Analytical Epidemiological Studies of Spotty Liver Disease in Australian Cage-free Laying Hens

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney

June 2025

This research reported in this thesis was supported by the award of an Australian Government Research Training Program (RTP) Off-set Scholarship, and the award of an Australian Eggs Limited Scholarship to the PhD Candidate.

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

During the preparation of this thesis, I used QuillBot and ChatGPT for the purpose of text enhancement. The use of both generative AI tools included spelling corrections, minor sentence restructuring, and clarity enhancement. I confirm that where text was modified by generative AI, the content was reviewed for possible errors, inaccuracies, and bias. I take full responsibility for the submitted thesis, confirm the work is my own, and have used generative AI in accordance with the University of Sydney guidelines and policies.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Yuanshuo (Karen) Gao Date: 30 June 2025

As the supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements below are correct.

Associate Professor Peter Groves Date: 30 June 2025

Author Contributions

The work contained in the body of this thesis, except otherwise acknowledged, is the result of my own investigations. In cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Chapter Three of this thesis is published as:

Gao, Y. K., M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Scratch area as an epidemiological risk factor for Spotty Liver Disease in cage-free layers in Australia. Poultry Science102:102922. doi https://doi.org/10.1016/j.psj.2023.102922

As the primary author, I developed the materials and methods under the supervision of Peter Groves, Mini Singh and Wendy Muir, as well as in consultation with the Australian Eggs Steering Committee, including the design of the questionnaires and sample collections. I was primarily responsible for organising farm visits, farmer interviews and sample collections, with the help of Mini Singh and Peter Groves during some of the visits. Mini Singh conducted the PCR tests documented in this paper. I was responsible for data organisation and analysis and the draft manuscripts, with Peter Groves providing advice on statistical analysis. All co-authors reviewed and provided suggestions and comments on the final manuscript.

Chapter Four is published as:

Gao, Y. K., M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Identification of epidemiological risk factors for spotty liver disease in cage-free layer flocks in houses with fully slatted flooring in Australia. Poultry Science 102:103139. doi https://doi.org/10.1016/j.psj.2023.103139

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and analysis and the draft manuscripts, with Peter Groves providing advice on statistical analysis. All co-authors reviewed and provided suggestions and comments on the final manuscript.

Chapter Five is published as:

Groves, P. J., **Y. K. Gao**, M. Kotiw, S. Eastwood, T. T. H. Van, R. J. Moore, and W. I. Muir. 2024. Descriptive epidemiology of spotty liver disease in Australian cage-free brown egg layer chicken flocks with a scratch area. Poultry Science 103:103941. doi https://doi.org/10.1016/j.psj.2024.103941

As a co-author, I developed the materials and methods under the supervision of Peter Groves, including designing the questionnaires and sample collections. I was primarily responsible for organising farm visits, farmer interviews, and sample collections. I conducted the PCR tests, and Sarah Eastwood and Thi Thu Hao Van carried out the ELISA tests documented in this paper. I contributed to the data organisation, analysis and manuscript write-up. All co-authors reviewed and provided suggestions and comments on the final manuscript.

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Co-supervisor: Dr Mini Singh Date: 04 October 2024

Co-supervisor: Dr Wendy Muir Date: 04 October 2024

Acknowledgements

Firstly, and most importantly, I would like to thank my primary supervisor, Associate Professor Peter Groves, who is also my mentor, my boss, my friend and a father figure. If it wasn't for you at the start of the journey, there would not have been a 'Karen, the chook vet' (and yes, I definitely would have gone down the vet surgeon path). If it wasn't for you throughout the journey, there would not have been a young poultry vet fascinated by poultry medicine and aspired to be a 'great poultry vet' like you. And now, finally, thanks to you, 'Karen, the chook vet' will soon complete a major life and career milestone! Once again, forever grateful for believing in me and supporting me unconditionally over the last ten years and for the many years to come!

I would like to thank Dr Mini Singh, my co-supervisor, for her great company, friendship and guidance through my PhD candidature, especially during the hours and hours we spent together cracking eggs (literally)! I would like to thank Dr Wendy Muir, my co-supervisor, for your kindness, guidance and friendship over the last few years, I know I can always count on you for a nice and supportive chat.

My *Salmonella* twin, Christine Clark, thank you for the countless days we spent together at Uni and outside Uni, usually up to no good. Together with Dan, I know you've always got me covered, and the same to you. All the best to your PhD, we'll get it done.

A big thank-you to my Birling family, especially Sue Sharpe, Sarah Williamson, and Taha Harris. You taught me everything I know, from microbiology to PCR to serology, and to look out for the 'spiders'. I would also like to thank the PRF family for your company over the years. And a thank you to Ruth Zadoks for keep pushing me to write!

I would like to acknowledge and thank Australian Eggs Limited for funding my projects and my scholarship, which allowed me to take a pause in my career and devote my time and attention to something I've always wanted to accomplish. I would like to thank the Australian Eggs Steering Committee, for your valuable advice that helped to shape our studies and ultimately benefited the greater layer industry. I am grateful to the farmers, veterinarians, researchers and industry colleagues who made this PhD possible by kindly sharing their time and knowledge.

A big shout-out to my support network, a.k.a all my friends, who have been there when I needed them the most and always encouraging (mostly within reasonable limits).

I would like to dedicate this thesis to my family. As always, I could not have made it thus far without Mom and Dad, and Tails. Thank you for the relentless confidence, support and patience you had in me.

Thesis Abstract

Spotty liver disease (SLD) is an infectious disease mainly reported in free-range laying hens, causing significant mortality and production losses. Since the isolation and identification of *Campylobacter hepaticus* and *C. bilis,* the causative agents of Spotty liver disease (SLD), significant advancement has been made in characterising the pathogen, developing vaccines, and understanding the transmission and spread of the disease. Yet, an in-depth understanding of SLD epidemiology is still lacking.

It has been found in several field situations that the presence of *C. hepaticus* in the laying hens was not always associated with the clinical cases of SLD. It has thus been hypothesised that there are potential risk or protective factor(s) associated with SLD. The overarching aim of a series of descriptive and analytical epidemiological studies in this thesis is to identify key determinants of the disease, i.e., risk factors that can be modified by management procedures, and ultimately provide guidance on the prevention and/or control of SLD in cage-free laying hens.

A preliminary cross-sectional survey was conducted in Australian cage-free laying flocks, as a scoping study, using an extensive questionnaire designed around the collective hypotheses of the author, the Australian veterinary community (**Chapter 2**), and available literature. This was followed by two retrospective case-control studies using a more focused questionnaire to examine various factors in sheds with fully slatted flooring and those with scratch areas separately.

In the preliminary cross-sectional (**Chapter 3**) in cage-free laying flocks (n=24) across different states of Australia, it was found that the presence of a scratch area inside the poultry house is a risk factor for Spotty Liver Disease ($P = 0.003$). Effectively, scratch areas significantly increase the animal's exposure to faecal matters contaminated with *C. hepaticus*, subsequently clinical SLD.

Notably, despite the absence of a scratch area, almost half of the laying flocks in fully slatted houses (barn and free-range) in the preliminary study developed clinical SLD. This highlighted that other putative risk factors could precipitate SLD occurrence, even when the faecal-oral transmission of the causative agents is greatly reduced in the poultry houses. The

second survey searched for further risk factors that may exist in houses with a floor fully covered by slats (n=49) (**Chapter 4**). The study indicated that layer houses with naturally ventilated environmental control systems are a key determinant of the risk for SLD, most likely the higher chance of laying hens experiencing heat stress in such sheds ($P = 0.002$). Further analysis showed that a higher nest density (birds per m^2 nest space) has a stronger risk of causing SLD ($P = 0.015$), it was thought that higher nest density may produce more stress on the birds as the flock approaches peak lay, and this could be a trigger for the emergence of clinical SLD.

A third field study was conducted to study putative risk factors for SLD in flocks (n=48) from sheds with a scratch area (**Chapters 5 and 6**), which subsequently confirmed the findings from earlier fully slatted survey. The absence of cool cells, i.e. less efficient shed cooling, was thought to be a statistically important risk factor for SLD ($P = 0.028$). Furthermore, though not statistically significant but approaching significance ($P = 0.08$), higher nest density was associated with higher risk of SLD in sheds with a scratch area and cool cells.

To the author's knowledge, this thesis is the first to report on risk factors, more importantly key determinants, of SLD based on a series of descriptive and analytical epidemiological studies. It was shown that, in the presence of the causative agent *C. hepaticus* (and most likely *C. bilis*), poultry houses with a scratch area are most likely to lead to SLD, followed by houses with natural ventilated systems, and then with high nest density.

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List of Publications and Presentations

	Published manuscripts
2024	Groves, P. J., **Y. K. Gao**, M. Kotiw, S. Eastwood, T. T. H. Van, R. J. Moore, and W. I. Muir. 2024. Descriptive epidemiology of spotty liver disease in Australian cage-free brown egg layer chicken flocks with a scratch area. Poultry Science 103:103941. doi https://doi.org/10.1016/j.psj.2024.103941
2023	**Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Scratch area as an epidemiological risk factor for Spotty Liver Disease in cage-free layers in Australia. Poultry Science102:102922. doi https://doi.org/10.1016/j.psj.2023.102922 **Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Identification of epidemiological risk factors for spotty liver disease in cage-free layer flocks in houses with fully slatted flooring in Australia. Poultry Science 102:103139. doi https://doi.org/10.1016/j.psj.2023.103139

Conference and oral presentations	
2024	**Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2024. Risk factors of Spotty Liver Disease, Australian Eggs Webinar, 5th March 2024
2023	**Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Spotty Liver Disease risk factors in sheds with scratch areas, Australasian Veterinary Poultry Association Scientific Meeting. Queenstown, 30th and 31st October 2023 **Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Risk factors of Spotty Liver Disease, Gen Egg, 5th September 2023 **Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2023. Spotty Liver Disease and risk factors, Australasian Veterinary Poultry Association Scientific Meeting. Sydney, 8th and 9th February 2023
2022	**Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2022. Spotty Liver Disease risk factors update, Australian Eggs Webinar, 11th July 2022 **Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2022. Spotty Liver Disease risk factors – starting from scratch, Poultry Information Exchange (PIX), Gold Coast, 15th to 17th May 2022
2021	**Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2021. Analytical Epidemiological study of Spotty Liver Disease, Australian Eggs Webinar, 7th July 2021 **Gao, Y. K.**, M. Singh, W. I. Muir, M. Kotiw, and P. J. Groves. 2021. Spotty Liver Disease and risk factors, Australasian Veterinary Poultry Association Scientific Meeting. Virtual meeting, 10th and 11th February 2021
2020	**Gao, Y. K.**, M. Singh, W. I. Muir, J. Toribio, and P. J. Groves. 2020. Analytical Epidemiological study of Spotty Liver Disease, 63rd American Association of Avian Pathologists Meeting, Virtual meeting, 30th July to 6th August 2020 **Gao, Y. K.**, M. Singh, W. I. Muir, J. Toribio, and P. J. Groves. 2020. Spotty Liver Disease Analytical Epidemiology, Australian Eggs Workshop, 10th June 2020 **Gao, Y. K.**, M. Singh, W. I. Muir, and P. J. Groves. 2020. Epidemiology of Spotty Liver Disease, Australasian Veterinary Poultry Association Scientific Meeting. Virtual meeting, 20th and 21st May 2020

List of Tables and Figures

List of Abbreviations

°C	degree Celsius
APVMA	Australian Pesticides and Veterinary Medicines Authority
AUD	Australian Dollar
AVH	Avian Vibrionic Hepatitis
bp	base pair
CTC	chlortetracycline
DAFF	Department of Agriculture, Fisheries and Forestry
DEFRA	Department for Environment Food & Rural Affairs
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EU	European Union
GK	glycerol kinase
H&E	Haematoxylin and Eosin
HD	hen day
HRM	high resolution melt
IQA	isoquinoline alkaloid
LAMP	Loop-Mediated Isothermal Amplification
m^2	square metre
MCFAs	medium-chain fatty acids
nov.	novel
NSW	New South Wales
PCR	polymerase chain reaction
QLD	Queensland
qPCR	quantitative polymerase chain reaction
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal ribonucleic acid
SA	South Australia
S&G	Standards and Guideline
SLD	Spotty Liver Disease
sp.	species

SPF	specific pathogen free
spp.	plural of species
TPE	total protein extract
UK	United Kingdom
μm	micrometre or micron
USA	United States
VBNC	viable but nonculturable
VOS	veterinarian opinion survey
VFL	Vitox, FBP and L-cysteine
VIC	Victoria
WA	Western Australia
WGS	whole genome sequencing

CHAPTER ONE: Literature Review

1.1 Overview of layer production systems globally

The production of commercial eggs has increased substantially over the past few decades, which was driven by food demand and provided an economical and dependable source of animal protein (McMullin, 2022). In 1990, the global layer industry produced 35 million tonnes of eggs, which increased by more than 100% to 87 million metric tons by 2022 (Shahbandeh, 2024). The Australian laying industry saw a similar growth pattern, where the Australian laying hen population increased from 12 million birds in 2000 to 22 million in 2023 (Australian Eggs Limited, 2023).

There are two main housing systems for laying hens: cage and cage-free, the former can be further categorised into conventional and enriched cages, whereas the latter encompasses barn and free-range systems (Scrinis*, et al.*, 2017). The enriched, furnished or colony cages when compared to the conventional cages, allow more space per hen and incorporate furnishing and enrichment to facilitate expression of natural behaviours of chickens. Barn systems are usually large flocks of hens housed in sheds and not allowed to range, whereas free-range systems provide hens with the choice between indoor and outdoor areas (Campbell*, et al.*, 2017; Scrinis*, et al.*, 2017).

Commonly, barn houses are either built as a single-level floor system, “a conventional barn”, or a multi-tier housing system, called an “aviary barn” (Singh and Groves, 2021). Aviary systems, irrespective of configurations, essentially consist of different levels of infrastructures, including perches, nests, drinkers and feeders, over a floored area (Frohlich*, et al.*, 2012). The advantages of aviary systems include the spatial separation of the essential husbandry and behavioural needs of the birds and allowing for the use of a third dimension to house additional birds per square metre of the floor space (Aerni*, et al.*, 2005). Free-range

housing systems also consist of an indoor "barn" component (except that of pasture-based systems), which is fitted with openings on the side(s) called "pop-holes" and allows birds to freely roam a range during daylight hours (Singh and Groves, 2021). Effectively, the common types of barn-based free-range operations could be classified as conventional (barn) free-range or aviary free-range.

