swabs ($n = 12$) positive for *C. hepaticus* by PCR in laying flocks surveyed.

		Number of cloacal samples positive ($n = 12$)			
Case definition	No. flocks positive	Mean	Minimum	Median	Maximum
Case	17	8.88	4	9	12
Control	6	0.67	0	0	2
Mann−Whitney U test $P = 0.0004$					

Table 2. Categorical variables association with Spotty Liver Disease occurrence for all flocks surveyed. Rearing flocks.

Variable - Rearing shed features	Variable Level	No. of flocks		Odds ratio	Pearson chi-square	Fisher's exact 2-tail[1]
		Case	Control		*P*=	*P*=
Hatchery	A	9	2	3.375	0.34	
	B	2	0	Undefined		
	C	4	3	Reference		
Other animals on property	Yes	9	6	0.00	0.07	0.12
	No	7	0			
Rear and laying location	Same farm	2	0	Undefined	0.41	1.00
	Remote farm	14	5			
Rearing shed type	Aviary	7	0	Undefined	0.07	0.12
	Barn	9	5			
Rearing ventilation style	Natural	7	3	0.52	0.53	0.64
	Tunnel	9	2			
Platforms in rearing shed	Yes	9	3	0.86	0.88	1.00
	No	7	2			
Perches in rearing shed	Yes	12	4	0.75	0.82	1.00
	No	4	1			
Feather pecking in rearing	Yes	1	0	Undefined	0.57	1.00
	No	15	5			
Smothering during rearing	Yes	7	2	1.17	0.88	1.00
	No	9	3			
Biosecurity plan in rearing	Yes	14	4	1.75	0.68	1.00
	No	2	1			
Vehicle disinfection on rearing farm	Yes	14	2	10.50	0.03	0.06
	No	2	3			
Rearing shed resting time	up to 14 d	5	2	0.45	0.88	1.00
	28 d or more	11	2			

[1]Where an expected value was <5, Fisher's exact test value is shown.

Table 3. Categorical variables association with Spotty Liver Disease occurrence for all flocks surveyed. Flocks in lay.

Variables in lay	Level	No. of flocks		Odds ratio	Pearson chi-square	Fisher's exact 2-tail[1]
		Case	Control		*P*=	*P*=
State	WA	1	0		0.22	
	SA	1	0			
	NSW	7	6			
	VIC	4	0			
	QLD	3	0			
Brown egg layer strain	Strain A	13	2	5.20	0.09	0.15
	Other	5	4			
Layer shed style	Barn	1	0		0.45	
	Aviary free range	3	0			
	Barn free range	14	6			
Shed ventilation system	Natural	13	3	3.25	0.23	0.32
	Tunnel	4	3			
Slats	Partial	13	0	Undefined	0.002	0.003
	Full	5	6			
Slat brand	A	1	0	Undefined	0.68	
	B	6	2	2.25		
	C	1	0	Undefined		
	D	6	1	4.50		
	E	4	3	*Reference*		
Pop hole position	Both sides	14	4		0.42	0.58
	One side	3	2			
Feeder type	Pan	7	4	0.32	0.24	0.36
	Chain	11	2			
Drinker type[2]	A	5	2		0.87	
	B	1	0			
	C	1	0			
	D	1	0			
	E	2	1			
Water source	Bore	6	2		0.89	
	Town	9	2			
	River	2	1			
	Dam	1	0			
Range vegetation type	Grass Yes	4	0	Undefined	0.30	0.55
	Grass No	14	4			
	Shrubs Yes	8	2	0.80	0.84	1.00
	Shrubs No	10	2			
	Tree Yes	6	2	0.50	0.53	0.6
	Trees No	12	2			

(*continued*)

Table 3 (*Continued*)

Variables in lay	Level	No. of flocks Case	No. of flocks Control	Odds ratio	Pearson chi-square *P*=	Fisher's exact 2-tail[1] *P*=
Access to water on range	Puddles Yes	12	1	6.00	0.15	0.22
	Puddles No	4	2			
Infectious Bronchitis vaccination in lay	Yes	11	4	0.92	0.93	1.00
	No	6	2			
Feed additives for SLD[3]	Additive(s)	14	1	17.50	0.007	0.15
	No additive(s)	4	5			
Light type	Warm White	9	1	4.50	0.10	
	Cool White	6	3			

[1]Where an expected value was <5, Fisher's exact test value is shown.
[2]Drinkers included Ziggitty, SKA, Lubing, Big Dutchman and Impex (not in order shown).
[3]Feed additives used as preventative for Spotty Liver Disease (SLD) including probiotics, organic acids, prebiotics, yeast extracts.

