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Descriptive epidemiology of spotty liver disease in Australian cage-free brown egg layer chicken flocks with a scratch area

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ABSTRACT Spotty Liver Disease (**SLD**), caused by *Campylobacter hepaticus* or *C. bilis* infection in adult female chickens continues to emerge as a major disease problem in cage-free production systems. Free range production has become the predominant system in Australian egg production and SLD is widespread in these farms. Previous studies have identified having a scratch area as a key determinant for SLD occurrence. An Australia-wide survey of egg production flocks with scratch areas was conducted regarding SLD including 48 individual flocks. Descriptive information on the facilities and flock management practices was reported. The incidence of SLD, age of first outbreak, initial mortality rate, duration of elevated mortality, and magnitude and duration of any associated egg production decline are described. Recurrence of SLD in the same flock was also reported and discussed. Therapies applied were recorded and assessed across SLD severity and duration. SLD occurred in 66.7% of layer flocks whose facility included a scratch area. Recurrent SLD outbreaks occurred in 31% of flocks experiencing SLD. Antibiotic medication reduced duration of mortality and egg production decline. Antibiotic therapy was associated with reduced duration of mortality and a less severe and shorter duration of egg production drops compared to untreated flocks. PCR detection of *C. hepaticus* in cloacal swabs and house dust samples and a serological ELISA test were compared and evaluated as diagnostic aids or as possible predictors of SLD outbreaks. The ELISA showed substantial agreement with detection of *C. hepaticus* in cloacal swabs by PCR. Examining composite house dust samples by PCR for *C. hepaticus* DNA appeared to be the most convenient and cost-effective aid to diagnosis and as a putative predictor for SLD outbreaks.

Key words: campylobacter hepaticus, epidemiology, recurrence, treatment

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INTRODUCTION

Spotty liver disease (**SLD**), caused by an infection with *Campylobacter hepaticus* (Van et al., 2016) or *Campylobacter bilis* (Phung et al., 2022), is an emergent, serious disease problem for free-range and barn egg production systems in Australia (Grimes and Reece, 2011), the UK (Burch, 2005) and is emerging in the USA (Becerra et al., 2023) and other countries such as Jordan (Hananeh and Ababneh, 2021), Germany (Courtice et al., 2018), Eastern Europe (Courtice et al., 2018) and Costa Rica (Quesada-Vásquez et al., 2023). Spotty liver disease affects adult hens in cage-free production systems and has been reported as capable of causing considerable mortality (10−15%) and a drop in egg production of up to 35% (Grimes and Reece, 2011; Courtice et al., 2018). Examples of egg production drops and mortality have been reported by Muralidharan et al. (2022).

Spotty liver disease appears identical to a disorder described in the 1950s as avian vibrionic hepatitis (Moore, 1958, Peckham, 1958) which disappeared following the introduction of intensive cage-based egg production systems (Shane et al., 2003). The route of spread of infection of SLD is regarded as fecal-oral (Van et al., 2017a; Phung et al., 2020; Phung et al., 2022; Gao et al., 2023a;Becarra et al., 2023) and this feature would explain the disappearance of the syndrome in cage systems. Courtice et al. (2023) reported the ability to find *C. hepaticus* DNA in diverse and plentiful sources in the farm environment, including hen feces, water and soil,

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and it was also detectable in flies, vermin, mites (*Dermanyssus gallinae*), beetles (*Alphatobius diaperinus*) and in the feces of wild birds and mammals. While a definitive source of infection for the hen is unknown, there are ample possible reservoirs of the organism in the environment.

From a base of almost complete cage egg production, the Australian egg industry has progressed to predominantly free-range production over the last 2 decades. This change in the production system has been accompanied by the continued emergence of SLD as a major disease problem in cage-free hens (Grimes and Reece, 2011). The Australian national egg layer flock consisted of 21.8 million hens in 2023 of which 71.7% were housed in cage-free production systems: 56.5% as free-range flocks and 15.2% used a barn-lay system (Australian Eggs, 2023). Free-range egg production continues to grow in Australia (EFA, 2023). Furnished cage systems, which are common in Europe, have not been adopted in Australia as the Australian climate is more conducive to cage free production and this has perceived consumer preference (Australian Eggs, 2023).

Clinical signs, gross pathology, and histopathology of SLD have been reviewed by Courtice et al. (2018). Most primary outbreaks are reported in the early laying period (22−28 wk of age) (Grimes and Reece, 2011). SLD is an acute disease with birds dying suddenly in good condition (Crawshaw et al., 2015). Soiling of the vent feathers is commonly seen. Moribund birds are usually febrile (Courtice et al. 2018). Gross pathological findings show hepatomegaly with a distinctive multifocal hepatitis with miliary white-grey or yellow necrotic spots throughout the liver. Hemorrhages in the liver have also been reported (Tudor, 1954; Hofstad et al., 1958; Courtice et al., 2018) There is often a fibrinous perihepatitis and peritoneal and/or pericardial effusion is common, as is a mild enteritis (Courtice et al., 2018). The ovary is commonly active, but the ova capsules are hyperemic. Affected birds often exhibit icterus (Grimes and Reece, 2011). Histologically, livers show general congestion, hemorrhages, a multifocal, acute hepatocellular necrosis displaying fibrin deposition and infiltration by inflammatory cells (or an acute coagulative necrosis) (Hofstad et al., 1958). Bacteria were not visualized in association with the lesions (Grimes and Reece, 2011). Previously, routine bacterial culture of the liver often detects no growth (Jenner 2001, Jennings et al., 2011). However, more recently, C. *hepaticus* isolated from the liver has been reported (Van et al., 2016). Spotty liver disease histopathology signs differ from other severe bacterial infections, inclusion body hepatitis and hepatitis/splenomegaly syndrome (avian hepatitis E virus) (Courtice et al., 2018).