Historically, housing systems for laying hens have undergone numerous changes and developments, in response to market and/or regulatory changes primarily driven by animal welfare improvement (Scrinis*, et al.*, 2017). The layer industry shifted from predominantly cage-free systems (free-range and barn) to cage systems in the 1970s and 1980s for more efficient production and improved health in response to the fast-growing demand for eggs (Australian Eggs Limited, 2024). It was largely achieved by the mechanisation of animal feeding and drinking, as well as egg collection and packing, and better control of diseases by minimising faecal-oral transmission (McMullin, 2022). This was reflected in the exponential growth in the proportion of cage systems in the United Kingdom, which increased from 19 per cent in the early 1960s to 93 per cent by the late 1970s, and a similar trend was observed in Australia (McMullin, 2022).

Several decades later, animal welfare in the intensive livestock industry has become a prominent social issue globally, including the call to end conventional cage systems for laying hens in most developed nations, including Australia (Campbell*, et al.*, 2017; Caputo, 2023; Hemsworth, 2021; Scrinis*, et al.*, 2017). The major drive was the public's perception that more space and freedom from enclosures means better welfare for the hens, especially in terms of their ability to express 'natural behaviours' (Pettersson*, et al.*, 2016). This led to the return of cage-free production systems in response to consumer concerns about the cage

system and the need for more ethical farming systems (Caputo, 2023; DAFF, 2022; EFSA, 2023). In this movement, both regulatory and market influences have been involved in the shaping of layer welfare policies and market developments. Regulation of animal farming tends to define the minimum standards of animal welfare, whereas some markets e.g. supermarkets, food service and retail corporations, are in a position to shape public expectations and demands, and to further define the parameters of animal welfare (Scrinis*, et al.*, 2017).

In 1999, the European Union (EU) enacted a directive to phase out battery (conventional) cages by 2012, while still allowing the use of 'enriched' or 'furnished' cages and alternative systems such as barns or free-range systems (Appleby, 2003). Enriched cages require the provision of 750cm^2 space per animal, equipment for feeding, drinking, egg collection and manure removal, claw shortening devices, perches, nest boxes and a pecking and scratching area (EFSA, 2023). In June 2021, the European Commission made a historic landmark decision to end the use of caged systems for several farm animals by the year 2027, as part of its Farm to Fork Strategy, including laying hens (EFSA, 2021).

In Australia, historically the welfare of layer hens had been governed by the largely non-mandatory *Model Codes of Practice for the Welfare of Animals: Domestic Poultry*, which was not legislated nor did it carry penalties for breaches (Scrinis*, et al.*, 2017). The *Model Codes of Practice* had allowed for the use of conventional cage housing systems and included minimum standards for such (Primary Industries Standing Committee, 2002). It was not until 2023, when the *Australian Animal Welfare Standards and Guidelines (S&G) for Poultry* replaced the *Model Codes of Practice*, that the first regulatory ban on conventional cages was established. According to the *Australian Animal Welfare Standards and Guidelines (S&G)*

77 *for Poultry*, conventional cages are to be phased out by 2036 while allowing the continuing
78 use of enriched cages (DAFF, 2022). Despite the absence of a government ban on
79 conventional cages, major Australian supermarket chains have been driving the expansion of
80 free-range systems for the past two decades, to associate their brands with higher animal
81 welfare standards, mirroring the strategies of overseas supermarkets and retailers (Scrinis, *et*
82 *al.*, 2017).
83
84 As a result, since the early 2000s, cage-free systems have gradually expanded in the layer
85 industry, particularly in the EU and Australia (Dikmen, *et al.*, 2016). In 2005, cage-free eggs
86 accounted for 24.8 per cent of Australia's retail sales by volume and by 2023, this figure
87 nearly tripled to 73.7 per cent (Australian Eggs Limited, 2005; Australian Eggs Limited,
88 2023). A similar trend was observed in the United Kingdom, where cage-free systems
89 accounted for 28 per cent of the nation's layer chicken production in 2000 and reached 76.5
90 per cent by 2023. (DEFRA, 2024; McMullin, 2022).

Table 1 Current percentage of different housing systems in laying hens between the EU, UK, Australia and the USA

	EU 2024[1] (hen housed)	**UK 2023 Q2[2] (egg throughput)**	**Australia 2023[3] (sales by volume)**	**USA 2023[4] (egg volume)**
Conventional Cage	0%	0%	26.3%	62%
Furnished Cage	39.2%	23.5%		
Barn	38.8%	11.2%	15.2%	38% (including barn, free-range and specialty eggs)
Free-range	15.2%	61.3%	56.5%	
Specialty eggs e.g. organic	6.8%	3.9%	2.0%	

[1] European Commission (June 2024) Eggs Market Situation Dashboard https://agriculture.ec.europa.eu/farming/animal-products/eggs_en

[2] Department for Environment Food & Rural Affairs (May 2024) https://www.gov.uk/government/statistics/historical-statistic-notices-on-uk-egg-production-and-prices-2023/united-kingdom-egg-statistics-quarter-2-2023#uk-egg-packing-volumes-methods-of-production-uk-country-breakdowns-and-price-statistics

[3] Australian Eggs (August 2023) https://www.australianeggs.org.au/who-we-are/annual-reports

[4] U.S. Department of Agriculture (October 2023) https://www.ers.usda.gov/data-products/chart-gallery/gallery/chart-detail/?chartId=107564

Expectedly, the ban on conventional cages and the transition to cage-free systems in laying hens were met with challenges and barriers along the way, especially on the potential impact on bird health, sustainability, food affordability, production efficiency, and environmental impact relative to cage-free production (Caputo, 2023).

1.2 The (re-)emergence of "Spotty Liver Disease"

1.2.1 Spotty Liver Syndrome or Spotty Liver Disease

Starting from the 1980s, a distinct clinical syndrome called 'summer hepatitis' or 'Spotty Liver Disease (SLD)' emerged in both the UK and Australia, following the increase in the free-range demands in both countries (Jenner, 2001; Swarbrick, 2003).. This condition causes acute mortality and production drops in affected flocks, predominantly in free-range lay hens, and it was named after its characteristic hepatic lesions, white-greyish spots (Crawshaw and Young, 2003; Grimes and Reece, 2011). SLD is one of the most significant infectious diseases in the Australian commercial layer flocks, directly impacting bird welfare, productivity, and require the use of therapeutic antibiotics (Courtice*, et al.*, 2018b; Noormohammadi, 2021). The aetiological agent was isolated and first identified as a novel *Campylobacter species* in 2015 by Crawshaw*, et al.* (2015), and further characterised and subsequently named *Campylobacter hepaticus* in 2016 by Van*, et al.* (2016). Following the advancement in *C. hepaticus* detection methods using microbiological and molecular techniques, SLD has been reported in other regions worldwide, including the United States, Jordan, Germany, New Zealand and Costa Rica (Becerra*, et al.*, 2023; Crawshaw*, et al.*, 2021; Gunther*, et al.*, 2023; Hananeh, 2020; Quesada-Vasquez*, et al.*, 2023). Interestingly, this clinical condition closely resembled Avian Vibrionic Hepatitis (AVH), reported worldwide in the 1950-60s, in Europe, America, Australia, and New Zealand, and disappeared shortly after (Delaplane*, et al.*, 1955; Pohl*, et al.*, 1969; Tudor, 1954).

1.2.2 Avian Vibrionic Hepatitis (AVH)

AVH was first described in the literature by Tudor (1954), who reported an increase in cases of liver degeneration of unknown origin in laying hens in the USA. The most striking feature of the disease was the gross liver pathology, 'where livers were mottled and injected, with some presenting with diffuse stellate white flecks or small necrotic foci'. According to Tudor (1954), the condition can be traced back in the USA as early as 1933, which was described as 'red flecks in the liver' by the clinicians at the time. Peckham (1972) suggested that AVH was also present in the UK in the 1930s, after identifying a paper that described similar gross and microscopic liver pathologies in chickens after an experimental transmission of Marek's disease.

Although suspected to be a bacterial causative agent, researchers failed to reliably isolate an agent from aerobic and anaerobic cultures using various media. Delaplane*, et al.* (1955) described a similar condition and was able to isolate a bacterial agent, which was found to be susceptible to streptomycin in chicken embryo assays. Hofstad*, et al.* (1958) was able to isolate an agent, termed a 'vibrio organism', found to be lethal for 7-day-old chicken embryos. Similar liver pathology can be reproduced in chickens by parenteral inoculation, and re-isolation of the agent in the livers of inoculated birds was achieved. After repeated isolation of a 'vibrio' agent in livers and bile of birds affected by hepatitis with the characteristic liver lesions, Peckham (1958) proposed the name 'avian vibrionic hepatitis' to distinguish it from other hepatitises. Clinical reports of AVH peaked in the early 1960s then declined, and virtually disappeared by the early 1980s, and as a result, the organism was never fully characterised (Crawshaw, 2019). The disappearance of AVH coincided with the industrialisation of the layer hen industry, marked by the global expansion of large-scale cage

systems, which, in turn, reduced or eliminated faecal-oral transmission and exposure to environmental sources of infection for diseases (Crawshaw, 2019; McMullin, 2022; Scrinis, *et al.,* 2017).

1.3. Spotty Liver Disease (SLD)

1.3.1 Clinical presentation

SLD is predominately reported in cage-free (barn or free-range) hens, including commercial laying and broiler breeder hens (Courtice*, et al.*, 2018b). Based on field observations, faecal-oral transmission is considered the primary transmission route. This is supported by the induction of disease in oral challenge models and the detection of *C. hepaticus* throughout the intestinal tract of infected birds (Courtice, *et al.,* 2023; Van, *et al.,* 2017). There have been rare reports of SLD outbreaks in caged birds; in these situations, soiling of the cage system was also reported, where birds can gain access to faecal matter (Gregory, *et al.,* 2018).

Affected birds can be found to be depressed and pyrexic, and dead birds are generally in good condition, although vent soiling due to faeces and urates can be regularly detected (Courtice*, et al.*, 2018b). Clinically, susceptible layer flocks may experience a drop in production ranging from 10 to 35% and a mortality increase of 10 to 15% (Courtice and Jenner, 2022). Based on a cost model presented by Courtice and Jenner (2022), in a flock of laying hens experiencing a typical SLD outbreak with a mortality of 6% and production drop of 7.2% at peak lay, the net cost of lost eggs, dead birds (and their associated eggs), cost of treatment and savings on feed could be calculated to a loss of up to AUD 4.29 per hen, which can be substantial at a commercial scale.

Experimentally, it has been reported that SLD can cause a transient reduction in the average egg mass for approximately one week but has no impact on yolk colour (Quinteros*, et al.*, 2021; Scott, 2021). Although clinical mortalities of SLD are often found to be in good condition, one study by Scott (2021) had found laying hens challenged by *C. hepaticus* experience a drop in body weight, together with a temporary depression in feed conversion efficiency.

The median age of onset for SLD is 28 weeks, ranging from 22 to 80 weeks (Scott, 2016). This is consistent with the observations that laying hens, regardless of age, are susceptible to the disease during initial outbreaks; nevertheless, subsequent outbreaks in replacement flocks typically occur in young hens around peak laying periods (Courtice*, et al.*, 2023).

Field reports have occasionally documented recurrent outbreaks of SLD within the same flock, a pattern consistent with the author's own observations in Australian free-range layer flocks (Courtice*, et al.*, 2018a; Gunther*, et al.*, 2023). One proposed hypothesis is that affected flocks may develop poor or partial protective immunity following an acute *C. hepaticus* infection, allowing the bacteria to (re-)colonise susceptible birds in the flock (Gunther*, et al.*, 2023). In some incidences, recurrent outbreaks have occurred despite prior antibiotic treatment, raising concerns about treatment failure or the emergence of antimicrobial resistance (Courtice, *et al.,* 2018; Crawshaw, 2019). Farms with a history of SLD appear to be at increased risk of future outbreaks, as *C. hepaticus* can persist and colonise incoming replacement flocks (Courtice*, et al.*, 2018a; Gunther*, et al.*, 2023). In a four-year study conducted across seven Australian free-range farms, Courtice *et al.* (2023) did not observe recurrent outbreaks within the same flock, but confirmed the presence of

asymptomatic carriers up to 51 weeks post-outbreak, a finding also supported by Gunther *et al.* (2023).

To date, the elimination of *C. hepaticus* from infected flocks has not been documented in scientific literature. The author speculates that this may be due to the ubiquity of the organism, its survival strategies, and the persistence of carrier states in infected hens. Theses aspects are further explored in later sections (Becerra, *et al.*, 2023; Courtice, *et al.*, 2023; Phung, *et al.*, 2020).

1.3.2 Gross and microscopic pathologies

The most common gross pathological findings of SLD are the presence of multifocal liver lesions consisting of numerous small, white-grey necrotic spots across the surface of the liver (Crawshaw*, et al.*, 2015; Grimes and Reece, 2011). The spots are sometimes seen as yellow or red and do not appear to be raised above or depressed below the liver surface level (Crawshaw et al., 2015; Scott, 2016). Excess peritoneal and pericardial fluid is often present together with fibrinous perihepatitis. At times, birds were observed to be jaundiced and had hepatosplenomegaly. Petechial haemorrhages are occasionally present in the abdominal fat as well as the heart (Crawshaw & Young, 2003; Grimes & Reece, 2011; Jenner, 2001).

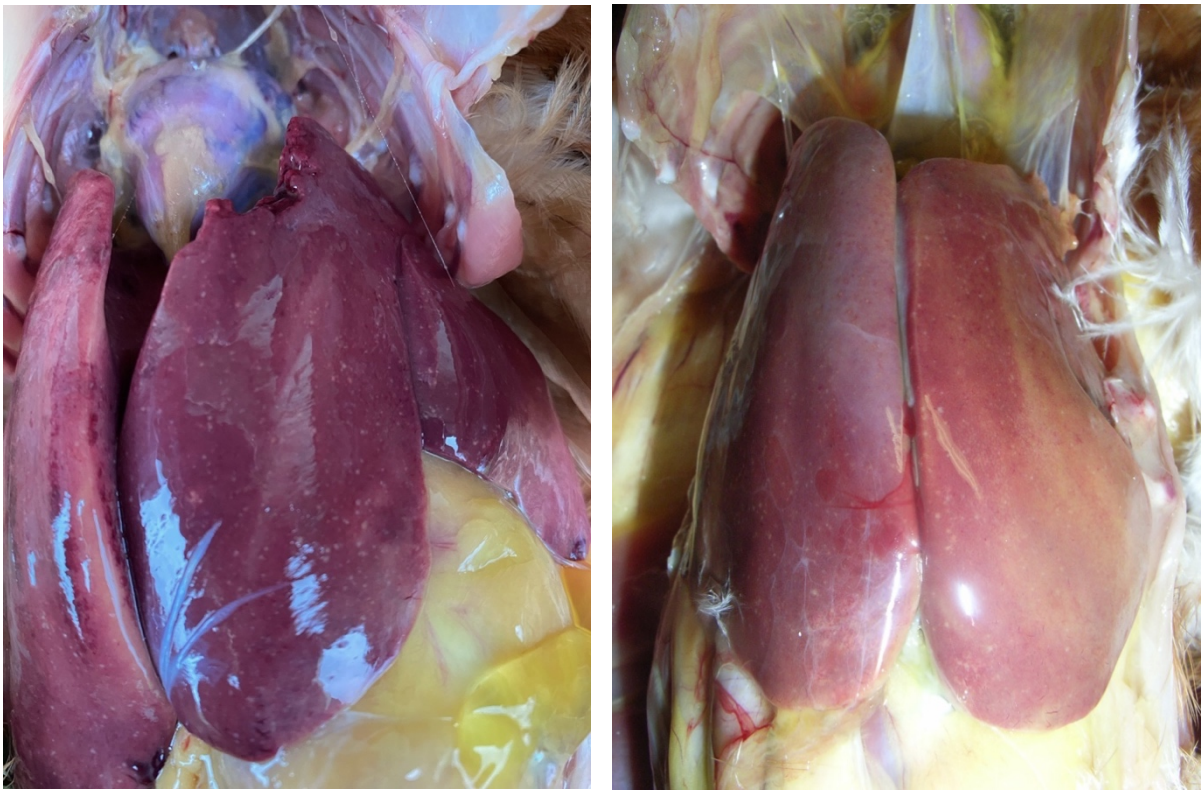


Figure 1 Classical gross pathological signs of SLD, including white greyish miliary liver lesions, with hepatomegaly and signs of jaundice and ascites. Photo courtesy of Peter Groves.