Table 4. Student's *t* test for continuous variables in rearing flocks.

Rearing flock variable	Case mean	Control mean	t-value	df	*P*	Valid N cases	Valid N controls
Property size (ha)	157.47	245.10	−0.777	15	0.449	12	5
Maximum age difference across farm (wk)	11.88	11.20	0.694	19	0.496	16	5
Shed floor area (m^2)	1421.07	1303.12	0.356	19	0.726	16	5
Platform area in house (m^2)	267.72	1.06	1.314	19	0.204	16	5
Total space available for birds (m^2)	1688.84	1797.72	−0.292	19	0.773	16	5
Total perch length (m)	1869.59	363.69	1.364	18	0.189	15	5
No. birds	24245.13	23095.80	0.192	18	0.850	15	5
Age birds delivered to laying (wk)	15.81	16.00	−0.326	19	0.748	16	5
Pan feeder space (cm/bird)	0.58	0.81	−0.526	20	0.605	17	5
Chain feeder (cm/bird 2 sides)	3.76	1.91	0.857	21	0.401	18	5
Feed space (cm/ bird)	5.18	2.72	1.301	18	0.210	15	5
Drinker space (birds/nipple)	8.36	9.63	−0.827	17	0.420	14	5
Perch space (cm/bird)	9.17	1.42	1.460	17	0.163	14	5
Stocking density (birds/m^2)	17.06	18.25	−0.334	17	0.742	14	5
Total number of light units/shed	303.42	32.00	0.615	12	0.550	12	2
Maximum temperature recorded in shed (°C)	51.61	47.20	0.266	18	0.793	15	5
Maximum temperature recorded outside shed (°C)	62.53	68.20	−0.362	18	0.722	15	5

[$P = 0.12$], vehicle disinfection in rearing [$P = 0.06$], total perch length in rearing [$P = 0.187$], nesting space [$P = 0.005$], strain of layer [$P = 0.15$], perch space in lay [$P = 0.153$], and range stocking density [$P = 0.112$]), all flocks with a scratch area could not be subjected to further analysis (see Discussion). This reduced the sample size to only 11 flocks, 5 (45.5%) of which were Cases.

Only factors from the univariate analyses with full data were considered further as the remaining sample size was small ($n = 11$). Continuous variables of interest (from Tables 4 and 5) were transformed into dichotomous categorical variables based on median values to simplify the analyses with this small sample size. One variable which showed high statistical significance ($P = 0.005$) in the layer flocks was the nest stocking density (Table 5). This variable did not have full data available: only 4 of the fully slatted flocks had provided information on this variable. Tables 6 and 7 were then constructed comparing categorical variables (rearing and layer flock data respectively) between the remaining

Table 5. Student's *t* test for continuous variables for flocks in lay.

Layer flock variable	Case mean	Control mean	t-value	df	*P*	Valid N cases	Valid N controls
Distance from rearing farm (km)	211.30	71.25	0.80	19	0.411	17	4
Shed slat coverage (%)	67.23	100.00	−2.24	17	0.038	13	6
First age of ranging (wk)	20.81	20.00	0.50	16	0.618	16	2
Duration of ranging (h)	11.09	11.25	−0.24	16	0.811	16	2
No. birds transferred to layer shed	19390	19900	−0.09	22	0.930	18	6
Feed space (cm/bird)	5.86	3.60	1.25	19	0.227	16	5
Drinker space (birds/nipple)	10.30	11.29	−0.46	19	0.649	16	5
Perch space (cm/bird)	9.41	4.86	1.48	21	0.153	18	5
Available nest space (birds/m^2)	121.68	72.68	3.33	15	0.005	14	3
House stocking density (birds/$m^{2)}$)	11.70	10.71	0.58	19	0.569	15	6
Range area stocking rate (birds/ha)	4605	1808	1.68	17	0.112	16	3
Feed intake at 5%HD[1] (g/bird/d)	78.86	74.40	0.67	10	0.521	7	5
Feed intake at 60%HD[1] (g/bird/d)	94.86	91.20	0.93	10	0.377	7	5
Feed intake at peak HD[1] (g/bird/d)	110.64	116.00	−0.69	14	0.500	11	5
Maximum age difference across farm (wk)	11.88	11.20	0.69	19	0.496	16	5