Spotty liver disease has been known to re-occur in the same flock after treatment (Courtice et al., 2018) but it is unknown whether this is due to insufficient spread through the flock to promote immunity or if initially affected birds remain susceptible. *C. hepaticus* can only be grown in culture from bile or gall bladder of infected birds as, due to its slow growing nature, it is swiftly overgrown by other organisms if culturing feces, intestinal contents or environmental samples (Van et al., 2017b). It has been shown to be present in the gastrointestinal tract and feces of infected birds by quantitative PCR with the highest populations residing in the caeca (Van et al., 2017b).

C. hepaticus is known to become endemic on properties after a primary outbreak and healthy birds may harbour the organism for long periods, perhaps for life (Courtice et al., 2018).

Spotty liver disease responds rapidly to antibiotic treatment (Grimes and Reece, 2011). The antibiotics of choice against campylobacteria are macrolides and fluoroquinolones (Wieczorek and Osek, 2013). In Australia, antibiotic use in food producing animals is regulated by the Australian Pesticides and Veterinary Medicines Authority (**APVMA**). Label restrictions limit the antibiotics which can be used in hens producing eggs for human consumption. These regulations and label restrictions can be searched on the APVMA PubCRIS database (APVMA, 2023). The only chemotherapeutics which can practicably be used in Australian commercial egg layers are chlortetracycline, amoxicillin, and a combination of lincomycin and spectinomycin (the latter being prohibitively costly). Wieczorek and Osek (2013) refer to tetracyclines as an alternative treatment for *Campylobacter* infections, but this class of drug does not find frequent selection for therapy of campylobacteriosis in humans. The majority of *C. jejuni* and *C. coli* strains are considered to be susceptible to amoxicillin (Wieczorek and Osek, 2013).

Previous epidemiological studies have identified some risk factors for the occurrence of SLD. Gao et al., (2023a) identified that the presence of a scratch area within the layer house (an area of solid flooring where the birds can dust bathe) is a strong risk factor for the occurrence of SLD in a flock, while having a fully slatted floor is somewhat protective. This makes biological sense as the infection is acquired via the fecal-oral route (Phung et al., 2020) and a scratch area affords close contact of the birds to fresh fecal material, while fully slatted flooring separates feces from the birds to some extent. A further study conducted in houses that have fully slatted floors (i.e., no scratch area) suggested that a higher number of birds per nest area increased the risk of SLD while having the ability to control environmental temperature gave a measure of protection against the disease (Gao et al., 2023b).

As the presence of a scratch area in a free-range or barn house has been identified as a strong risk factor for SLD, the present study has been conducted across cage-free houses that have a scratch area. Attention was focused on the severity of the SLD outbreak observed in the studied flocks. Observations are reported on the incidence of outbreaks in the study group including severity of mortality and egg production effects, antibiotic and non-antibiotic treatments, and the occurrence of recurrent outbreaks. In some flocks, cloacal swabs and house dust were collected for the detection of *C. hepaticus* by PCR and compared between flocks which did not

experience clinical outbreak with those which did experience SLD. The study was restricted to descriptive epidemiology, focusing on animal-health related findings and limited attempts were made to assess associations of exposure factors and disease occurrence where these explain the distribution of SLD in the target population (Dohoo and Stryhn, 2003). Paired serum samples and cloacal swabs were collected from a subset of flocks. *C. hepaticus* specific antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) and the analysis method was assessed for its diagnostic value. The serological results were compared with cloacal swab detection by PCR.

MATERIALS AND METHODS

Animal Ethics

The survey was supervised by the Animal Ethics Committee of the University of Sydney (approval number 2022/2014). All animal procedures were conducted in accordance with the Australian Code for the care and use of animals for scientific purposes, 8th Edition (NHMRC, 2013), the Australian Code for the Responsible Conduct of Research (NHMRC, 2018), the NSW Animal Research Act 1985 the NSW Animal Research Regulations 2010 and other relevant legislation.

Epidemiological Survey

Forty-eight houses were included in the survey, 31 in New South Wales (**NSW**), 5 in Victoria (**VIC**), 8 in Western Australia (**WA**), 3 in Queensland (**QLD**) and one in South Australia (**SA**). The intended interstate survey was inhibited by government instituted COVID-19 travel restrictions during 2021- early 2023, restricting the visitation study to NSW. The WA and SA participants were interviewed remotely by telephone or virtual link. Locations and farms were selected from those who participated in an earlier survey (Gao et al., 2023a) and further producers who were recruited during extension meetings held by Australian Eggs. This is not a fully random selection of farms but does cover a range of free-range egg producers from areas previously experiencing SLD outbreaks.

Where physical farm visits were made, a wide-ranging questionnaire was completed with the manager/ producer and entered into an MS Excel file (a copy of the questionnaire is included in S1 in Supplementary Information). Questions covered poultry house design including slat set up, nest box type and number, feeder and waterer facilities and ventilation system, range structure and use, husbandry practices, occurrence of other conditions in the flock, occurrence of SLD and its severity and duration and any treatments administered. On each visit in NSW, a cloacal swab was collected from 12 randomly selected birds in the house and a pooled dust sample was collected. These samples were subjected to qPCR analysis, proceeding as described by Gao et al., (2023a) for the detection of DNA of *C. hepaticus*. The qPCR was designed and described by Van et al. (2017a) and has been shown to be capable of detecting both *C. hepaticus* and *C. bilis* (Van et al., 2023). Dust was brushed off surfaces (nest box tops, ledges at side walls, tops of feeder lines) from multiple random locations around the house into a sterile 70 mL sample plastic jar.

On a separate set of farms, sequential sampling of paired cloacal swabs and serum samples (from each of 12 birds randomly selected per house at each visit) and a pooled house dust sample was collected 2 to 3 times between 21 and 37 wk of age. Birds were selected at random on each farm visit. Two rearing farms were also included in this section of the survey. Cloacal swabs and dust were assayed for the presence of *C. hepaticus* DNA as described above. The sera samples were subjected to a *C. hepaticus* antibody ELISA, as described by Muralidharan *et al.*, (2022).