Microscopically, affected livers consistently show 'an acute, randomly distributed, focal, necrotic, hepatitis with a fibrous exudate', usually accompanied by minimal heterophil and macrophage infiltration in the necrotic foci, and with variable levels of fibrin deposition in the sinusoids or central veins (Crawshaw, 2019; Grimes and Reece, 2011; Scott, 2016). Liver lesions were not associated with specific hepatic cell types or structures but instead seem randomly located (Scott, 2016). The random foci of hepatic necrosis are the most described changes associated with acute cases of SLD and Crawshaw*, et al.* (2021) further characterised signs of more chronic changes in SLD-affected birds, affecting the biliary system. It is characterised by marked lymphoplasmacytic infiltration around ducts of varying sizes with hyperplastic epithelium (Crawshaw*, et al.*, 2021).

Crawshaw*, et al.* (2020) was the first to document the potential, direct visualisation of *C. hepaticus* colonies associated with the pathological lesions in the liver on Warthin-Starry

staining, which was not visible on Haematoxylin and Eosin (H&E) or Gram-Twort stains. It was described as 'small numbers of spiral or gull-wing shaped bacteria' in the bile lakes of the liver, the lumina of affected bile ducts and the bile of the gallbladder lumen.

Fibrinoid necrosis and fibrin thrombi have been described in spleens and inflammation of the small and large intestine has been reported (Crawshaw, 2019; Grimes and Reece, 2011; Scott, 2016). Crawshaw*, et al.* (2021) reported that few remarkable histological changes were present in the heart, spleen, kidney and pancreas of affected hens. To date, there has been no published literature on any changes to the organs of the central nervous sytem.

1.3.3 Similarity to AVH

According to the review by Crawshaw (2019), it was identified that AVH and SLD shared similar, but not identical, disease conditions such as clinical signs and gross and microscopic pathologies, and that both conditions were highly associated with birds housed on the floor but extremely rare in caged layers. Further comparison between the biochemistry and growth characteristics of the hard-to-isolate, unnamed causative agent for AVH and *C. hepaticus*, Crawshaw (2019) was confident that *C. hepaticus* was, in fact, the causative agent for AVH prevalent globally decades ago.

1.4. Pathogen isolation and characterisation

1.4.1 *Campylobacter hepaticus*

Crawshaw*, et al.* (2015) reported on the first successful isolation of a novel thermophilic *Campylobacter* sp. in 2009 from multiple SLD-affected laying hens in the UK. This was achieved following enrichment in broth and prolonged incubation on non-selective solid

media from uncontaminated samples (Crawshaw, 2019). The novel bacterium was subsequently re-isolated from 4 weeks old specific pathogen free (SPF) chickens inoculated intraperitoneally with the novel *Campylobacter* sp., which showed microscopic pathology resembling that of SLD lesions seen in natural SLD infections (Crawshaw*, et al.*, 2015). The following year, Van*, et al.* (2016) isolated the novel *Campylobacter* sp. in Australian layer flocks, further characterised the bacterium and named it *Campylobacter hepaticus* sp. nov., after the organ it was first isolated from, the liver.

1.4.1.1 Microbiological properties

C. hepaticus is a Gram-stain-negative, curving or spiral rod with a typical S-shaped *Campylobacter* spp. morphology, 0.3 to 0.5μm wide and 1 to 3 μm long on electronic micrographs (Crawshaw*, et al.*, 2015; Van*, et al.*, 2016). The bacterial cells are predominately motile in young cultures and possess single, unipolar or bipolar, unsheathed flagella, with occasional isolates described as non-flagellated (Crawshaw, *et al.,* 2015; Gregory, *et al.,* 2018; Van, *et al.,* 2016). Cells typically grow in Preston broth or blood agar under microaerophilic conditions at 37 and 42°C (Crawshaw*, et al.*, 2015; Van*, et al.*, 2016). Growth on agar plates would typically form a thin spreading film on the agar surface or as wet, round, cream-coloured colonies, the variation in colony morphology can occur after long incubation (Van*, et al.*, 2016).

Due to the lack of highly selective media for primary isolation and the fastidious growth conditions required by *C. hepaticus*, it is most reliably isolated from chicken tissue samples where it is generally present as a monoculture, such as liver and bile from SLD-affected birds, provided that samples are collected aseptically (Crawshaw*, et al.*, 2015; Phung*, et al.*, 2022a). Isolation of *C. hepaticus* from microbially complex samples such as environmental

samples, faeces and gastrointestinal contents had been difficult, where faster-growing bacterial contaminations readily overgrow the slow-growing *C. hepaticus*. To overcome this, Phung*, et al.* (2020) used a filter penetration method and successfully recovered *C. hepaticus* from fresh faeces of SLD-affected birds. Motile organisms such as *C. hepaticus* can move through a filter membrane, where most contaminants will be trapped due to a lack of motility. Motile-filter method alone, however, could not recover viable *C. hepaticus* from PCR-positive environmental samples, leading to the speculation that *C. hepaticus* can enter a viable but nonculturable (VBNC) state, like some *Campylobacter* species (Phung*, et al.*, 2020).

VBNC is a survival mechanism that non-spore-forming bacteria possess to withstand adverse physico-chemical and ecological conditions that otherwise may be lethal to the cells, such as light, temperature, high or low salinity, change in pH, oxygen stress, and starvation (Babu*, et al.*, 2014; Ramamurthy*, et al.*, 2014). Under this mechanism, bacteria enter a state of dormancy and show low levels of fluctuating metabolic activity and reduced synthesis of nucleic acids and protein biomolecules (Sakur*, et al.*, 2024). Morphological changes, such as from rods to coccoid forms, are other characteristics of VBNC. Crawshaw*, et al.* (2015) identified that on sub-culture *C. hepaticus* was shown to be a Gram-negative coccoid, almost exclusively. This morphological change was confirmed by Van*, et al.* (2016) that *C. hepaticus* became coccoid after 12 days of incubation. Interestingly, Gregory, *et al*. (2018) found that serial subculturing of 7-day-old colonies over a 9-week period did not change the morphology of *C. hepaticus*. This reported variability in bacterial morphology may suggest that *C. hepaticus* could enter VBNC states depending on exposure to different stress conditions.

297 More importantly, once entering the VBNC state, bacteria cannot be cultured by conventional
298 laboratory methods. Theoretically, resuscitation of bacteria from VBNC status can occur
299 upon removing stress factors and providing nutrients and favourable growth conditions
300 (Babu*, et al.*, 2014; Phung*, et al.*, 2022a). Phung*, et al.* (2022a) were the first to report on the
301 successful resuscitation from VBNC state for *C. hepaticus*. The authors found that *C.*
302 *hepaticus* became nonculturable, using the normal culturing conditions, after being in the
303 water at 25°C for 3-4 days or at 4°C for 21 days. The live but unculturable cells changed cell
304 shape into a coccoid form, and a significant proportion remained intact and alive. The
305 resuscitation of *C. hepaticus* was achieved by adding a mix of supplements, including Vitox,
306 FBP, and L-cysteine (VFL), to either a Brucella broth, Bolton broth, or brain heart infusion
307 broth media base (Phung*, et al.*, 2022a).
308
309 The ability to enter a VBNC state potentially plays a significant role in the epidemiology and
310 pathogenesis of bacterial pathogens, including *C. hepaticus*. Firstly, VBNC state allows
311 pathogens to elude detection using traditional culture methods. VBNC cells generally have
312 higher physical and chemical resistance than culturable cells and can show changes in
313 adhesion and virulence properties (Li*, et al.*, 2014). Data indicate that certain bacteria,
314 including *Campylobacter jejuni*, develop VBNC cells within biofilms in the face of water-
315 related environmental stresses, resulting in heightened biocide resistance (Li*, et al.*, 2014;
316 Newell and Fearnley, 2003). Furthermore, organisms such as *C. jejuni* can be resuscitated to
317 a culturable and fully virulent form under specific conditions, such as ingestion by
318 susceptible hosts (Colwell*, et al.*, 1996).
319

320 *1.4.1.2 Molecular detection and characterisation*

321 Following the initial microbial isolation of the novel *Campylobacter* sp. isolates in the UK,
322 Crawshaw*, et al.* (2015) confirmed all isolates shared an identical 1,290 bp 16S rRNA
323 sequence, which appeared to represent a previously undescribed lineage within the
324 *Campylobacter* genus. The isolates also tested negative for *Campylobacter jejuni* and
325 *Campylobacter coli* using PCR, ruling out the common *Campylobacter* spp. affecting or
326 found in poultry (Crawshaw*, et al.*, 2015).

327

328 Van*, et al.* (2016) subsequently confirmed the new bacterium isolated from livers of SLD-
329 affected laying hens in Australia shared identical 16S rRNA gene sequences with the novel
330 *Campylobacter* sp. reported by Crawshaw*, et al.* (2015). Based on a 16S rRNA gene
331 sequence similarity of ≤ 98.54% to other *Campylobacter* species, which is lower than the
332 threshold of 98.65% for differentiating two species, Van*, et al.* (2016) agreed that this is a
333 novel species and named the bacterium *Campylobacter hepaticus*. According to Van*, et al.*
334 (2016), *C. hepaticus* has a genome size of 1,482,384 bp with a DNA G+C content of 27.9
335 mol% and its sequence is closely related to that of *C. jejuni*.

336

337 Van, *et al.* (2017) designed an end-point polymerase chain reaction (PCR) assay using
338 primers to amplify the *C. hepaticus* specific glycerol kinase gene (*GK*), which has been
339 widely used for *C. hepaticus* detection by researchers (Gregory, *et al.,* 2018; Gunther, *et al.,*
340 2023; Phung, *et al.,* 2020). Compared to the bacterial isolation of *C. hepaticus* from bile or
341 liver samples, the PCR assay was non-invasive and demonstrated higher sensitivity and
342 specificity, especially in microbially complex samples e.g. faeces and environmental samples.
343 A quantitative PCR (qPCR) was subsequently designed and optimised from the end-point
344 PCR using melt curve analysis, allowing for the enumeration of *C. hepaticus* and a faster

post-PCR confirmation of amplified DNA products (Van, *et al.*, 2017). The same primers (*GK)* had been used in the design of a multiplex PCR, with primers specific for *C. jejuni* (hipO) and for *C. coli* (glyA), for simultaneous detection and differentiation of three *Campylobacter* spp. in clinical samples and environmental samples (Van*, et al.*, 2018).

Courtice*, et al.* (2023) reported on an end-point PCR assay using a different primer set targeting *C. hepaticus* specific *GntP* permease gene, which had been used in the molecular detection of *C. hepaticus* in biological and environmental samples. There is no published study or data comparing the two primer sets available, particularly regarding sensitivity or specificity.

Table 2 Primers for *Campylobacter hepaticus* PCR detection

Gene	Primers (5' to 3')	Amplicon size (bp)	Reference
GK	F: CAGGAGTTTTACCACAATTC R: CAAGCTAAAACAGGTTTGG	463	Van, *et al.* (2017)
GntP	F: AGCTCTTCCAGGTAGTCCTCAAATTC. R: CCTGCTAATAAATTAACTGCTATACCCTC	537	Courtice*, et al.* (2023)

Ghorashi*, et al.* (2022) developed a Loop-Mediated Isothermal Amplification (LAMP) assay for *C. hepaticus* detection as an alternative to the PCR method using the *GK* primer set. The assay had a shorter result turn-around time relative to the PCR assay and did not require sophisticated laboratory equipment for interpretation. Nonetheless, it was found to have much lower sensitivity and lower specificity than the PCR test.

To date, there are only a few publicly accessible whole genome sequences of *C. hepaticus*, hence a limited number of whole genome sequencing (WGS) studies, phylogenetic studies and comparative analyses using isolates from the United Kingdom, Australia, the United States, New Zealand and Germany, as well as comparisons with other *Campylobacter*

365 species. Analyses were carried out to study the phylogeny of *C. hepaticus* and to identify
366 potential genetic components contributing to pathogenicity, such as virulence traits, survival
367 mechanisms and antimicrobial resistance (Crawshaw*, et al.*, 2020; Gunther*, et al.*, 2023;
368 Ienes-Lima*, et al.*, 2023; Petrovska*, et al.*, 2017; Van*, et al.*, 2019). The *C. hepaticus*
369 genomes were considered highly conserved, ranging from a match level of 95.19% to 99.95%
370 to the reference strain HV10, and the C. *hepaticus* clonal populations are geographically
371 confined (Ienes-Lima*, et al.*, 2023; Phung*, et al.*, 2020; Van*, et al.*, 2019). Van*, et al.* (2019)
372 identified five *C. hepaticus* phylogroups, two within Australia and three in the UK, based on
373 the analysis of 23 isolates. Subsequently, Gunther*, et al.* (2023) found the seven German
374 isolates were closely clustered with the British isolates, forming a 'European' clade and
375 distinct from the Australian clades and the U.S and New Zealand strains. Ienes-Lima*, et al.*
376 (2023) found that six American isolates are closely associated with the Australian clade and
377 Crawshaw*, et al.* (2020) reported that two sequenced isolates from New Zealand formed a
378 monophyletic clade with several Australian isolates.
379
380 Courtice*, et al.* (2023) reported on the genomic characterisation of *C. hepaticus* using
381 Restriction Fragment Length Polymorphism (RFLP) as an 'ease-of-use' alternative to WGS
382 analysis. It was based on the amplification of a single, *C. hepaticus* specific and highly
383 conserved flagellin gene (*fla*A), which was subsequently digested into DNA fragments using
384 restriction enzymes. Genotyping was achieved by analysing the different gel electrophoresis
385 blotting patterns, based on the molecular weights of the restriction fragments. Similar to the
386 aforementioned phylogenetic studies using whole genome sequencing, two different clades of
387 *C. hepaticus* were identified, from different Australian farms and within a single farm during
388 separate outbreaks.