[1]HD – HenDay % egg production.

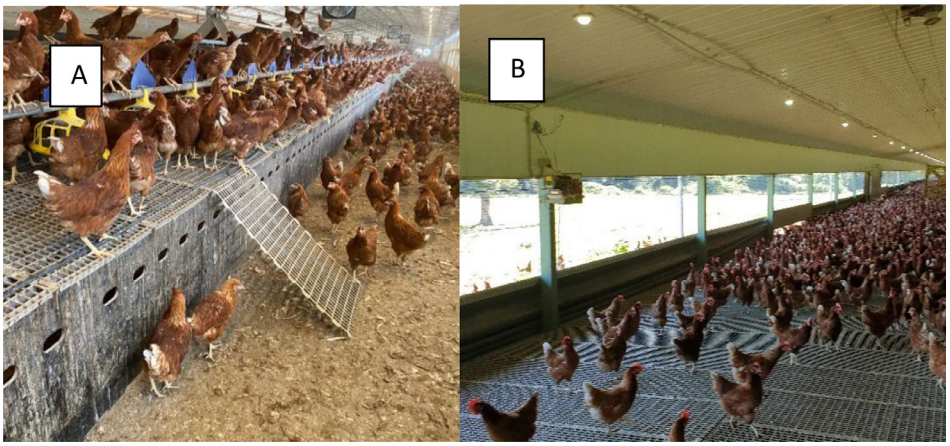

Figure 1. (A) Free-range house with partial slats showing scratch area; (B) free-range house with full slat coverage.

Case and Control flocks with only fully slatted flooring. Variables which showed little or no association with the occurrence of SLD ($P > 0.70$) were beak trimming frequency, probiotic use, water supply disinfection, transport hygiene resting time for the rearing house between batches, distance between the rearing and layer facilities, water source in lay, total available floor space in lay, feeder type, vegetation type in the range area and feed additives (data for these variables are not shown in Tables 6 and 7). From Tables 6 and 7, the factors with a Fisher's exact test $P < 0.20$ with full data include hatchery and layer strain (these 2 factors are identical as strains originate from their own hatchery, hence only strain was considered), flock size, ventilation system and slat type and nest box type (the latter 2 factors are always supplied by the same manufacturer and hence are the same). From this analysis SLD in fully slatted sheds occurred more frequently in flocks of less than 16,080 birds of layer strain A in sheds with natural ventilation systems using nests/ slats of type X. However, these factors are highly confounded in their occurrence. All the Case flocks were smaller flocks ($n = 5$) which were naturally ventilated and used layer strain A and 4 used nest /slat type X. This high level of confounding made discernment of the priority of importance of any of the factors impossible and attempts at multiple factor analysis gave unstable estimates and failed to provide statistical validity. Attempted multivariate analyses produced unstable estimates.

DISCUSSION

Finding the presence of *C. hepaticus* in control flocks, although at lower prevalence than the Cases, is suggestive that the pathogen is widespread, possibly ubiquitous. Phung et al. (2020) have shown that birds can be infected for some time before disease occurs, and in some cases *C. hepaticus* was identified as being present in fecal swabs in rearing age flocks. This is suggestive that factors along with the presence of the organism in the environment or management affecting the birds are necessary for the disease to manifest. When clinical SLD occurs, however, *C. hepaticus* can be detected in a greater proportion of cloacal swabs from randomly selected birds, perhaps indicating a more active spread

Table 6. Categorical variables association with Spotty Liver Disease occurrence for flocks with full slat cover ($N = 11$). Rearing flocks.