Only a subset of the data has been considered in this report, dealing with description of the SLD outbreaks regarding bird type, age, SLD effects on the flock, cloacal swab and dust PCR detections, treatments and any recrudescence of SLD in the flock and comparative cloacal swab and dust detections with serological *C. hepaticus* ELISA results. A further analytical epidemiology analysis will be published separately.

Statistical Analyses

Descriptive statistics are presented as mean, 95% confidence intervals of the mean, range, including minimum, lower quartile, median, upper quartile and maximum values. Where comparisons are made, these were conducted using Student's t-test where the dependent variable was binary or one-way ANOVA where there were multiple dependent variables. Odds ratios were assessed using Pearson's X^2 or Fisher's exact test if an expected value was <5. Significance was determined at ≤ 0.05. Epidemiological sensitivity and specificity were calculated as per Martin et al. (1987). Test agreement was assessed using Cohen's Kappa (κ) as described in Martin et al. (1987). Data entry was carried out using MS Excel and statistical tests were completed in STATISTICA ver 6.1 (StatSoft Inc, 2003).

RESULTS

Survey Findings

There were 3 basic house types participating in the survey: "conventional free-range houses," "barn style" houses, and aviary houses.

There were 48 flocks included in the survey of which 32 experienced at least one outbreak of SLD. The incidence risk of SLD was estimated at 66.7 cases per 100 flocks at risk, 95% confidence interval of the incidence risk estimate was 52.54 to 78.32 cases per 100 flocks at risk.

Descriptive information in Tables 1 and 2 is provided to describe the background of the differences in housing and management systems existing across the survey.

Table 1. Descriptive data for categorical variables from all chicken layer flocks surveyed.

Categorical variable	Level	No. flocks	% of contribution to survey
State	NSW	31	64.6%
	Victoria	5	10.4%
	Western Australia	8	16.7%
	South Australia	1	2%
	Queensland	3	6.2%
Breed	ISABROWN	13	27%
	HyLine Brown	31	64.6%
	Lohmann Brown	4	8.3%
Ventilation in rearing house	Natural	9	28%
	Mechanically assisted	10	31%
	Tunnel ventilated	13	42%
Layer House style	Conventional free-range	28	58.3%
	Barn	5	10.4%
	Aviary	15	31.3%
Cooling system in layer house	Foggers	25	68%
	Cool cells	12	32%
Perches in layer house	Yes	48	100%
	No	0	
Feeder type	Chain	27	56.3%
	Pan	21	43.8%
Light colour	Warm white	33	68.8%
	Cool white	15	31.3%

Table 1 lists categorical factors. The predominant number of flocks were located in New South Wales (**NSW**) while participants in other states were limited by COVID-19 travel restrictions in place during 2019-2022. When the survey took place, Hyline Brown and ISABROWN were the major breeds involved, which was typical of the industry at the time. Ventilation system used in rearing varied from "natural" (open sided house with curtains or shutters, circulation fans and fogger cooling systems) to those with mechanically assisted airflow (including extraction fans in the roof or tunnel ventilation systems (evaporative cool cells and extraction fans at the end of the house)). Laying houses were predominantly of the type described as "conventional free-range" (older style houses with open sides, curtains or shutters with internal circulation fans and fogging systems for cooling and with doors which allow the birds to access and outside range area), "barn style" which are similar houses to "conventional" but where the birds are not permitted to leave the house, and "aviary" systems, which are modern complex houses which make use of vertical space by having a multilevel deck system incorporating sections with nesting boxes, feeding levels and resting levels. Aviaries may have natural ventilation or mechanically assisted in ventilation. Conventional and barn style houses are also called "flat deck" houses. All styles have a central automated nest box system allowing eggs to roll onto a conveyor belt for collection at the end of the house. All houses in this survey had a scratch area within the house.

Table 2 shows statistics for continuous variables within the survey across all flocks surveyed, describing the distribution of age of transfer to laying quarters, flock size, floor area in the house, stocking density, scratch area proportion of floorspace, perch space per bird, nest density, age of access to nests, drinker space allowance, feed space, range area and range stocking density.

Table 3 displays some descriptive data (mean with 95% confidence intervals, range, median and upper and lower quartiles) for flocks participating in the survey that experienced an outbreak of SLD. Thirty-two flocks experienced at least one SLD occurrence. The first outbreaks of SLD in the surveyed flocks occurred between 20 and 35 wk of age. The mean age of the first outbreak of SLD was 28.5 wk and the median outbreak age was 28 wk. The maximum daily rate of mortality in an outbreak prior to any treatment ranged between zero and 4.17 birds per thousand per day with a mean of 1.23 birds per thousand per day prior to any antibiotic treatment being administered. The duration of increased mortalities ranged between zero and 70 d with a mean of 17.1 d. Declines in Hen Day egg production (%HD = the percentage of birds laying an egg per day averaged over a week) during an outbreak of SLD averaged 7.68% and ranged between zero and 24% with a mean duration of the production drop of 27 d (ranging between zero and 91 d). Duration of mortality was not correlated with the duration of the egg production decline ($r = 0.06$, $p = 0.75$) but the extent of the egg production drop and the duration of the production drop were strongly positively correlated (Figure 1: $r = 0.75$, $p < 0.001$).

Table 2. Descriptive data for continuous variables from all chicken layer flocks from the survey.