Table 3 Primers for *Campylobacter hepaticus* RFLP genotyping

Gene	Primers (5' to 3')	Amplicon size (bp)	Reference
***fla*A**	F: GGATTTCGTATCAATACTAATGTCGC R: TTGCAATAATCTTAACACATTTTG	1,722	Courtice*, et al.* (2023)

389 *1.4.1.3 Serological detection*

390 Muralidharan*, et al.* (2020) reported on the first immunoassay for detecting *C. hepaticus*
391 specific antibodies from infected bird sera, using an enzyme-linked immunosorbent assay
392 (ELISA). To minimise the cross-reactivity to other common *Campylobacter* spp. found in
393 poultry as commensal bacteria, such as *C. jejuni* and *C. coli*, the reported method included a
394 pre-absorption step using *C. hepaticus* total protein extract (TPE) to increase its specificity to
395 *C. hepaticus* antibodies. Although highly specific (95.5%) and highly sensitive (97.6%), this
396 method was time-consuming and challenging to standardise. A second ELISA was developed
397 using a pure, recombinant fragment of *C. hepaticus* protein as the capture antigen, which is a
398 quicker test and has better quality control, with 95% specificity and 100% sensitivity when
399 tested with sera of naturally infected birds (Van*, et al.*, 2022a). *C. hepaticus* ELISA had been
400 used to study SLD epidemiology in field investigations and experimental settings and to
401 evaluate SLD vaccines in development (Scott, 2021).
402
403 1.4.2 *Campylobacter bilis*
404 In 2022, a second novel *Campylobacter sp.* was isolated from the attempted isolation of *C.*
405 *hepaticus* from bile samples of SLD-affected birds in Australia (Phung*, et al.*, 2022b; Van*, et*
406 *al.*, 2023). WGS analysis showed that the new isolates constitute a robust clade separate from
407 other *Campylobacter* species, including *C. hepaticus* (Phung*, et al.*, 2022b). Furthermore, the
408 novel isolates shared 99% similarity in 16S rRNA gene sequences with *C. hepaticus*, which
409 satisfies the cut-off of less than 99.7% similarity required to differentiate distinct bacterial
410 species within the same genus (Ienes-Lima*, et al.*, 2023; Phung*, et al.*, 2022b). The novel

411 bacterium was subsequently named *Campylobacter bilis*, reflective of the location from
412 which the bacterium was first isolated, the bile (Phung*, et al.*, 2022b). *C. bilis* was confirmed
413 as a second bacterial aetiological agent for SLD due to its capacity to induce liver lesions like
414 those caused by *C. hepaticus* and its re-isolation from experimentally challenged birds (Van*,*
415 *et al.*, 2023).
416
417 Van*, et al.* (2023) found that *C. bilis* was readily detected by the established *C. hepaticus*
418 PCR using GK primers. This meant both species have a glycerol kinase gene, sharing 90.55%
419 identity of this gene, which was absent in most *Campylobacter* species. A *C. bilis* specific
420 PCR assay was developed using a different GK primer pair, derived from divergent
421 sequences between the two species, and varying annealing temperatures.

Table 4 *C. hepaticus* and *C. bilis* PCR primers based on GK gene

Organism	Gene	Primers (5' to 3')	Amplicon size (bp)	Reference
C. hepaticus	*GK*	F: CAGGAGTTTTACCACAATTC R: CAAGCTAAAACAGGTTTGG	463	Van, *et al.* (2017)
C. bilis	*GK*	F: CGTTTTAGCTTGTATTAAATTTG R: GATTTTACCCAAATTTATCCAC	258	Van*, et al.* (2023)

422 While it was hypothesised that *C. bilis* might induce comparable clinical manifestations to *C.*
423 *hepaticus*, including production losses and mortalities, long-term studies are necessary for
424 validation and to better understand the novel bacterium's role in the pathogenesis of SLD.
425

426 1.5 Current mitigation strategies for SLD

427

428 1.5.1 Antimicrobial therapy

429 SLD responds readily to therapeutic antibiotics, and the following antimicrobials have been
430 reported in the treatment of SLD, including chlortetracycline, lincomycin and spectinomycin

combination, amoxicillin, tiamulin, neomycin-sulfate, and erythromycin (Courtice*, et al.*, 2018b; Gray*, et al.*, 2021; Gunther*, et al.*, 2023). The most common antimicrobial used to treat SLD in Australia was Chlortetracycline (CTC) at 60mg/kg body weight in drinking water for five days, whereas the second-choice treatment was lincomycin-spectinomycin in drinking water for 3-5 days (Gray*, et al.*, 2021). Both drugs of choice were preferred based on field response and nil withholding periods for table eggs (Courtice*, et al.*, 2018b), and in the author's experience, CTC was preferred over lincomycin-spectinomycin due to a much lower treatment cost. Following the registration change of an amoxicillin medication in Australia in 2019, allowing nil egg withholding, amoxicillin has also been prescribed for treating SLD recently (Gray*, et al.*, 2021); however, its response was not well-documented.

While treatment with antimicrobials has been the most effective treatment option in controlling SLD outbreaks, it cannot prevent outbreaks from occurring (Crawshaw*, et al.*, 2021). In addition, treatment failures due to incomplete dosing and/or increasing CTC resistance have been reported, leading to the recurrence of SLD post-antimicrobial treatments in some cases (Courtice*, et al.*, 2018b; Grimes and Reece, 2011). On occasions, depending on the severity of the SLD outbreak, 1 to 2 weeks of in-feed treatment may be required (Scott, 2016). On the other hand, in the cases of low background SLD mortality within standard expectations based on the history of the flock or farm, some veterinarians would elect not to use antimicrobial therapies to avoid promoting antimicrobial resistance and disrupting the microflora of the animals (Scott, 2016).

Sakur*, et al.* (2024) examined the antimicrobial susceptibility of five Australian *C. hepaticus* isolates against fourteen antimicrobial agents by using a disc diffusion methodology. Like other *Campylobacter* spp. (e.g. *Campylobacter jejuni* and *Campylobacter coli*), *C. hepaticus*

was found to show intrinsic resistance to trimethoprim, vancomycin, rifampicin, and bacitracin (Sakur*, et al.*, 2024; Wieczorek and Osek, 2013). Three out of five *C. hepaticus* isolates were found resistant to tetracycline by showing a mean annular radius of less than 6mm, whereas all 5 isolates were found to be sensitive to amoxycillin and spectinomycin (Sakur*, et al.*, 2024). Van*, et al.* (2019) demonstrated that a single antibiotic resistance gene, *tetO*, was found in plasmids in a selection of Australian and UK *C. hepaticus* isolates, and the plasmid harboured a tetracycline-resistant gene. It was speculated that other *Campylobacter* species might act as a genetic reservoir for *C. hepaticus* and vice versa, and consequently, this antibiotic resistance plasmid could potentially spread to other bacteria, which is concerning. For example, *Campylobacter jejuni* tetracycline resistance plasmid, *pTet*, has already been detected in three *C. hepaticus* isolates from separate UK layer farms (Petrovska*, et al.*, 2017). Notably, Ienes-Lima*, et al.* (2023) did not find any genes, including *tetO* gene, or plasmids associated with antimicrobial resistance in four *C. hepaticus* isolates from the United States. It was speculated this could be attributed to the lower selection pressure for antimicrobial resistance on those isolates, as they originated from an organic farm where antibiotics were not used for several years. These findings were consistent with the field observation of increasing CTC resistance reported by veterinarians due to decades-long, repeated usage to combat SLD in laying hens, and highlighted the growing necessity to identify alternative therapies for SLD (Grimes and Reece, 2011; Quinteros*, et al.*, 2021; Scott, 2021).

1.5.2 Alternatives to antimicrobials

Alternatives to antimicrobials, including various short and medium-chain fatty acids (MCFAs), phytobiotics, and probiotics, have been trialled experimentally and in the field to manage SLD, with limited success (Moore*, et al.*, 2019; Scott, 2018). It has been reported

that experimental *C. hepaticus* infection adversely affected the gut (caecal) microbiota of chickens based on a microbial diversity study and that the overall structure of the microbiota could vary depending on the pathogenicity of the *C. hepaticus* strains (Van*, et al.*, 2022b). It was hypothesised that such changes in the composition of the gut microbiota could trigger or potentially result from the induction of SLD due to *C. hepaticus*; the latter could be attributed to the activation of the host's innate immune response, such as antimicrobial peptides (Scott, 2018; Van*, et al.*, 2022b). Using feed additives to modify microbiota populations or activities towards a 'healthy' direction was thought to reduce the incidence and severity of the disease potentially (Scott, 2018).

MCFAs and some phytogenic compounds have been found to subjectively delay and/or modify the course of SLD in the field, provided they are introduced well before the onset of lay i.e. the onset of disease (Scott, 2018). They had no significant therapeutic value if they were introduced after the outbreak. In the field studies by Scott (2018), the author found that a combination of oregano and sanguinarine-based products showed efficacy in reducing SLD mortality and production losses. A similar trend was noted when using medium-chain fatty acids in conjunction with phosphorylated monosaccharides, but to a lesser degree.

Despite the favourable response reported in some field observations and field studies, Scott (2018) found no class of feed additives (MCFAs, organic acids, novel botanical products, oregano-based products, or prebiotic yeast extract) was able to demonstrate a statistically significant reduction in SLD liver lesions in the experimental model or to show any advantages or at least equivalence to antimicrobial therapy. However, in an experimental study to assess a phytogenic feed additive containing isoquinoline alkaloid (IQA) in the control of SLD, the authors found statistically significant lower number of liver lesions and a

reduction in inflammatory response in the birds of IQA treatment group, but no reduction in the number of birds positive to SLD (Quinteros*, et al.*, 2021). Based on field trials, dietary biochar was another anti-pathogenic additive that may potentially reduce *C. hepaticus* carriage (7.7%) compared to untreated challenged birds (50%) (Willson, *et al.*, 2019). Although the exact mechanism was unknown, similarly, it was speculated that biochar could modulate the intestinal microbiota of laying hens.

1.5.3 Vaccination

Since the isolation of the bacterium, the development of vaccine strategies against *C. hepaticus* has been a prioritised focus of Australian researchers and the layer industry. Scott*, et al.* (2019) reported on the first case of vaccination against SLD, in a flock of 20,000 commercial pullets. The authors trialled an off-label application of an inactivated vaccine, which was commercially available and registered for use in sheep to control reproduction losses caused by *Campylobacter jejuni* and *Campylobacter fetus fetus*. It was hypothesised that due to the genetic similarities between *C. jejuni* and *C. hepaticus*, the vaccine might provide immunological protection to the laying hens in case of SLD challenge. The vaccine used was found to be safe for administration in commercial pullets at 8 and 11 weeks of age. However, the vaccine was expensive and failed to protect against a naturally occurring SLD outbreak, where both the control and treatment groups experienced similar production and mortality losses.

Courtice (2020) reported on a trial of three experimental *Campylobacter hepaticus* vaccines, where nine layer pullets were immunised in each vaccine group at 16 and 18 weeks of age and orally challenged with *C. hepaticus* isolates at 21 weeks of age. Vaccine efficacy was assessed based on post-mortem liver lesion scores, histopathology, *C. hepaticus* bacterial

isolation and PCR detections in various organ tissues. The majority of vaccinated birds had normal gross and histological findings in liver samples. In contrast, most birds in the positive control group showed gross and histological changes consistent with SLD, which was encouraging and warranted further research.

Killed autogenous *C. hepaticus* vaccines have been tested in experimental (challenge model) and field trials (natural challenge) and were proven safe to be used in chickens, with variable and sometimes promising protection results (Courtice and Jenner, 2022; Scott, 2021). Scott (2021) reported on a series of experimental and field trials using a killed autogenous *C. hepaticus* vaccine and studied its effects on gross and histological findings, serology and production parameters (bird weight, egg production, egg weight, feed intake and feed efficiency). Overall, a moderate reduction in liver lesions of vaccinated birds and an improvement in certain production parameters were reported, which were promising, but inconsistent throughout experimental and field trials. Courtice and Jenner (2022) documented a different killed autogenous *C. hepaticus* applied in field trials, which did not prevent the occurrence of SLD, but led to reduced mortality and egg production loss compared to unvaccinated birds.

Yet, to date, no experimental or commercial vaccines have been proven to be fully protective against SLD in laying hens, especially in preventing mortality, production losses and the need for antimicrobial therapies.

1.6. Aims and hypothesis

1.6.1 Epidemiological studies of SLD

Epidemiology is the study of the occurrence and distribution of health-related events, states, and processes in specified populations, including the study of the determinants influencing such processes, and the application of this knowledge to control relevant health problems. Four major approaches are recognised in epidemiological investigations: descriptive, analytical, experimental, and theoretical epidemiology (Thrusfield and Christley, 2018).

Descriptive epidemiology, typically the first step of an investigation, aims to characterise the occurrence and distribution of disease in terms of incidence and prevalence across host, pathogen and environmental factors in space and time; essentially the "who, what, when and where" of disease. Analytical epidemiology, by contrast, seeks to understand the "why and how" of disease by identifying and quantifying factors that influence disease risk, often using statistical and diagnostic procedures. Experimental epidemiology involves the use of experimental models, where researchers can manipulate variables under controlled environment, to test hypotheses derived from observational studies. Lastly, theoretical epidemiology uses mathematical modelling to stimulate disease dynamics and predict patterns of occurrence (Martin*, et al.*, 1987; Thrusfield and Christley, 2018).

Over the past two decades, although somewhat limited, clinicians and researchers have attempted to describe the epidemiology of SLD, largely through descriptive observations (Becerra*, et al.*, 2023; Courtice*, et al.*, 2018a; Crawshaw, 2019; Grimes and Reece, 2011; Jenner, 2001; Phung*, et al.*, 2020; Scott, 2016). SLD has been primarily reported in laying hens around peak lay, and is rarely observed in male birds or during the rearing phase of

laying hens (Grimes and Reece, 2011). The primary route of infection for SLD is believed to occur via faecal-oral transmission of *C. hepaticus* or *C. bilis* in susceptible birds, predominately affecting cage-free systems such as barn and free-range flocks (Van*, et al.*, 2023; Van*, et al.*, 2016).