		No. of flocks			
Variable - Rearing farm features	Level of variable	Cases	Controls	Odds ratio	Fisher's exact P (2-tailed)
Hatchery	Hatchery A	5	3	Undefined	0.18
	Hatchery B	0	3		
Rearing house style	Aviary	1	0	Undefined	1.00
	Barn	4	5		
Rearing house ventilation system	Natural	5	0	Undefined	0.44
	Tunnel	0	2		
Rearing lights able to be dimmed	Yes	1	4	0.06	0.21
	No	4	1		
Perches in rearing house	Perches present	1	4	0.06	0.21
	No perches	4	1		
Rearing feeder type	Pan	4	3	2.67	1.00
	Chain	1	2		
Rearing feed type	Mash only	3	2	Undefined	0.14
	Crumbles only	2	0	Undefined	
	Crumble starter, then mash	0	2	Reference	
Rearing feeding program	Ad libitum	3	0	Undefined	0.17
	Restricted	2	4		

Table 7. Variables association with Spotty Liver Disease occurrence for flocks with full slat cover ($N = 11$). Flocks in lay.

Variable - Layer shed features	Level of variable	No. of flocks		Odds ratio	Fisher's exact P (2-tailed)
		Cases	Controls		
State	NSW	4	6	0.00	0.45
	WA	1	0		
Flock size (bird number)	Flock < 16,080	5	0	Undefined	0.002
	Flock >= 16,080	**0**	6		
Brown egg layer strain	Breed A	5	2	Undefined	0.06
	Breed other	**0**	4		
Layer shed type	Barn	1	0	Undefined	0.45
	Free range	4	6		
House ventilation system	Tunnel ventilation	**0**	3	0.00	0.18
	Natural ventilation	5	3		
Slat type in fully slatted house	Slat type E [1]	**0**	3	0.00	0.18
	Other slat types	5	3		
Platforms in house	Platforms in shed	0	2	0.00	0.45
	No platforms	5	4		
Perches in house	Perches in shed	3	5	0.30	0.54
	No perches	2	1		
Automatic nest type	Brand X	4	1	20.00	0.08
	Other brands	1	5		
Drinker type	Nipple drinkers	4	6	0.00	0.45
	Bell drinkers	1	0		
Drinker space	<10.7 birds/ nipple	8	2	2.00	0.63
	≥10.7 birds/ nipple	6	3		
Water chlorination	Water treated	3	5	0.00	0.44
	Water not treated	2	0		
Pop hole position	Pop hole one side	2	2	2.00	
	Pop hole both sides	2	4		0.45
	No pop holes	1	0		
Light types in house	Cool white lights	**0**	3	0.00	0.14
	Other lights[2]	4	1		
Infectious bronchitis vaccination during lay	Vaccination in lay	1	4	0.17	0.53
	No vaccine in lay	3	2		
Feeding regime	Varied feed run times	1	3	0.17	0.52
	Feed times not varied	4	2		

[1]Slat types present in the study include Salmet, SKA, Big Dutchman, Vencomatic and Roxel.
[2]Includes warm white or a mixture of warm and cool white lights in the house.

of infection when the disease is occurring or has occurred.

Identifying the role of having a scratch area on SLD occurrence has significant implications for the industry. Scratch areas are included at the discretion of the farm owner, relating to a more natural flooring media on welfare grounds and the provision of a scratch area is considered a future requirement of cage-free housing in Australia (DAFF, 2022). All flocks with scratch areas (i.e., with only partial slat coverage of the floor) in the survey became cases within the survey time frame.

Free range and barn houses which have a scratch area are at much higher risk of clinical SLD occurrence than those which have a floor area fully covered by slats. As a scratch area allows much closer contact of the birds with their feces and a slat coverage can restrict this access, this observation does make biological sense and is an important effect. Presence of a scratch area can therefore be considered a "Key Determinant of SLD" (i.e., a factor strongly associated with a disease that is amenable to management; Martin et al., 1987). A "sufficient cause" of a disease is defined as a set of factors, including any "necessary cause(s)," which when they occur together will always cause the disease (Martin et al., 1987). These data indicate that the presence of a scratch area in a free range or barn layer house when *C. hepaticus* is present (the Necessary Cause), may comprise a Sufficient Cause of SLD. There may be more than one sufficient cause in

the ecology of any disease and others obviously exist, as we observed SLD in 45% of houses with full slat cover over the floor.