Continuous variable	Valid n	Mean	Median	Minimum	Maximum	SEM[1]
Age at transfer (wk)	43	15.37	15	12	17	0.15
No of birds transferred	46	19,769	15,252.5	6000	45,313	1,579
Total floor area (m^2)	46	1,391.8	1,318.5	304.0	3,101.0	72.1
Total usable space (m^2)	37	1,512.5	1,320.0	304.0	2,860.0	97.6
Stocking density (birds/m^2)	33	11.7	11.00	6.0	20.0	0.5
Perch space in lay (cm/bird)	41	110.4	104.18	19.1	265.0	8.6
Scratch area coverage of shed (%)	40	63.45%	42%	25%	100%	5.61%
Nest density (birds/m^2)	45	109.3	110.95	36.0	270.0	7.0
Age of first access to nest boxes (wk)	32	15.7	16.0	14.0	17.0	0.13
Drinker space (birds per nipple)	35	11.4	10.8	4.0	24.0	0.73
Feed space - Chain feeder (birds per m)	14	15.14	16.50	7.0	18.0	0.804
Feed space - Pan feeder (birds per pan)	26	46.1	44.19	29.5	84.0	2.5
Range size (ha)	29	5.14	3.71	0.90	16.10	0.80
Range density (birds/ha)	33	4783	3780	1206	10140	492

[1]Standard error of the mean.

Table 3. Descriptive statistics on characteristics of spotty liver disease (**SLD**) outbreaks for clinically affected flocks within the survey.

SLD case flocks only	Valid n	Mean	95% confidence interval		Minimum	Lower Q[1]	Median	Upper Q[1]	Maximum
Age of 1st outbreak (wk[2])	31	28.5	27.0	30.1	20	25	28	31	35
Highest % SLD mortality b/1,000/day	32	1.232	0.825	1.640	0.000	0.552	0.678	1.667	4.167
Mortality duration (days)	32	17.1	11.3	22.9	0	7	14	21	70
Max %HD[3] lost in outbreak	32	7.68%	5.40%	9.96%	0.00%	0.00%	8.00%	10.16%	24.00%
Duration of production decline for 1st outbreak (days)	32	27.0	16.6	37.5	0	0	14	42	91
Age of second outbreak (wk[2]), if occurred	10	40.6	37.1	44.0	36	37	39	46	47

[1]Quartile.
[2]Week of age.
[3]Hen day percent production.

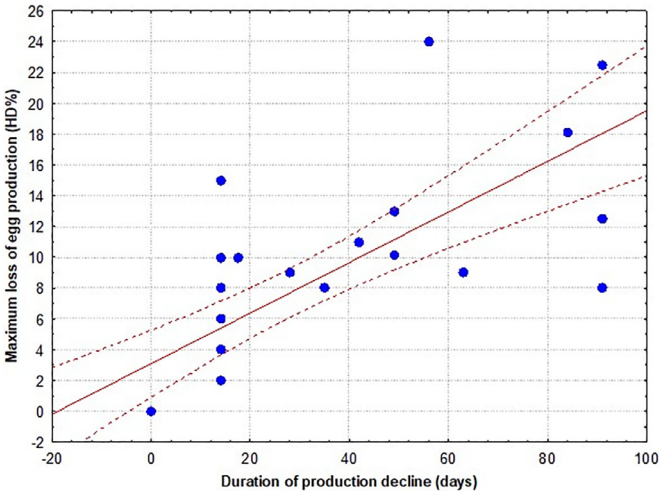

Figure 1. Correlation of maximum loss in egg production (HD%) with duration of the production decline (days).

Ten of the SLD affected flocks experienced a second occurrence of the disease at a mean age of 40.6 wk (Table 3: ranging between 36 and 47 wk of age). Flock identity that showed a recurrence was highly confounded by farm identity as six of these recurrences occurred on a single farm. Where a second outbreak occurred in a flock, it did so with a mean of 9 wk after the initial occurrence of SLD in treated flocks.

Twenty of the 32 SLD affected flocks were treated with antibiotics. Eleven flocks were treated with non-antibiotic products, such as essential oils (oregano), organic acids, combinations of short chain and medium chain organic acids and mushroom extract. Table 4 compares onset of SLD, mortality rate and duration and Hen Day production decline and its duration in flocks that were treated or not treated with antibiotics or non-antibiotic products in response to the initial outbreak. Flocks which were treated with antibiotics by a veterinarian tended to have a later age of onset compared to those that were left untreated (30.4 wk compared to 25.1 wk respectively). Those flocks treated with antibiotics had approximately double the maximum mortality prior to treatment than did the non-antibiotic treated flocks (1.64 birds/100/day compared to 0.84 birds/100/day, which approached significance, $P = 0.07$).

Table 4. Treatment of flocks at first outbreak of SLD with antibiotic or non-antibiotic treatments.

	Mean (SE) for treated flocks	95% confidence range	Mean (SE) for nontreated flocks	95% confidence range	$P =$
Antibiotic treatment	(n = 20)		(n = 12)		
Age of outbreak (wk)	30.4[A] (0.88)	28.6 – 32.2	25.1[B] (0.67)	23.6 – 26.7	<0.001
Highest % SLD mortality (birds/1,000/day)	1.64 (0.26)	1.09 – 2.18	0.84 (0.33)	0.11 – 1.57	0.07
Mortality duration (days)	13.4 (2.67)	7.8 – 19.0	24.6 (5.91)	11.6 – 37.6	0.06
Max %HD[2] lost in outbreak	5.4%[B] (1.19)	2.87 – 7.89	11.3%[A] (1.97)	6.97 – 15.64	0.011
Duration of production decline for 1st outbreak (days)	20.5	6.2 – 34.8	39.1	22.8 – 55.3	0.09
Recurrence of SLD (n)	9 (OR[1]=9.00)	OR 0.97 – 83.6	1		0.05*
Time (wk) between first and second outbreak	9.0 (2.12)	3.98 – 14.0	13.0 (0.0)		0.55
Non-antibiotic treatment	(n=11)		(n=17)		
Age of outbreak (wk)	27.6 (1.25)	24.8 – 30.3	29.3 (1.10)	26.9 – 31.6	0.32
Highest % SLD mortality (birds/1,000/day)	1.49 (0.43)	0.53 – 2.45	1.36 (0.24)	0.83 – 1.86	0.77
Mortality duration (days)	17.9 (4.53)	7.82 – 28.00	19.5 (4.46)	10.02 – 28.92	0.82
Max %HD[2] lost in outbreak	6.95 (2.80)	0.71 – 1.32	8.27 (1.12)	5.76 – 10.8	0.63
Duration of production decline for 1st outbreak (days)	24.8 (10.1)	2.3 – 47.3	31.1 (7.28)	15.7 – 46.5	0.61
Recurrence of SLD (n)	7[A] (OR[1]=8.17)	OR 1.42 - 47.0	3[B]		0.02*
Time (wk) between first and second outbreak	11.9[A] (1.40)	8.4 – 15.3	1.0[B] (0.0)		0.006