Since the isolation and identification of *C. hepaticus* (Van, *et al.*, 2016), much of the research focus has been on bacterial characterisation and molecular epidemiology of SLD (Petrovska, *et al.*, 2017; Phung, *et al.,* 2020; Van, *et al.*, 2019). While culturing *C. hepaticus* is challenging, viable organism have been successfully isolated from the faeces of infected birds (Phung*, et al.*, 2020). In addition, PCR-based detection of *C. hepaticus* DNA in environmental samples, including wild bird faeces, rat faeces, flies, red mites, poultry house dust, soil and stagnant water in the range, suggests a wide range of potential reservoirs and environmental sources for the faecal-oral transmission (Becerra*, et al.*, 2023; Courtice*, et al.*, 2023; Phung*, et al.*, 2020). Comparative genomic analyses have also revealed geographic clustering of *C. hepaticus* strains from multiple countries, including the UK, USA, Germany, Australia and New Zealand (see Section 1.4.1.2) (Gunther, *et al*., 2023; Ienes-Lima, *et al.*, 2023; Petrovska, *et al.*, 2017; Van, *et al.*, 2019).

Field studies have shown that birds can become colonised with *C. hepaticus* as early as 12 weeks of age and up to eight weeks before a clinical outbreak of SLD (Phung, *et al.*, 2020). Importantly, *C. hepaticus* has been shown to persist in asymptomatic carriers for up to 51 weeks post-outbreaks (Courtice, *et al.*, 2023; Gunther, *et al.*, 2023). Although oral challenge models using *C. hepaticus* and *C. bilis* can reproduce mild to moderate gross and microscopical hepatic lesions, they have so far failed to reliably induce the production drops or mortality levels typically observed in field cases of SLD (Scott, 2021; Van, *et al.,* 2017).

These findings were suggestive that infection with *C. hepaticus* or *C. bilis* alone may not be sufficient to induce clinical onset of SLD and that additional predisposing factor(s) are likely involved (Courtice*, et al.*, 2018a; Gunther*, et al.*, 2023; Moore*, et al.*, 2014; Phung*, et al.*, 2020).

A range of potential predisposing factors have been proposed by researchers and clinicians. Host-related factors include flock sizes, physiological stress, endoparasitic infections, and behavioural issues such as cannibalism. Environmental factors include geographical location, seasonal variation, weather condition, and access to feed. Lastly, pathogen- factors may involve virulence mechanism such as strain variability and toxin production, for example, *C. hepaticus* is thought to have higher metabolic requirement for iron when comparing to other *Campylobacter* spp. (Courtice, *et al.,* 2018; Crawshaw, 2019; Grimes and Reece, 2011; Groves, 2010; Scott, 2016).

1.6.2 Knowledge gap

To date, most epidemiological studies on SLD have primarily focused on descriptive and molecular investigations of the sources, transmission, survival and effects of the aetiological agent *C. hepaticus*, and less so of *C. bilis* due to its recent discovery (Petrovska*, et al.*, 2017; Phung*, et al.*, 2020; Van*, et al.*, 2019). However, effective epidemiological investigations require a holistic approach, incorporating not only the characteristics of the aetiological agents but also host and environmental factors that may influence occurrence and severity of the disease (Martin*, et al.*, 1987).

Current understandings of SLD epidemiology remain largely based on descriptive studies, which focuses on documenting disease patterns and proposing plausible causal factors

(Thrusfield and Christley, 2018). Analytical epidemiology studies, which are essential for testing these hypotheses and identifying statistically significant risk factors, are lacking. Designing and implementing such studies can be challenging, particularly owing to the logistically complexity for reliable field data collection and the need for sufficient sample size for valid study design and analysis.

This thesis seeks to address these challenges by conducting the first large-scale analytical epidemiological investigations into the risk factors associated with SLD in commercial layer flocks. The overarching aim is to better understand the epidemiology of SLD through the application of both descriptive and analytical epidemiological studies, to identify key determinants of the disease, i.e., risk factors that can be modified by management procedures, and to ultimately provide evidence-based guidance for the prevention and control of SLD in cage-free laying hens.

1.6.3 Thesis outline and objectives

Chapter 2: Veterinary opinion survey on putative risk factors of Spotty Liver Disease

This chapter discusses the findings from a veterinarian opinion survey used to gather the opinions and hypotheses of the Australian veterinary community on SLD and potential risk factors.

Chapter 3: Scratch area as an epidemiological risk factor for Spotty Liver Disease in cage-free layers in Australia

This chapter has been published in *Poultry Science*, detailing a preliminary epidemiological investigation for SLD using a cross-sectional study, the aim is to explore an extensive collection of plausible factors that may impact the expression of SLD in cage-free hens.

Chapter 4: Identification of epidemiological risk factors for Spotty Liver Disease in cage-free layer flocks in houses with fully slatted flooring in Australia

This chapter has been published in *Poultry Science*, presenting the findings from a more tailored, retrospective case-control study in flocks housed in sheds with fully slatted floors and on associated risk factors for SLD.

Chapter 5: Descriptive epidemiology of Spotty Liver Disease in Australian cage-free layer flocks with a scratch area

This chapter has been published in *Poultry Science*, describing the findings from a retrospective case-control study on cage-free layer flocks housed in sheds with a scratch area, using a descriptive epidemiological method.

Chapter 6: Analytical epidemiology of Spotty Liver Disease in Australian cage-free layer flocks with a scratch area

This chapter focuses on the findings from the same retrospective case-control study on cage-free layer flocks housed in sheds with a scratch area (Chapter 5), using an analytical epidemiological method

Chapter 7: General discussion

This chapter summarises and further discusses the significant findings identified in this thesis.

CHAPTER TWO: Veterinary opinion survey on putative risk factors of Spotty Liver Disease in Australian free-range layer poultry, 2019

2.1 Introduction

Spotty Liver Disease (SLD) is a significant bacterial disease of the Australian commercial layer flocks, caused by the infection of *Campylobacter hepaticus* or *Campylobacter bilis*, and negatively impacts bird welfare, productivity and antimicrobial sensitivity (Crawshaw*, et al.*, 2015; Van*, et al.*, 2023). The disease is mostly associated with birds housed on the floor, mainly, in barn and free-range systems (Crawshaw, 2019). Following the advancement of microbiological, molecular, and serological techniques it is apparent that the presence of *C. hepaticus* alone is not sufficient to cause SLD outbreaks. Namely, *C. hepaticus* can be detected in birds as early as eight weeks prior to disease manifestation and up to 51 weeks post-outbreak in asymptomatic carrier birds, highlighting the necessity for predisposing or risk factors to elicit a clinical SLD infection (Courtice, *et al.,* 2018; Gunther*, et al.,* 2023; Phung, *et al.,* 2020).

The aim of this study was to use an opinion survey to capture existing opinions and hypotheses on potential risk factors for SLD amongst the Australian poultry veterinarian community. Its findings would guide the development of an extensive, preliminary questionnaire for an observational epidemiological study to identify putative risk factors of SLD.

2.2 Materials and Methods

Ethics Statement

The veterinary opinion survey (VOS) was conducted under the supervision of the Human Ethics Committee of the University of Sydney (protocol number 2019/662).

Methodology

An opinion survey was designed using REDCap, which aimed to capture the opinions of the veterinarians working in the Australian poultry industry (Harris*, et al.*, 2009). The target population for this survey are the veterinarians who are or have been actively looking after the health of laying hens in Australia and who have clinical experience with SLD. Forty veterinarians working or have worked in the Australian poultry industry were approached during the Australasian Veterinary Poultry Association Scientific Meeting in Adelaide, November 2019. These veterinarians volunteered to participate in the survey and were asked to respond to the survey.

The survey had 11 yes-or-no questions on the respondent's opinions, firstly on whether they believe potential risk factor(s) exist with SLD, then on whether they consider feed or nutrition, weather conditions, shed environmental conditions, physiological stress (associated with egg laying), water (contamination), bird health status, poor biosecurity, behavioural issues, and/or other factors, to be a potential risk factor(s) for SLD. These categories were designed to be as broad as possible and to cover potential predisposing factors described in the existing literature (Courtice, *et al.*, 2018; Crawshaw, 2019; Grimes and Reece, 2011; Groves, 2010; Scott, 2016). The respondents were asked to provide explanations for the yes-or-no answers. A copy of the opinion survey is available as Appendix I.

The survey was conducted under the supervision of the Human Ethics Committee of the University of Sydney (protocol number 2019/662).

2.3 Results

In total, 13 out of 40 Australian poultry veterinarians responded to the VOS, which was deemed a satisfactory response rate. The respondents represent nearly all veterinarians currently working or having worked in the Australian layer industry in all major states, including QLD, NSW, VIC, SA and WA. Several poultry veterinarians have declined the survey due to the lack of experience with the layer industry and/or Spotty Liver Disease. Table 5 summarises the keywords from the responses (n=13), which served as the basis for developing subsequent field study questionnaires.

All respondents (n=13) believed there are additional risk factors associated with SLD other than the infection of *C. hepaticus*. All respondents (n=13) concurred that weather conditions, shed environmental conditions, and physiological stress can be potential risk factors for SLD. Keywords such as “heat stress”, “summer”, “high humidity”, “pressure on shed (cooling) conditions”, and “rainfall” were commonly referred to in the question on **weather conditions** as potential risk factors. When asked about the impact of the **shed environment** on SLD, “poor ventilation or air movement”, “overcrowding”, “wet litter”, “high temperature or humidity”, “reduced nest space”, “empty drinker line or feeders” are some of the keywords identified. Keywords such as “Peak lay”, “between onset and peak”, “mainly at 22-28 weeks of age”, “stress hormone”, “sexual maturity”, “immunosuppression or drop in cell-mediated immunity” and “not seen in rearing birds” were found in responses on the association between **physiological stress**, especially around the time of lay, and SLD outbreaks.

Majorities of the respondents believed that feed and/or nutrition (11 out of 13), poor biosecurity (10 out of 12), other health issues (8 out of 12), or behavioural issues (8 out of 12) were associated with SLD occurrence. When asked about the influence of **feed or nutrition**

on SLD, two respondents believed that SLD could occur irrespective of nutritional or feed changes, whereas the rest of the responses mentioned “nutritional availability”, “palatability” and “feed disruption or restriction” can potentially influence SLD occurrence. Regarding **poor biosecurity** impact on SLD, two respondents answered “no” and one commented that “biosecurity is only important in a naïve site/farm, considering how ubiquitous the organism is". The responses favouring poor biosecurity as a risk factor mentioned vectors such as “wild birds, rodents and insects”. Most of the respondents believed that **concurrent health issues** could be a trigger for SLD, such as ongoing bacterial diseases, e.g. fowl cholera, intestinal worms, Big liver spleen disease (BLS), or immunosuppressive diseases. Some differed in their opinions and experience on this and stated that SLD is a “disease of healthy, well-performing birds” and can occur “despite the occurrence of coccidiosis, enteritis, cholera and colibacillosis”. Just over half of the respondents believed **behavioural issues** such as cannibalism or smothering can be associated with SLD, as a source of “stress” that can “induce endogenous neurotransmitters” and that cannibalism may “enhance the movement of bacteria between birds”. Others commented on the lack of association based on clinical observations and that SLD and behavioural issues could result from “a third stress” rather than having a causal relationship.

Lastly, only 5 out of 12 respondents considered **water** important in SLD outbreaks, mostly referring to contaminated water in the range area, such as puddles, as a potential source for *C. hepaticus*. In addition, the lack of water and “unpalatable water” were mentioned as other potential triggers for SLD.

Other notable comments from the survey include that “various feed additives seem to have (positive) effects on SLD”, “strain variations” may affect the ability of *C. hepaticus* to cause

disease and the level of clinical severity, and one last, intriguing (yet controversial) comment that "flocks infected with *Salmonella* Typhimurium do not get SLD!".

Table 5 Veterinarian Opinion Survey (VOS) on potential risk factors of SLD

Question	Yes	No	Key words or comments
Do you think there are risk factor(s) for SLD other than the infection of *Campylobacter hepaticus*?	13	0	
Do you consider the following to be a risk factor for SLD?			
Weather condition	13	0	Yes: summer disease; high humidity; rainfall
Shed environment	13	0	Yes: worst in some shed type; poor ventilation; feed distribution; nest box space and availability
Physiological stress	13	0	Yes: peak lay and associated weight loss; stress hormone; no clinical diseases in rearing; nutrient metabolism at peak lay
Feed/nutrition	11	2	Yes: net nutrient intake; nutritional availability; feed disruptions; palatability; malnourishment
			No: SLD occurs despite changes in nutritional specifications
Poor biosecurity*	10	2	Yes: only if flock/site is naïve to *C. hepaticus*
			No: organism found on farms that never had SLD
Suboptimal health or concurrent disease*	8	4	Yes: enteric disease; intestinal worms; other viral or bacterial infections
			No: disease of healthy and well-performing flocks
Behavioural issues*	8	4	Yes: interrelated; enhance movement of bacteria
			No: occurs in both flocks with and without cannibalism
Water (contamination)*	5	7	Yes: rainwater; water availability; changes in drinking pattern
Other potential factors			Various feed additives had anecdotal effects on controlling SLD;
			Strain variation in pathogenicity;
			Flocks infected with *Salmonella* Typhimurium do not get SLD!

*One respondent did not provide answers to these questions.

2.4 Discussion

This survey confirms the consensus amongst Australian poultry veterinarians on the existence of predisposing factors of SLD, apart from the presence of *C. hepaticus*, as documented by clinicians and researchers elsewhere (Courtice, *et al.,* 2018; Crawshaw, 2019; Grimes and Reece, 2011; Groves, 2010; Scott, 2016).

Most recorded potential risk factors are considered to be common stressors to laying hens, which can cause disruptions in the animal's homeostasis and physiological and behavioural responses to overcome the challenge (Tilbrook and Fisher, 2021). For example, SLD has been mostly associated with hot and/or humid environmental conditions or weather due to birds suffering from heat stress, which is common during the Australian summer months (Courtice, *et al.,* 2018; Grimes and Reece, 2011). In addition, the author speculates that poor husbandry practice could enhance the infection of *C. hepaticus* by creating favourable conditions for the bacterial survival and spread or causing physiological stress on the bird's immunity. Studies have shown that heat stress can cause reduced appetite and feed intake, lower body weight, decreased egg production and suppressed gastrointestinal or immune function of laying hens, thus impacting their productivity, health and welfare (Fouad*, et al.*, 2016; Tilbrook and Fisher, 2021).

Scott (2016) and Phung*, et al.* (2020) have previously discussed how nutrition (feed and water) can impact the occurrence or outcome of SLD outbreaks, where disruptive feeding or drinking patterns would cause undue stress on the hens and should be avoided. Changes to feeding patterns could also impact hens' gut microbiota, which may inadvertently promote

colonisation and/or proliferation of C. *hepaticus* or make birds more susceptible to the bacteria (Phung*, et al.*, 2020).