Having a variable which displayed a zero-cell value n its contingency table of association between the factor and the disease, creates analytical problems for any multiple factor analysis as it seriously inflates standard error values. Hosmer et al. (2013) suggested methods for dealing with this problem statistically, including collapsing the categorization within the levels of the exposure factor or removing the members of the data with the zero value. The separation in Cases and Controls between full and partially slatted houses (i.e., those without or with a scratch area respectively) was complete, and there was no obvious way to collapse the categories any further. Some Cases and Controls were observed in fully slated sheds. Hence our only solution was to remove all houses with a scratch area for further analysis and continue analyses on the reduced number of remaining flocks in fully slatted houses.

Unreliable data, due to strong confounding of factors within the remaining small sample size of fully slatted sheds, might, speculatively from this data, point to ventilation system as being important. In this study, naturally ventilated houses which were smaller on average, may be more at risk of SLD in fully slatted facilities. This may make some sense as SLD was often noted in warmer weather (Business Queensland, 2017) and Courtice and Jenner (2022) reported that keeping the house cooler (by 8°C in their estimation) during an outbreak of SLD can decrease disease severity, an option which is more obtainable with tunnel ventilation (mechanically ventilated system) than in naturally ventilated houses. But a larger study is needed to confirm this contention.

A further study with a much larger sample size, focused on houses with no scratch area, is needed to identify further risk factors and key determinants under these conditions.

We observed that SLD can become clinical in barn houses and also in free-range operations prior to birds being allowed access to the range area. As *C. hepaticus* has a fecal-oral mechanism of spread (Courtice et al., 2018; Phung et al., 2020), this finding regarding the scratch area makes biological sense, as there would be much higher chance of bird access to fresh feces in the scratch area than it would be on a fully slatted floor where feces pass through the slats efficiently. We can propose then that the existence of a scratch area in a barn or free-range flock where *C. hepaticus* (or perhaps *C. bilis*) is present constitutes a "sufficient cause" for SLD. As 45% of houses without a scratch area (full slats) also developed SLD, there are obviously other factors besides a scratch area that contribute to cases as part of other sufficient cause scenarios and a search for these needs to continue.

Study Limitations

Sample size was an obvious limitation to the outcome, with the majority of the sampled flocks unable to provide further information with the detection of a major over-riding risk factor (scratch area presence). Controlling for the presence or absence of this factor will assist in further studies. This is the first analytical epidemiological study of SLD reported and as such, much was unknown and made sample size selection a limitation. COVID-19 travel restrictions further hampered the ability to expand sample size.

Further studies should focus on houses with or without scratch areas to further identify important management factors that may contribute to the occurrence or severity of SLD in cage-free layer flocks.

CONCLUSIONS

Within the framework of this survey, all flocks with only a partially slatted floor in the layer house (hence those that have a scratch area within the house) when *C. hepaticus* was present showed the occurrence of clinical SLD. Thus, the presence of a scratch area in the layer house can be considered to be a key determinant (Martin et al., 1987) of SLD.

Spotty Liver Disease will also occur in sheds with full slat coverage and further factors which contribute to this need to be identified. Other factors were considered as potential risk factors under fully slatted flooring but could not be satisfactorily assessed due to strong correlation between them with this sample size. However, for new cage-free housing having a fully slatted floor covering should be considered to decrease the risk of SLD occurring.

A preliminary multivariate data analysis did indicate that of the factors revealing some statistical interest, natural ventilation may place flocks more at risk of SLD than would tunnel ventilation systems, but this needs to be confirmed by further studies.

A further survey examining possible factors under fully slatted conditions was subsequently undertaken and results will be provided in a further paper.

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DISCLOSURES

I declare that the authors specified for this paper do not have any conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2023.102922.

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