[A,B]Means within the same row with different superscripts differ significantly ($P < 0.05$) by Student's t-test or ¶ Mann-Whitney U-test, or * Fisher's exact test, 2-tailed.
[1]OR = Odds ratio of treated compared with untreated flocks.
[2]Hen Day % production.

Table 5. Mortality and production parameters with different therapies used for SLD affected flocks.

Treatment administered	n	Age of 1st outbreak (wk[2])	Highest daily mort during SLD, prior to treatment birds/ 1000/ day	Mortality duration (days)	Max %HD[3] lost in outbreak	Duration of production decline for 1st outbreak (days)	No. flocks where SLD recurred
No. flocks treated with antibiotics	20	30.4	1.64	13.4	5.4%	20.5	
Chlortetracycline	10	30.1	1.66	11.7	8.16	35.4	2^{B}
Amoxicillin	9	31.3	1.76	16.0	2.67	4.67	8^{A}
Lincomycin	1	25.0	0.57	7.0	2.00	14.0	0
	P=	*0.31*	*0.66*	*0.63*	*0.06*	*0.08*	*0.005*[2]
No. flocks treated with non-antibiotic products	11	27.6	1.49	17.9	6.95%	24.8	
Essential oil + organic acids	4	24.3^{B}	0.29^{B}	17.8	$17.1\%^{A}$	64.8^{A}	1^{B}
SC and MC fatty acids[1]	6	31.0^{A}	1.84^{AB}	19.8	$0\%^{B}$	0^{B}	6^{A}
Mushroom extract	1	20.0^{C}	4.17^{A}	7.0	$8.0\%^{AB}$	14.0^{A}	0
	P=	*<0.001*	*0.012*	*0.77*	*0.001*	*<0.001*	*0.03*[2]

[1]Combined short and medium chain fatty acids
[A,B,C]- means within a grouping without common superscripts differ significantly (ANOVA, P<0.05, separated by Tukey's unequal n HSD test)
[2]Fisher's exact test, 2-tailed.

Consequently, the duration of mortality in antibiotic treated flocks was reduced from 24.6 d for untreated flocks to 13.4 d for treated flocks ($P = 0.06$). The maximum loss of egg production in antibiotic-treated flocks was significantly reduced compared to the untreated flocks 5.4%HD compared to 11.3%HD respectively, $p = 0.011$) and the duration of the production drop was numerically reduced by treatment (20.5 d compared to 39.1 d respectively, $P = 0.09$).

Ten antibiotic treated flocks showed recurrence of SLD whereas this only occurred in 1 untreated flock (odds ratio = 9.0, $P = 0.05$).

The lower section of Table 4 shows the same parameters for flocks treated with a non-antibiotic treatment (mostly essential oils and organic acids were used). Eleven flocks were treated with these products (Table 5) and six of these were also treated with antibiotics simultaneously (Table 6), so any individual effects are difficult to separate statistically. Considering non-antibiotic treatment as a main effect, there were no significant differences in age of the first SLD outbreak, maximum mortality prior to treatment, duration of mortality, egg production drop or duration of production decline (Table 4). Recurrence of SLD was higher in non-antibiotic treated flocks than those that were not given these products (odds ratio = 8.17, $P = 0.02$).

Table 5 lists the antibiotics and non-antibiotic products used across the survey. The distribution of age of first outbreak, highest mortality prior to treatment, mortality duration, egg production drop and its duration did not differ between the antibiotics used. Recurrence of SLD was significantly more likely if amoxicillin was used compared to chlortetracycline ($P = 0.005$). SLD recurrence was more likely if a short and long chain fatty acid mixture (**SC MC**) was used compared to essential oil plus organic acids ($P = 0.03$). These results are confounded however as the same flocks which used amoxicillin also used the SC MC fatty acid mixture.

Table 6. Outcomes of treatments for SLD outbreaks.

Treatment	n	Age of 1st outbreak (wk[1])	Highest daily mortality during SLD, prior to treatment b/ 1,000 / day[2]	Mortality duration (days)	Max %HD[3]lost in outbreak	Duration of production decline for 1st outbreak (days)	No. flocks where SLD recurred
None	4	26.8^{AB}	0.91	39.2^{A}	$9.8\%^{A}$	29.4^{AB}	0^{B}
Antibiotic alone	13	29.8^{A}	1.53	10.9^{B}	$7.7\%^{AB}$	30.4^{AB}	4^{B}
Non-antibiotic alone	5	23.4^{B}	1.07	15.6^{AB}	$15.3\%^{A}$	54.6^{A}	1^{B}
Both	6	31.0^{A}	1.84	19.8^{AB}	$0.00\%^{*B}$	0.00^{*B}	6^{A}
	P=	*0.005*	*0.54*	*0.007*	*0.0002*	*0.02*	*0.003*

[A,B,C]means within a grouping without common superscripts differ significantly (ANOVA, P<0.05, separated by Tukey's unequal n HSD test)
[1]week of age
[2]Birds / 1000 / day
[3]Percent HenDay production

Table 6 shows the usage of antibiotic, non-antibiotic and the combination of both treatments across flocks with SLD outbreaks. Flocks which received antibiotics, alone or combined with non-antibiotics experienced more secondary outbreaks than those which had only non-antibiotic treatment and the magnitude and duration of egg production drops were higher for flocks only receiving non-antibiotic treatment. Recurrence of SLD was higher in flocks treated with both types of medication.