Husbandry-related stressors, including high stocking densities, reduced nest spaces and poor air quality, are linked to suboptimal production and disease occurrence due to immunosuppression (EFSA, 2023; Tilbrook and Fisher, 2021). Therefore, it is logical to speculate that the occurrence of SLD can be attributed to any of the husbandry challenges. It is known that laying hens undergo physiological and hormonal changes, especially around onset and peak lay periods, which coincide with the usual age of onset for SLD-affected hens (Phung*, et al.*, 2020; Scott, 2016). It is hypothesised that these changes could result from a rapid increase in egg production and ensuing nutritional deficit, potentially affecting the liver metabolism of the hens (Phung*, et al.*, 2020). Crawshaw (2019) has speculated that the rapid increase in blood iron level in hens at early lay could increase the likelihood of *C. hepaticus* infection, leaving these hens more susceptible. It was based on the finding that *C. hepaticus* has a notable reduction in iron acquisition and metabolism when compared to *C. jejuni* (Petrovska *et al*., 2017).

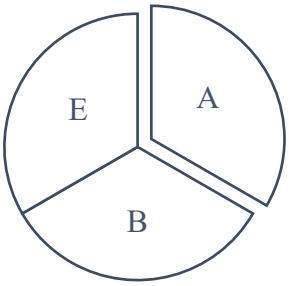
Finally, poor biosecurity practices and contamination of the environment, including water access, are thought to be predisposing factors for SLD. Although one response has indicated that SLD occurs in farms with both good and bad biosecurity, according to Courtice, *et al.* (2018), numerous hygiene and biosecurity measures have been deployed by farmers and veterinarians attempting to prevent or minimise SLD, including showering, change of attire, limiting vector movements, sanitisation of equipment and vermin control. While their efficacy is yet to be determined, it is essential to investigate the relationship between biosecurity and the incidence of SLD.

153

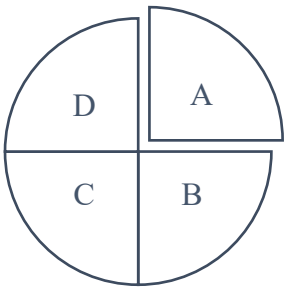
154 The findings from this VOS have been summarised into causal diagrams (Figure 2) and will
155 form the basis of the questionnaire used in a preliminary observational epidemiological study
156 designed to identify putative risk factors for SLD.

157

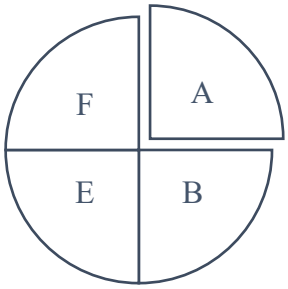
Figure 2 Speculative sufficient causes for SLD – causal diagrams based on VOS



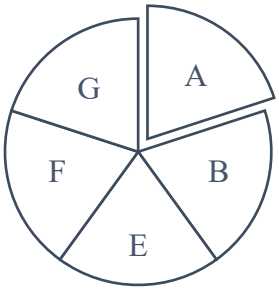
Speculative sufficient cause # 1 = A+B+E



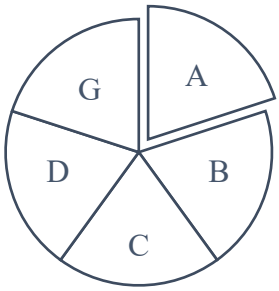
Speculative sufficient cause # 2 = A+B+C+D



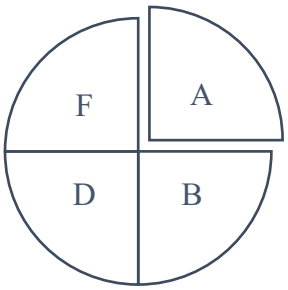
Speculative sufficient cause # 3 = A+B+E+F



Speculative sufficient cause # 4 = A+B+E+F+G



Speculative sufficient cause # 5 = A+B+C+D+G



Speculative sufficient cause # 6 = A+B+D+F

Components of some speculative sufficient causes from the VO will include (based on consensus or at least 10 responses)

A = *C. hepaticus* (necessary cause)
B = non-cage system (necessary cause)
C = summer – high humidity; rainfall
D = shed environment – poor ventilation
E = physiological stress – peak lay and weight loss
F = Poor biosecurity
G = nutrition – feed disruptions; net nutrient intake

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

Poultry Science

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

2023 Poultry Science 102:102922
https://doi.org/10.1016/j.psj.2023.102922

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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Received April 4, 2023.
Accepted July 2, 2023.

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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 *P*=	Fisher's exact 2-tail[1] *P*=
		Case	Control			
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 *P*=	Fisher's exact 2-tail[1] *P*=
		Case	Control			
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.

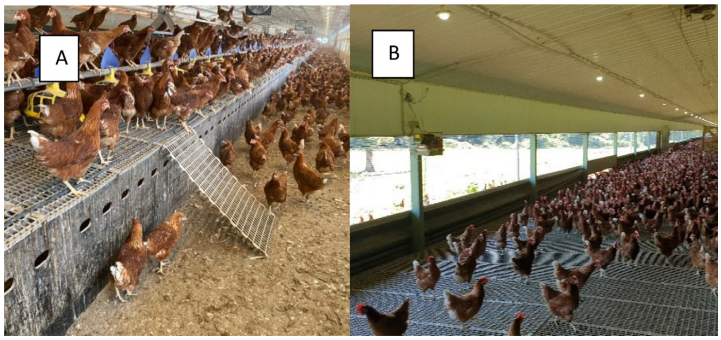

Figure 1. (A) Free-range house with partial slats showing scratch area; (B) free-range house with full slat coverage.

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.

ACKNOWLEDGMENTS

The authors would like to thank the layer companies, layer farmers, and veterinarians for their participation in and support of this project, especially through the challenging periods of COVID-19. This research was funded by Australian Eggs Limited (grant number 1BS004US). The first author is a recipient of a postgraduate scholarship from Australian Eggs Limited.

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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Addendum to Chapter Three: Scratch area as an epidemiological risk factor for Spotty Liver Disease in cage-free layers in Australia

This addendum provides additional details to the published paper.

Table 6 Hierarchical structure table for the cross-sectional epidemiological study in cage-free laying hens in Australia

Level	n	Number at next highest level		
		Median	Minimum	Maximum
Farm	18			
Flocks	24	1[a]	1	3

[a] Interpretation: The median number of flocks per farm (i.e. the next highest level) was 1.

CHAPTER FOUR: Identification of epidemiological risk factors for Spotty Liver Disease in cage-free layer flocks in houses with fully slatted flooring in Australia

Poultry Science

Identification of epidemiological risk factors for spotty liver disease in cage-free layer flocks in houses with fully slatted flooring in Australia

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ABSTRACT Spotty liver disease (**SLD**) is recognized to be caused by infection with *Campylobacter hepaticus* in adult layer hens farmed in cage-free environments. SLD is an emerging disease as cage-free egg production increases in popularity in response to desires for improved welfare of poultry. Outbreaks of SLD are frequently experienced around peak egg production in flocks, commonly between 25 and 40 wk of age. The disease becomes manifest with increased exposure and access of the birds to the feces of the flock. This study follows from a previous epidemiological survey of free-range and barn flocks in Australia which identified the presence of a scratch area within the laying house as a major risk factor for the occurrence of SLD. However, that survey also observed SLD occurrence in 45% of houses with a fully slatted floor (no scratch area). The present study describes a further analytical survey aimed at identification of risk factors for SLD in houses with fully slatted flooring. A comprehensive questionnaire was completed for 49 cage-free flocks from point of lay until 40 wk of age across Australia, retrieving information on house design, bird breed, flock size, stocking densities, bird growth, and performance and the occurrence of SLD.

Multiple logistic regression model building was used to separate factors and identify important management factors that may be amenable to modify the occurrence of SLD in egg layers.

Key determinants of SLD identified from the analyses were that houses with mechanical ventilation (such as tunnel ventilation) have some protection from SLD and an increase of an extra 1 bird/m^2 of nest space increased odds of occurrence of SLD by 1.172 times. A recommendation to not exceed 112 brown egg layer hens/m^2 of nest space in naturally ventilated houses with a full slat floor was suggested. A delay in birds reaching 60% hen day production (**HD**) by 1 wk is suggested as a possible predictor for a subsequent outbreak of SLD.

Key words: spotty liver disease, cage-free production, epidemiology, risk factors

2023 Poultry Science 102:103139
https://doi.org/10.1016/j.psj.2023.103139

INTRODUCTION

Spotty liver disease (**SLD**) is a serious disease affecting adult egg-laying hens in commercial cage-free situations (free-range and barn lay systems). The condition is considered to be identical with an historic disease known as Vibrionic Hepatitis (Moore, 1958; Peckham, 1958) which disappeared with the introduction of cage layer systems. SLD has re-emerged with the increase in cage-free egg production in Australia. The systems of keeping laying hens by developed countries have been changing over the last 20 yr. Guyonnet (2022) reported that following the banning of conventional cages in Europe in 2012, housing systems for layers have changed variably, with some European states, such as France and Spain, adopting furnished cages as their predominant method of egg production. Others, such as Germany, chose to move to barn systems (indoor cage-free housing) while the United Kingdom has prompted more for free-range systems. Guyonnet (2022) also notes that in North America, colder conditions in Canada encouraged the adoption of furnished cages while the United States has moved toward cage-free aviary systems with the birds remaining inside the house. Aviary systems allow birds to have free access to a solid floor and although these do have slatted areas, they all essentially have a scratch area. In Asia and Latin America however, conventional cages still predominate (Guyonnet, 2022). Hence the Australian move toward predominantly free-range production is somewhat unique, mainly due to the more amenable year-round climate. Hence the relevance of the present project would be mostly applicable to free-range

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Received June 21, 2023.
Accepted September 18, 2023.

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production in Australia and the United Kingdom. A meta-analysis by Weeks et al. (2016) described much higher mortality in free-range flocks in the United Kingdom compared to conventional cage systems although the reasons for this were not detailed, some of this could have been due to SLD. More recently Schuck-Paim et al. (2021) found that mortality rates in barn style cage-free production in Europe has reduced to levels similar to caged flocks, but this dealt only with raw mortality not cause-specific conditions and the differences between Australian and European operations and climate are substantial. A review by Bonnefous et al. (2022) noted that free-range management increases the risk of some bacterial, viral and parasitic diseases, and notes that *C. hepaticus* infections are increasing in Europe and hence the present study will be of relevance in regions where SLD is emerging.

The Australian commercial egg industry uses brown egg layers (Rhode Island breeds) almost exclusively. SLD is now known to be caused by an infective process with the recently identified and characterized bacteria, *Campylobacter hepaticus* (Crawshaw et al., 2015; Van et al., 2016; Phung et al., 2020) and the more recently identified *Campylobacter bilis* (Phung et al., 2022). Mortality rates during untreated SLD outbreaks can reach 10 to 15% and egg production may drop as much as 35% (Grimes and Reece, 2011; Courtice et al., 2018). The epidemiology of SLD is not well studied, with only some descriptive findings reported (Courtice et al., 2018). No analytical epidemiological studies on SLD have been published prior to Gao et al. (2023). Analytical studies aim to identify risk factors associated with the occurrence of a disease in a population of animals (Martin et al., 1987). This study follows on from information presented in a precursor study (Gao et al., 2023). The initial epidemiological survey identified that cage-free houses that included a scratch area were at much higher risk of developing SLD than those with the floor area fully covered by slats. However, almost half of the flocks with fully slatted flooring still developed clinical SLD. Hence a second survey examining flocks in lay in cage-free houses with fully slatted floors was undertaken to identify other factors which might modify the expression of SLD in this type of housing. The aim of the present study was to identify further factors that could be subject to intervention and thus allow a change in the risk of SLD. Factors that have a significant effect on a disease and that are under the control of farm management are termed "key determinants" (Martin et al., 1987).

MATERIALS AND METHODS

The survey was conducted under the supervision of the Animal Ethics Committee of the University of Sydney (protocol number 2021/1898).

Survey Design

Farms were sought to participate from those who participated in the original SLD epidemiological survey (Gao et al., 2023), from egg producers that attended seminars on SLD conducted by 2 of the authors which were organized by Australian Eggs Ltd. and from farms serviced by interested poultry veterinarians. All producers approached cooperated willingly as SLD is of high concern in the Australian cage-free egg industry.

A questionnaire was designed based on the preliminary findings of the first cross-sectional survey (Gao et al., 2023), with a heavy focus on shed infrastructure, resource availability, and bird production data. A copy of the survey questionnaire is included as supplementary information. The survey was carried out retrospectively during 2020 to 2021. The introduction of travel restrictions in Australia due to the COVID-19 pandemic disrupted the visitation plans for the survey. Interviews for flocks in New South Wales (**NSW**) were able to be carried out in person, whereas information for all interstate flocks was obtained by interview by phone or by online discussion. Data were collected retrospectively. An example of the questionnaire is included supplementary material.

All farms involved used birds reared on separate farms. All birds had beak treatment (infrared) administered by the hatchery (there are only 2 hatcheries producing commercial egg layers in Australia). Birds were all vaccinated against Marek's Disease, Newcastle Disease, Infectious Bronchitis, Fowl Pox, Infectious Laryngotracheitis, Avian Encephalomyelitis, Egg Drop Syndrome '76, Fowl Cholera, and *Salmonella* Typhimurium at the hatchery and at the rearing farms under standard industry practices using Australian registered vaccines.

Participating flocks were categorized as "Cases" or "Controls." The case definition used was that the 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. SLD in all "Case" flocks was diagnosed by the veterinarian consulting to the particular farm. Collection of cloacal swabs, feces, or dust was only achieved on only 13 houses; 8 of which were classified as Controls and 5 as Cases. Of the 8 Control flocks, 7 returned dust samples which were positive for the presence of *C. hepaticus* and all 5 of the cases also had positive cloacal swab or dust samples by PCR (as developed by Van et al., 2017). As the survey was conducted retrospectively and most farm visits were made impossible due to strict government travel restrictions during the COVID-19 pandemic.

Statistical Analyses

Data were transferred to MS-Excel and uploaded into JMP v16,1,0 (SAS Institute Inc, 2022, www.jmp.com) for analysis.

All survey variables were assessed in a univariate analysis using a contingency table analysis for categorical variables or Student 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 high probability value is used as a screening level for selection of possibly important factors the significance of which may be hidden within the complexities of the problem. Pearson χ^2 or Fisher's exact 2-tailed tests (the latter when an expected value was <5) were used to assess probability of associations being due to chance. Continuous variables expressing a probability of being due to chance of <0.20 were selected for further analysis.