Table 7 shows the quantitative PCR results for *C. hepaticus* detection from cloacal swabs and from dust collected from the house at the time of the survey visit, compared between flocks that did and did not experience an SLD outbreak. Sample collection was made during farm visits which in all cases occurred several weeks after the SLD outbreak if it had occurred in that flock. Flocks experiencing an SLD outbreak had significantly higher numbers of positive *C. hepaticus* PCR cloacal swabs and higher DNA copies of *C. hepaticus* in house dust than flocks which did not have a clinical outbreak of SLD. The lower quartile for flocks experiencing SLD was 5 positive cloacal swabs/ 12 sampled. Table 8 performs an epidemiological sensitivity and specificity analysis between cloacal PCR and dust qPCR results. The dust qPCR test showed an epidemiological sensitivity of 90% for detecting that a flock would have ≥5 positive cloacal PCR swabs from 12 samples and a specificity of 100%.

Evaluation and Assessment of a Serological Test for C. hepaticus

Thirteen flocks across six farms were included in the serological comparison survey. These flocks had paired serum and cloacal swabs collected from 12 birds per flock and a house dust sample collected. Nine flocks were sampled during rearing at 15 to 18 wk of age. Two of these flocks had received injections of an autogenous *C. hepaticus* bacterin (Spotvax, Trèidlia Biovet Pty Limited, Seven Hills, NSW, Australia) at 7 and 11 wk of age. These vaccinated flock exhibited positive ELISA serology and remained serologically positive for the remaining sampling ages. Of the unvaccinated rearing flocks three returned negative ELISA results by 18 wk of age. All of the flocks in the rearing age group were found to have negative cloacal swab PCR results but one flock delivered a positive house dust result for presence of *C. hepaticus.* This dust-positive flock was also ELISA positive (Table 9).

Unvaccinated flocks were then used to compare the serological ELISA to paired cloacal swab samples and dust detection through the laying period. Table 9 shows the percentage flocks showing positive ELISA, percentage flocks with positive *C. hepaticus* cloacal swab PCR and number of houses positive for *C. hepaticus* PCR in dust. Not all flocks were sampled at the same ages. Out of five flocks sampled at 21 to 22 wk of age, 60% had positive *C. hepaticus* ELISA while 40 % had positive cloacal swab results. Four of the five flocks however had detectable *C. hepaticus* DNA by PCR at this age. None of the flocks had experienced clinical SLD by this age. Between 27 to 29 wk of age nine flocks were sampled and 44% showed positive ELISA results while 33.3% gave detectable cloacal swab PCR results. Four of these nine flocks had PCR positive house dust at this age, but none had yet experienced clinical SLD. Of 4 flocks sampled at 32 wk, 75% were ELISA positive and 50% were cloacal swab positive for *C. hepaticus.* All 4 flocks had positive house dust and 1 flock experienced clinical SLD. The final sampling was conducted between 36 and 39 wk of age involving seven flocks of which 71% were ELISA positive and 57% were positive for *C. hepaticus* on cloacal swabs. All seven had positive house dust PCR for *C. hepaticus* and all seven had experienced clinical SLD by this age.

Using cloacal PCR results as the standard test, the epidemiological sensitivity and specificity of the *C. hepaticus* ELISA test from this sample was calculated as 66.7% and 89.9% respectively from this sample of flocks (Table 10). The predictive value of a positive ELISA test for this sample of flocks was 76.4%.

Table 8 also allows calculation of agreement between the 2 tests, in this case revealing a Kappa of 0.606, which is regarded as a substantial level of agreement (Statology, 2021).

Farmer Observations

Some astute farmers offered useful comments on SLD outbreaks, coming from keen observations and experience with their flocks. One farmer had noted that at 2 to 3 d prior to an outbreak of SLD, the flock often showed a slight increase in flightiness and increased feather pecking activity. This increased observed activity may also often be associated with an occurrence of piling or smothering. Another offered that during the onset of an SLD outbreak, some birds tended to move to a less populated area of the house and appeared slightly depressed, with their tails drooping slightly (Figure 2). On one visit, a number of these birds were culled and necropsied, and they showed liver lesions typical of SLD. It is thought that these birds would progress to death by the following day. Such observations, although not as yet scientifically evaluated, offer valuable insights for others experiencing SLD and may be warning signs of an impending disease outbreak.

DISCUSSION

Descriptive epidemiological surveys are conducted to explore the frequency and distribution of selected observations within a defined population (Dohoo and Stryhn, 2003). The present study was an observational study of the field situation, and no interventions were undertaken by the research team. The use of antibiotic and non-antibiotic treatments were instituted on some of the farms. as determined by the attending veterinarian or farm owner.

Table 7. PCR[1] tests for presence of C. hepaticus within flocks with and without clinical SLD.

Tests for detection of *C. hepaticus*	SLD Occurrence	Mean	95% Confidence limits		n	Std Err	P=	Minimum	Lower quartile	Median	Upper quartile	Maximum
PCR Positive cloacal swabs/12 Mean	Control	0.55[B]	-0.32	1.41	11	0.40	**0.0004**	0	0	0	0	4
	Case	8.21[A]	5.46	10.95	14	1.27		0	5	10.5	12	12
Dust PCR DNA copies/ mg	Control	19.8[B]	-16.4	55.9	16	16.96	**0.0001**	0	0.0	0.0	8.3	273.6
	Case	145.1[A]	79.5	210.6	18	31.06		0	27.1	110.7	230.8	460.0

[1]Van et al., 2017a.
[A,B]- means within a grouping without common superscripts differ significantly ($P < 0.05$ by Mann-Whitney U test).