Multiple logistic regression is an iterative statistical test that allows assessment of the effect of factors after controlling for the presence of other factors, thus assisting in eliminating extraneous effects. It also allows assessment of confounding and interaction between factors (Dohoo et al., 2003; Hosmer et al., 2013). Confounding exists when "the results of 2 or more factors cannot be separated" from each other (Blood and Studdert, 1999). All selected variables were considered in a univariate logistic regression which is able to consider several levels of a variable (e.g., slat brands). Variables showing a statistical association with occurrence of clinical SLD were then added to a multivariate logistic regression analysis along lines suggested by Dohoo et al. (2003). Variables that showed a probability due to chance of <0.05 were then examined for confounding and then subjected to a forward stepwise analysis including interaction terms to identify the key factors. A final multivariate analysis was then performed to define the major factors involved in risk of SLD in fully slatted houses. Odds ratios ($\boldsymbol{\Omega}$) for significant variables were calculated from the regression coefficients ($\boldsymbol{\beta}$) such that $\boldsymbol{\Omega} = e^{\beta}$.

RESULTS

A total of 49 flocks contributed to the survey. Of these, 20 flocks (41%) were categorized as "Cases" and 29 (59%) were regarded as "Controls." Table 1 shows means, median, and range of values for a number of descriptive statistics. Table 1 also summarizes the extent of the effects of SLD in some of the farms which experienced the disease. Mean age of an SLD outbreak was 31 wk with a range of 22 to 47 wk of age. All flocks experiencing SLD were treated promptly by veterinarians so the full effects would have been modified. The duration of the disease was up to 7 wk and mortality on affected farms due to SLD ranged between 0 and 9% and depression in egg production varied between 0 and 14% over the outbreak periods.

House ventilation system was flagged as of interest from the small sample number of fully slatted sheds examined in the previous survey (Gao et al., 2023). Out of 49 sheds enrolled in the present survey, 8 used mechanical ventilation control (tunnel ventilated) and the remaining 41 were of conventional, naturally ventilated design. This study's observations identified no Cases occurring in tunnel ventilated houses. Hence a tunnel ventilated fully slatted house was identified as a putative protective factor against SLD, although a strong conclusion here was limited by the low sample number. As was found in the previous survey (Gao et al., 2023), the zero cell value for Cases confounds all other factors within tunnel ventilated facilities. Hence only naturally ventilated houses could be considered further for the analysis. This reduced the sample size to 41. Layer breeds were not evenly distributed across the house ventilation types and only 1 layer breed was present in 39 of the naturally ventilated flocks. This distribution negates any breed comparison in this study as representation of other breeds was negligible.

Table 2 presents the remaining categorical variables in contingency tables identifying the number of naturally ventilated houses experiencing SLD ("Case") or not ("Control"). Hosmer et al. (2013) recommend an initial selection of variables for further evaluation as those that the univariate analyses produce a probability of being due to chance as less than 0.25 or 0.20. The use of a P value of 0.05 at this early stage of the analytical process may not detect important variables which may be confounded or involved in interactions with other variables. Hence, based on $P < 0.20$ as a screening level, categorical variables were selected for further assessment, which included nest/slat brand, closure of nests at night, and nutritionist used. Only 4 flocks did not close their nests at night, making this variable difficult to interpret and this variable was not considered further.

Table 3 shows outcomes of Student t tests conducted using Case or Control as the dependent variable and all continuous variables measured in the study in naturally ventilated sheds. Tables 2 and 3 were used to select

Table 1. Descriptive statistics for houses included in the survey ($N = 49$).

Variable	Mean	Minimum	Median	Maximum
Date flock transferred to laying house	7 Jan 2021	9 Oct 2019	19 Feb 2021	28 Jan 2022
Age transferred to laying house (wk)	16.28	14	16.14	20.14
Stocking density (birds/m^2)	10.14	7.44	10.50	12.11
Nest space (birds/m^2)	116.99	58.30	118.77	195.36
Age first access to nest boxes (wk)	16.73	15.43	16.64	20.00
Range stocking density (birds/ha)	3497.4	1153.9	2222.2	9662.6
For SLD Case flocks ($N = 20$)[1]				
Age at SLD outbreak (wk)	30.97	22.29	31.21	47.00
Total percentage of flock lost due to SLD	4%	0%	4%	9%
Duration of mortality due to SLD (wk)	3.71	0	3.00	7.00
Percentage drop in hen day egg production during SLD	8%	0%	8%	14%

[1]Medication was administered to all SLD-affected flocks, affecting mortality and egg production outcomes variably.

Table 2. Categorical variables distributed across Case or Control sheds in naturally ventilated houses.

Variable	Level	No. Case houses	No. Control houses	χ^2 or Fisher's exact 2-tailed test[1] $P=$
Season chicks hatched	Autumn	6	10	0.25
	Winter	6	3	
	Spring	5	5	
	Summer	1	5	
Age of house	Converted from older	7	6	0.50
	Purpose built	11	17	
Perches in rearing shed	Yes	7	9	0.80
	No	11	12	
Nest and slat brand[2]	A	7	13	**0.04**
	B	4	5	
	C	6	1	
	D	0	4	
	E	1	0	
Slat shape	Rectangular	5	3	0.34
	Square	6	5	
	Oval	7	13	
Lights type	Cool white	10	8	0.61
	Warm white	7	8	
Nests closed at night	Yes	16	17	**0.12**[1]
	No	0	4	
Nutritionist	A	8	8	**0.02**
	B	1	5	
	C	3	8	
	D	5	0	
Feed mill	W	8	8	0.37
	X	6	5	
	Y	3	8	
	Z	1	0	
Water additives used till 40 wk	Yes	4	5	0.91
	No	14	16	
Number of rations used in lay	1	12	13	0.76
	>1	6	8	
Perches in laying shed	Yes	14	14	0.48[1]
	No	4	7	

[1]Pearson χ^2 test but if an expected value was <5, Fisher's exact 2-tailed test was used.
Bold P values indicate factors considered worthy of further statistical evaluation ($P < 0.20$).
[2]A, Vencomatic; B, Roxell; C, Big Dutchman; D, Facco; E, Salmet.

factors for further analysis if the probability of the association of being a Case was <0.20. There were some missing data points in many variables due to differences in the information that farmers generally record and the information that could be provided. Obtaining full records was hampered by travel restrictions due to COVID-19 during 2021, especially with regard to flocks outside of NSW. Continuous variables considered

Table 3. Continuous variables distributed across Case or Control sheds in naturally ventilated houses.

Natural ventilation ONLY $N = 41$ Student t tests, independent variables*	Control Mean	Case Mean	t value	df	$P=$	Control Valid N	Case Valid N	Total N
Age transferred (wk)	16.09	16.10	−0.03	39	0.974	23	18	41
Stocking density (birds/sq. m)	10.17	9.61	1.44	39	**0.158**	23	18	41
Perch space in lay (cm/bird)	45.63	86.51	−1.77	37	**0.084**	21	18	39
Total nest space on record (sq. m)	153.74	144.93	0.70	36	0.490	21	17	38
Nest space on record (birds/sq. m)	101.41	115.86	−2.44	36	**0.020**	21	17	38
Age of first access to nest boxes (wk)	16.63	16.57	0.21	35	0.836	21	16	37
First let out age (wk)	22.12	22.71	−0.69	32	0.497	17	17	34
Range size (ha)	7.06	5.65	1.07	36	0.291	21	17	38
Range density (birds/ha)	3232	4666	−1.59	36	**0.122**	21	17	38
Number of rations from arrival to 40 wk of age	1.87	1.61	0.82	39	0.416	23	18	41
Body weight at arrival (kg)	1.38	1.39	−0.34	38	0.735	23	17	40
Age at first egg (wk)	19.02	18.84	0.49	32	0.629	18	16	34
Age at 5−10% HD[1] (wk)	19.65	20.16	−1.34	35	**0.190**	21	16	37
Age at 60% HD[1]	21.71	22.38	−1.59	34	**0.122**	20	16	36
Body weight at 60%HD[1] (kg)	1.75	1.80	−2.06	28	**0.049**	17	13	30
Age at peak lay (wk)	28.86	27.55	0.86	34	0.394	19	17	36
Body weight at peak lay (kg)	1.85	1.90	−1.30	20	0.207	11	11	22
Time since transfer to 60% HD[1] (wk)	5.40	6.16	−1.71	34	**0.097**	20	16	36
Body weight gain from transfer to 5−10% HD (g)	251	275	−0.72	29	0.477	18	13	31
Body weight gain from transfer to 60%HD (g)	341	418	−106.6	28	**0.042**	17	13	30
Body weight gain from transfer to Peak lay (g)	487	494	−0.19	20	0.854	11	11	22

Bold factors deemed worthy of further statistical evaluation ($P < 0.20$).
*Continuous value variables.
[1]HD, hen day production.

further included stocking density (birds/m^2 useable floor space in shed), perch space in lay, nest space (birds/m^2 of nest space), the age at which birds were first allowed to range (weeks), range density (birds /ha), age (weeks) at which the flock reached 5% hen day production (**HD**), age they reached 60% HD, the body weight of the birds at 60% HD, age at peak lay, and the number of weeks between transfer to the laying shed and the flock reaching peak HD production.

Several of these variables are autocorrelated (i.e., occurrence of 1 variable was strongly associated with the occurrence of another). Notably, factors related to bird age were correlated with body weights at that age, as birds continue to gain weight throughout lay: older birds are heavier. Five of the factors selected for further study fall under this association and data for all of them was incomplete as hen body weights were not always recorded every week. Dohoo et al. (2003) suggested a valid technique to deal with this type of autocorrelation would be to intuitively select 1 factor as representative of all of the related factors, based on the investigator's knowledge of the husbandry system and the relative P values of each variable with the dependent variable. In this case, the time since transfer until the flock reached 60% HD production was selected as the best representative, as this factor had the highest complete data set and the lowest P value of being due to chance in its association with the flock being a "Case." Thus, this variable provides a representation of age at transfer, age at initiation of egg laying, and rapidity of the rise in egg production. This gave 8 remaining variables to further assess within naturally ventilated sheds.

Each identified variable was then entered into a univariate logistic regression model. For continuous variables, logistic regression evaluates the effect of a change of 1 unit on the likelihood of an effect on the dependent variable. The outcomes for the univariate logistical regression of each of the variables selected for further analysis are presented in Table 4. This analysis showed that nest/slat brand and nutritionist produced large standard errors giving unstable estimates of regression coefficient ($\boldsymbol{\beta}$) and extreme odds ratios ($\boldsymbol{\Omega}$) between types, indicating that including them would make the model overfitted (Hosmer et al., 2013).

The associations of nest and slat brand and nutritionist delivered unstable coefficient estimates with extreme standard error values (Table 4) and were removed from further consideration. Perch space in lay, stocking density in the house, range density, nest space density, and time from transfer to 60% HD produced statistically valid regression coefficients and odds ratios of the association between the variables and the SLD outcome. These remaining terms were entered into a multiple logistic regression model (Table 5). The multiple logistic regression model assessed each variable, controlling for the presence of each other variable in the model. Using this approach, perch space in lay and range density exhibited nonsignificant associations and were removed from further consideration in the model. The remaining 3 variables were examined for confounding by sequentially entering each variable into a multiple logistic regression model and evaluating major changes made to the estimate of the regression coefficient for each. Variables were considered likely to be confounded if their regression coefficient changed by more than 30% by the addition of another variable to the model (Dohoo et al., 2003).

Stocking density was found to be strongly confounded by both the addition of nest space (41% change in coefficient estimate) and also by time from transfer to 60% HD (66% change) to the model. Nest space and transfer time to 60% HD were not confounded with each other. Nest space and transfer time to 60% HD showed acceptable fit of the models. Hence the final model for assessment of risk factors for SLD in naturally ventilated fully slatted-floor sheds contained only nest space (birds/m^2) and time (weeks) from transfer to the laying quarters until the flock reached 60% HD. A term for the interaction between these remaining 2 variables was added to the model. The final model is shown in Table 6. The means and 95% confidence limits for nest space allowance for Control and Case flocks is shown in Figure 1. The main findings resulting from these analyses were that a delay in birds reaching 60% HD by 1 wk increased

Table 4. Univariate logistic regression for selected factors for risk of SLD.

Variable	Level	β[1]	Standard error of β	Ω[2]	Wald's test	$P=$
Nest/slat brand[3]	C	1.60	737.7	11.14	4.19	0.38
	D	−16.39	1475.5	1.7×10^{-7}		
	B	−0.41	737.7	1.49		
	E	16.01	2660.0	2.02×10^{7}		
	A	Reference	Unstable			
Nutritionist	A	−5.26	608.2	5.00	2.53	0.47
	B	−3.65	608.2	1.47×10^{8}		
	C	Reference	Unstable			
	D	13.55	1824	1.875		
Perch space in lay	cm/bird	0.008	0.0046	1.01	2.94	0.087
Range density	1000 birds/ha	0.0002	0.0001	1.00	2.37	0.12
Stocking density in shed	Birds/m^2	−0.381	0.270	0.68	2.00	0.157
Nest space	Birds/m^2	0.086	0.057	1.09	2.297	0.130
Time from transfer to 60% HD	Weeks	0.476	0.305	1.61	2.437	0.119

[1]Logistic regression coefficient.
[2]Odds ratio for risk of SLD.
[3]A, Vencomatic; B, Roxell; C, Big Dutchman; D, Facco; E, Salmet.

Table 5. Multivariate logistic regression for valid factors for risk of SLD.

Variable	Level	β[1]	Standard error of β	Ω[2]	Wald's test	$P=$
Intercept		−28.499	15.74			
Perch space in lay	cm/bird	0.007	0.013	1.01	0.30	0.581
Range density	1000 birds/m^2	0.340	0.331	1.40	1.06	0.304
Stocking density in shed	Birds/m^2	−1.532	0.608	0.216	6.34	**0.012**
Nest space density	Birds/m^2	6.628	3.026	756.3	4.80	**0.029**
Time from transfer to 60% HD	Weeks	0.664	0.428	1.94	2.40	**0.121**

[1]Logistic regression coefficient.
[2]Odds ratio for risk of SLD.

Table 6. Final multivariate logistic regression analysis of identified variables associated with SLD.