Gao et al. (2023a) had identified having a scratch area as a major risk factor for the occurrence of SLD and the present survey estimated SLD prevalence among flocks living in houses with scratch areas at 66.7%. Two earlier surveys showed a prevalence of 45% and 43.5% respectively where houses had fully slatted floors (Gao et al., 2023a; Gao et al., 2023b). A study by Muralidharan et al. (2022) reported 6 out 12 flocks had experienced clinical SLD, but the individual house designs were not declared. The objective of the present study was to provide descriptive epidemiological information on the occurrence of SLD in Australian cage-free flocks where the house incorporated a scratch area.

Some descriptive information on geographic location, bird breed, house design characteristics and some management features were presented to provide a background into the existing bird environment within the study. No attempt to analyze the association of any exposure factors with the occurrence of SLD has been made in the present report. Descriptive results include the age of occurrence of SLD, maximum mortality rates observed prior to the institution of any treatment, the duration of the mortality, the magnitude and the duration of the decline in egg production associated with an SLD outbreak and also if SLD recurred in the same flock after the initial outbreak. The outcomes following treatment with antibiotics or non-antibiotics were observed. Detection of *C. hepaticus* by qPCR on cloacal swabs and house dust were examined as possible aids to diagnosis and a recently developed serological ELISA for detection of antibody to *C. hepaticus* was compared with qPCR on paired cloacal swabs.

Conventional free-range houses, also called "flat deck" design, have a central automated nest box system (either a single tier or double tier structure) with a slatted area extending laterally and with an exposed floor area ("scratch area") at floor level extending to the walls. This scratch area may be concrete or dirt flooring. No litter material is usually used and the litter which accumulates is dried fecal material. Aviary houses have a vertical "system" composed of cage-type areas at different levels, one of which is a nest box system, others contain feed lines or are available for birds to rest/ sleep. The floors in aviary style houses are concrete and fecal material does build up here and is usually scraped out at intervals (often fortnightly).

The main treatment and control approaches used against SLD is antibiotic medication through drinking water. In Australia the antibiotics used in egg layers are chlortetracycline and amoxicillin, and this was reflected in the survey. The flocks which were selected for antibiotic treatment by attending veterinarians generally had outbreaks which occurred slightly later and were somewhat more severe in their initial mortality than flocks that were left without antibiotic treatment. Antibiotic treatment however was associated with flocks subsequently having a shorter duration of mortality, a lower maximum egg production loss and a shorter time before the flock returned to standard egg production levels.

Table 8. Epidemiological sensitivity and specificity of qPCR on house dust compared to that on cloacal swabs on a flock basis.

	Cloacal swab PCR			
House dust qPCR	No. flocks ≥ 5 cloacal swabs positive / 12[1]	No. flocks < 5 cloacal swabs positive/ 12	Total	Apparent prevalence
No. flocks qPCR ≥ 27.1 DNA copies/ mg dust[1]	9	0	9	0.375
No. flocks qPCR < 27.1 DNA copies/ mg dust	1	14	15	
Total	10	14	24	
Apparent prevalence	0.417			

Sensitivity (ability to detect cloacal swab PCR positive flocks) = 90%
Specificity (ability to detect cloacal swab PCR negative flocks) = 100%
Predictive value of a positive test = 100%
Predictive value of a negative test = 93.3%

[1]Positive cut-off values selected as the lower quartile value of PCR results for SLD case flocks.

Table 9. Sequential study of detection of positive serology, cloacal swab detection and house dust detection by age and SLD outbreak status.

	Age:	15−18 wk	21−22 wk	27−29 wk	32 wk	36−39 wk
	No. flocks tested	7	5	9	4	7
ELISA[1]	% flocks positive	57.1	60	44.4	75	71.4
Cloacal swabs[2]	% flocks positive	0	40	33.3	50	57.4
House dust[3]	No. flocks positive	1	4	4	4	7
SLD status[4]	No. flocks with SLD	0	0	0	1	7

[1]*C. hepaticus* antibody detection ELISA, positive cutoff 0.224 optical density
[2]qPCR for *C. hepaticus* detection from 12 cloacal swabs per flock
[3]qPCR for *C. hepaticus* detection from composite house dust sample
[4]Flock's previous experience of clinical SLD

Hence the use of antibiotics appeared to have a beneficial effect on the course of the disease. Historically, Winterfield et al. (1958) found that chlortetracycline was protective against the unidentified agent of avian hepatitis when inoculated into chicken embryos. Courtice et al. (2018) noted that egg production is usually restored to standard production levels after treatment. The findings from the present survey show agreement with these publications.

The use of non-antibiotic treatment was often in association with antibiotic treatment, so the separation of any effect is difficult. However, when compared to no treatment there did not appear to be a perceptible difference in mortality or egg production effects provided by these non-antibiotic products. There are few publications concerning non-antibiotic therapy of SLD. Quinteros et al. (2021) reported that administration of an isoquinoline alkaloid could provide some protection

Table 10. Epidemiological sensitivity and specificity of paired ELISA serological test for *C. hepaticus* compared with PCR of cloacal swabs.

	Cloacal swab PCR result			
ELISA test result	No. birds POSITIVE	No. birds NEGATIVE	Total	Apparent prevalence
No. birds POSITIVE	42	13	55	0.286
No. birds NEGATIVE	21	116	137	
Total	63	129	192	
Apparent prevalence	0.388			

Sensitivity (ability to detect PCR positive birds) = 66.7%
Specificity (ability to detect PCR negative birds) = 89.9%
Predictive value of a positive test = 76.4%
Predictive value of a negative test = 84.7%
Tests of agreement:
Observed proportion agreement $(\mathbf{p_o})$ =0.823
Chance proportion agreement (both positive) =0.111
Chance proportion agreement (both negative) =0.440
Chance proportion agreement $(\mathbf{p_e})$ =0.551
Observed minus chance agreement $(\mathbf{p_o - p_e})$ =0.272
Maximum possible agreement beyond chance level $(\mathbf{1 - p_e})$ =0.449
Kappa[1] $= (\mathbf{p_o - p_e})/(\mathbf{1 - p_e}) = \mathbf{0.606}$

[1]Cohen's Kappa statistic = Quotient of (Observed − chance agreement)/(maximum possible agreement beyond chance.

Figure 2. Typical posture of a hen in early or mild stages of *C. hepaticus* infection. The hen moves to lesser populated areas of the house and shows signs of depression, particularly drooping of the tail.

against experimental SLD. This class of compound was not, however, used by any of the participants in the present survey.

Recurrence of clinical SLD in flocks was relatively common (10 out of 32 flocks with SLD experienced a recurrent outbreak). These tended to be flocks that had been treated with antibiotics, but this group also experienced the more severe initial outbreak levels. The reasons that afford recurrence of clinical disease in a previously seriously affected flock have yet to be elucidated. This may however indicate that the known and unknown risk factors which precipitated the initial outbreak were still operating within these flocks which experienced recurrent outbreaks.

It is evident that *C. hepaticus* may be present and circulating in a flock of hens well before an outbreak of SLD occurs and may be present in flocks that never show a disease outbreak. This has also been observed by Phung et al. (2020) and Muralidharan et al. (2022). This was shown first by detection of positive *C. hepaticus* antibody serology, some weeks before cloacal swabs began to expose its presence, within the small 12 sample size employed in this study. However, detection of the organism's presence occurs much earlier in composite house dust samples in many cases. Physiological changes at this developmental stage of the hen may be associated with the onset of detectability of *C. hepaticus*. Stresses instigated by transfer of the birds to the laying facility, onset of lay and the suppression of the hen's cell mediated immunity which is known to occur at sexual maturity (Johnston et al., 2012) may be contributing factors to proliferation of the organism in the intestinal tract and increased presence in the environment.

The ELISA test is a recent development and there was interest in evaluating its usefulness as an aid to diagnosis or prediction of an outbreak. Muralidharan et al. (2022) concluded that this ELISA test would be of value in detecting mild or subclinical SLD. For this purpose, paired serum and cloacal swab samples were compared, with cloacal swabs currently being considered a standard detection method. The ELISA can detect both current and past infections while the PCR can only detect DNA during current infections. However, it has been observed that *C. hepaticus* infected birds can remain asymptomatic carriers for long periods after infection (Courtice et al., 2023). Hence on a flock level we would expect that cloacal swab PCR test to continue to detect the organism for many weeks following an outbreak and this should align with serological evidence of earlier infection. The level of agreement for the two tests was compared using calculation of Cohen's Kappa statistic (κ). The determined value of κ was 0.606 for the level of agreement between cloacal swab PCR and the serological *C. hepaticus* ELISA, which is interpreted as substantial (κ >0.6), but not strong (where κ would exceed 0.7), agreement (Statology, 2021).

In an epidemiological sense, the sensitivity calculated in Table 7 describes the ability of the ELISA test to detect birds that also have positive cloacal swabs by PCR, while the epidemiological specificity describes the ELISA's ability to detect birds with a negative cloacal swab (Martin et al., 1987). The predictive value of the ELISA test is defined as the proportion of cloacal swab positive birds that tested positive on the ELISA test (Martin et al., 1987). Predictive value describes the likelihood that a bird with a positive serological ELISA test would also have a positive PCR test on a cloacal swab (i.e., that it has the infection). From this sample the predictive value of a positive test is 76.4%. Predictive value is affected by prevalence of the disease (in the sample of birds with paired samples tested here the apparent prevalence which in this sample was 0.286 (Table 7). The predictive value would be higher in a flock with higher cloacal swab positive prevalence. Given that the serological and PCR tests showed a substantial level of agreement, and the predictive value of a positive serological test was 76.4%, the serological test can be considered useful in determining the level of birds with fecal *C. hepaticus* shedding. The ELISA may also provide an earlier detection of exposure of the flock to *C. hepaticus*.

The most cost effective and simple test studied however was composite house dust submitted for a single qPCR for *C. hepaticus*. Positive results were obtained very early in the adult life of the flock. It is hoped that the quantitative measure of DNA copies of *C. hepaticus* per mg dust may provide some degree of prediction of a subsequent outbreak but the benefit of this needs to be much further researched.

CONCLUSIONS

The survey estimated the incidence of SLD (*C. hepaticus*) outbreaks in brown egg layer flocks housed in cage-free facilities with a scratch area in Australia to be 66.7% of flocks.

Detection of *C. hepaticus* was possible by qPCR of cloacal swabs or composite house dust samples in flocks prior to an outbreak occurring and was detectable in some flocks which did not experience a subsequent outbreak of SLD.

Antibiotic treatment was used in outbreaks that appeared more severe in terms of initial mortality but such treatment decreased the duration of mortality and the extent and duration of the associated decrease in egg production compared to flocks with milder initial outbreaks which were left untreated.

Recurrence of a SLD outbreak occurred in 31.3% of flocks which experienced SLD, and tended to occur in those which had a more severe onset and had been treated with antibiotics.

Examining composite house dust samples by qPCR for *C. hepaticus* was an efficient method of detection of the organism in the house environment and may provide a tool for diagnosis and possibly prediction of an outbreak.

A serological ELISA for detection of antibody to *C. hepaticus* appears to be a useful tool for early detection of the exposure of the flock to the organism.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

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