			Likelihood ratio tests				Ω confidence limits	
Term	β[1]	SE	χ^2	$P=$	LogWorth[2]	Ω[3]	Lower 95%	Upper 95%
Intercept	−28.396	15.97						
Time from transfer to 60% HD[4] (wk)	1.747	1.042	3.841	0.050	1.301	5.736	1.000	63.645
Nest space (birds/m^2)	0.158	0.100	5.910	0.015	1.822	1.172	1.017	1.471
Nest space (birds/m^2) × Transfer to 60% HD (wk)	−0.136	0.102	2.079	0.149	0.826	0.873	0.694	1.043

Whole model test

Model	−LogLikelihood	DF	χ^2	$P=$	R^2	**0.2401**
Difference	5.937	3	11.873	0.008		
Full	18.793				BIC[5]	51.922
Reduced	24.731				Observations	36

[1]Logistic regression coefficient.
[2]A statistic based on χ^2 to evaluate a contribution due to chance in the full model (=(−log$_{10}$(P value)). Higher values signify more contribution to the model.
[3]Odds ratio for risk of SLD.
[4]HD, hen day % production.
[5]Bayesian information criteria—minimum BIC used for selection of best model fit.

the odds of occurrence of SLD by 5.736 times. These analyses indicated that an increase of an extra 1 bird/m^2 of nest space increased odds of occurrence of SLD by 1.172 times). Hence, using the means, the increase in the risk of SLD occurring when the number of birds per nest m^2 is increased from 101 to 115 is $(1.172)^{14} = 9.23$ (i.e., a 923% increase in risk) (Dohoo et al., 2003). The sample means here are an estimate of the actual population mean and the 95% confidence limits for the Control mean were between 90.5 and 112 birds/m^2 of nest space,

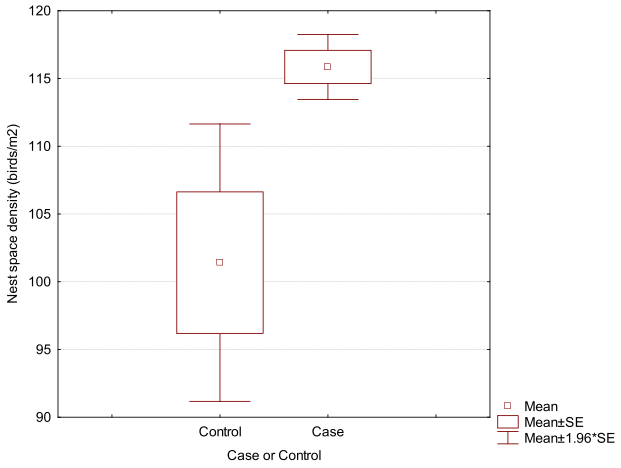

Figure 1. Box and whisker plot of distribution of birds/m^2 of nest space for Control and Case flocks for SLD occurrence. Bars denote ± 95% confidence intervals.

while the Case 95% confidence limits were from 113.3 to 118.5 birds/m^2.

DISCUSSION

An earlier cross-sectional survey of SLD in cage-free egg layer flocks in Australia (Gao et al., 2023) identified the presence of fully slatted flooring in the house as a partially protective factor against the disease, compared to houses which gave access to the solid floor area (scratch area) within the house. The present study was undertaken to examine other factors that may be involved in determining the likelihood of an outbreak of SLD in flocks which had the floor completely covered by slats. A broad questionnaire was administered across 49 cage-free (free range or barn style) flocks across Australia and the results examined through a multiple logistic regression analysis technique.

Hosmer et al. (2013) suggest a cutoff of probability due to chance association between putative factors and the outcome variable well above the usual value of <0.05, as variation, confounding and interaction in field data can often obscure associations which may actually be significant risk factors. These relational problems often produce aberrant analyses. These interrelationships needed to be assessed to determine the most meaningful associations between factor presence and the disease outcome. Entering each variable into a logistic regression model can reveal erroneous issues where extreme values of standard errors lead to biased or unstable associations as observed in this study between stocking density with regard to floor space in the house and nest space availability. Some variables had minimal valid numbers for either Case or Control (e.g., nest closure at night) and could not be validly analyzed.

The data indicated that shed with tunnel ventilation have some protection against the occurrence of SLD. Courtice (2022) reported that the severity of SLD can be much reduced if the shed temperature can be reduced by 8°C at the start of an outbreak. This intervention will obviously be more possible in mechanically ventilated sheds than in those relying on open sided ventilation (termed "natural ventilation" in the present report). The finding in the present study would suggest that protection against an outbreak of SLD may be possible if temperature control is optimal before an outbreak develops. Other possible factors could also contribute to the ameliorating value of a tunnel ventilation system. Tunnel ventilation systems provide forced air movement through the length of the house, achieving cooling by the wind chill factor of air speed, but this also introduces more fresh air, increases air flow and helps remove noxious gasses such as ammonia and carbon dioxide. The more effective fresh air movement may also possibly reduce SLD transmission and promote general health.

Further, this observation of the protective role of tunnel ventilation for SLD was consistent with putative findings from the previous study (Gao et al., 2023). The absence of clinical SLD in the present study in tunnel ventilated sheds meant that further analyses could only be considered across naturally ventilated sheds within the database.

In continuing the analysis for naturally ventilated houses only, identified putative factors were then subject to multiple logistic regression techniques to draw out the most likely contributors and develop a parsimonious model for risk of SLD. Once the confounders were removed, acceptable odds ratios were developed for the remaining important variables.

Nest space (number of birds/m^2 of nest) emerged as the most important remaining factor ($P = 0.015$) in the model for naturally ventilated sheds within this survey. An odds ratio of 1.172 for higher number of birds/m^2 of nest space was estimated. That is, for every increase of 1 bird/m^2 of nest space, the risk of SLD rises by 1.17 times. This was consistent with the preliminary findings from the previous study (Gao et al., 2023). The mean nest space for Control flocks was 101.4 birds/m^2 of nest space compared with 115.9 for flocks which experienced SLD (Cases). The suggested maximum nest space allocation suggested by a free-range brown egg layer breed management manual (HLB, 2021) is 120 birds/m^2 in colony nests. Bessei et al. (2013) noted that competition for nest space, especially early in lay, may be a considerable stressor for commercial hens and that brown egg layers have a higher requirement for nest space than do white egg layer breeds as brown egg breeds spend more time in the nests. If hens compete more for nest space, this could be a trigger for the expression of SLD in flocks. The current study would lead to a recommendation that it would be advisable to not exceed 112 birds/m^2 of nest space (the upper 95% confidence limit for unaffected flocks) in naturally ventilated houses with fully slatted flooring to avoid a higher risk of SLD occurring.

There were also associations of several variables involving the age of the birds to begin and continue into egg production and an association with the occurrence of SLD in the flock. These included bird age at 5% HD ("point of lay"), age at 60% HD, body weight at 60% HD, time (weeks) from the birds' arrival in the laying shed ("transfer") until the flock reached 60% HD, body weight at 60% HD and weight gain from transfer to 60% HD. There was missing data for some of these factors as farmers do not consistently weigh birds at each week. These factors however are linked, as weight increases with age. In all the factors considered in this group, the mean age for Control flocks was about 0.5 to 0.6 wk younger at point of lay and at 60% HD and in terms of the time from bird transfer to the layer quarters until they reached 60% HD, than for Case flocks. In this situation, 1 variable was chosen to represent the correlated variables (Dohoo et al., 2003). The time from transfer to 60% HD was selected in this instance as that variable contained the least number of missing values. The association of this factor, the time between transfer to layer quarters and when the flock reached 60% HD production, approached statistical significance ($P = 0.050$) in its association with the occurrence of SLD in naturally ventilated flocks with fully slatted flooring. It is not clear whether the observed significant association of birds reaching 60% HD production later (Controls at 21.71 wk

compared with Cases at 22.38 wk: a difference of 0.67 wk or 4.7 d) with a higher risk of occurrence of SLD represents a cause or an effect. Arguably flocks coming into lay slightly later may be more likely to experience SLD resulting from physiological effects on their development and maturity, or, conversely, flocks being affected by early subclinical infection with SLD may have their onset of lay delayed. It is not possible to determine which of these is a critical component for SLD occurrence from the data available in this study. What can be understood, however, is that flocks that are later and slower to come into lay (say point of lay at 20 wk rather than 19 wk of age), may be more at risk of a subsequent SLD outbreak. In this sense, the observed delay in onset and continuance of lay may be a predictor for SLD.

The confounding between stocking density and nest space allowance was interesting. While higher numbers of birds per nest space area was a risk factor for SLD, higher numbers of birds/m^2 of available floor space was protective (odds ratio <1.0). The Hyline Brown breed management manual recommends 7 to 9 birds/m^2 of useable space in free-range houses (HLB, 2021). The stocking density observed in this study had a mean of 9.92 birds/m^2 with a range of 7.44 to 11.92 birds/m^2 of useable floor space. There was no correlation between nest space and shed stocking density (correlation coefficient, $r = -0.05$, $P = 0.766$). However, nest space (birds/m^2) was strongly negatively correlated with the ratio of total nest space (m^2) in the shed to the total available floor space (m^2) ($r = -0.92$, $P < 0.0001$). From this it is obvious that where nest space makes up a greater proportion of the shed area, there will be a lower number of birds/m^2 of nest space but a higher number of birds/m^2 of available floor space. This would explain the confounding between the 2 measures of nest space and floor space. Statistically, the measure of fit (Deviance) of the model of stocking density as a risk factor for SLD showed that the stocking density parameter did not fit the data well and thus nest space was more significant and is the risk factor identified here for SLD.

CONCLUSIONS

In layer sheds that have a full slat coverage of the floor area, a further key determinant of the risk for SLD occurrence in the flock is having a natural ventilation system (i.e., an open sided house with curtains or shutters and only air circulation fans inside the house). Tunnel ventilation systems may provide some protective effect against SLD.

In houses with fully slatted floors and a natural ventilation system, increasing the number of birds/m^2 of nest space results in an increased risk of SLD occurrence. Based on the available data for this survey, for every increase of 1 extra bird/m^2 nest space increases the risk of SLD by 1.172 times (i.e., by 17.2%). Highest recommended nest space allocation developed from this survey data would be 112 birds/m^2 of nest space for colony nest systems.

A delay in the onset of egg production and a later achievement of 60% HD production by about 5 d may be predictive of a later SLD occurrence in the flock.

As ventilation system and number of birds placed in the house/m^2 of nest space are amenable to modification by management, these factors can be declared as key determinants (Martin et al., 1987) for SLD in fully slatted houses.

ACKNOWLEDGMENTS

This project was funded by Australian Eggs Limited (project 1BS004US).

We express appreciation for all assistance provided by owners and staff of all cooperating farms and their attending veterinarians.

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.2023.103139.

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Addendum to Chapter Four: Identification of epidemiological risk factors for Spotty Liver Disease in cage-free layer flocks in houses with fully slatted flooring in Australia

This addendum provides additional details to the published paper.

Table 7 Hierarchical structure table for the epidemiological study in flocks housed in sheds with fully slatted floors in Australia

Level	n	Number at next highest level		
		Median	Minimum	Maximum
Farm	13			
Flocks	49	4[a]	1	3

[a] Interpretation: The median number of flocks per farm (i.e. the next highest level) was 4.

CHAPTER FIVE: Descriptive epidemiology of Spotty Liver Disease in Australian cage-free layer flocks with a scratch area

Poultry Science

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

2024 Poultry Science 103:103941
https://doi.org/10.1016/j.psj.2024.103941

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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Received April 16, 2024.
Accepted May 29, 2024.

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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.

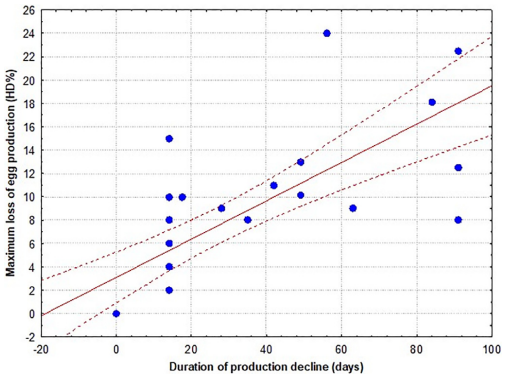

Figure 1. Correlation of maximum loss in egg production (HD%) with duration of the production decline (days).

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] $= (\mathbf{p_o - p_e})/ (\mathbf{1 - p_e}) =$**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.

ACKNOWLEDGMENTS

The project was funded by Australian Eggs Limited (project 1BS004US). The authors express great gratitude to all egg producers and veterinarians who assisted with the survey.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103941.

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Addendum to Chapter Five: Descriptive epidemiology of Spotty Liver Disease in Australian cage-free layer flocks with a scratch area

This addendum provides additional details to the published paper.

Table 8 Hierarchical structure table for the case-control study on cage-free layer flocks housed in sheds with a scratch area

Level	n	Number at next highest level		
		Median	Minimum	Maximum
Farm	18			
Flocks	48	2[a]	1	3

[a] Interpretation: The median number of flocks per farm (i.e. the next highest level) was 2.

CHAPTER SIX: Analytical epidemiology of Spotty Liver Disease in Australian cage-free layer flocks with a scratch area

6.1 Introduction

Scratch areas have been identified as a significant risk factor for the occurrence of Spotty Liver Disease (SLD), especially in cage-free laying flocks (Gao*, et al.*, 2023b). A subsequent analytical epidemiological study in fully slatted sheds, i.e. in the absence of a scratch area or reduced access to faecal matter, highlighted natural ventilation systems and high nest density (birds per m^2) as likely contributors to SLD outbreaks (Gao*, et al.*, 2023a). A third survey of cage-free layer flocks housed with scratch areas was conducted, and the descriptive epidemiological analyses for this survey were discussed in Chapter 5 (Groves*, et al.*, 2024). This chapter is focused on the analytical epidemiological analyses of the survey data to identify management and environmental factors (key determinants) that are associated with the risk of SLD occurrence in sheds with a scratch area.

6.2 Materials and Methods

Ethics Statement

The survey was supervised by the Animal Ethics Committee of the University of Sydney (approval number 2022/2014). All animal procedures were conducted following the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition (NH&MRC 2013), the Australian Code for the Responsible Conduct of Research (NH&MRC 2007), the NSW Animal Research Act 1985 the NSW Animal Research Regulations 2010 and other relevant legislation. The study methodology was also approved by the Human Ethics Committee of the same institution (protocol number 2019/662).

Scratch area survey

A retrospective case-control study of forty-eight flocks (n=48) from eighteen Australian

cage-free farms (barn and free-range), that have a scratch area in the poultry house, was conducted in this study. The target population was all cage-free flocks with >8,000 birds with scratch areas in Australia, and the flocks are to be older than 40 weeks of age to allow for potential delayed SLD onset. The source population were all farms from the target population identifiable for contact through previous studies. The eligible population were all members of the source population that could have been enrolled in the survey, in face of the COVID travel restrictions. Farms included in the survey were from New South Wales (NSW), Queensland (QLD), Victoria (VIC) and Western Australia (WA). The survey was conducted between July 2022 and January 2023. Due to COVID-19 pandemic travel restrictions, face-to-face survey visits were conducted only in NSW, and the remainder of the farms were interviewed remotely by phone or digitally.

Table 9 Hierarchical structure table for the case-control study on cage-free layer flocks housed in sheds with a scratch area

Level	n	Number at next highest level		
		Median	Minimum	Maximum
Farm	18			
Flocks	48	2[a]	1	3

[a] Interpretation: The median number of flocks per farm (i.e. the next highest level) was 2.

The survey questionnaire covered questions relating to farm or shed infrastructures, environmental conditions and host-related factors, and a summary of the factors examined is listed in Table 10. The questionnaire had been refined to examine factors and records that are readily available and recorded consistently across most layer operations, based on the experience of previous studies, this is to avoid recall bias in a retrospective study. A copy of the survey questionnaire is included in Appendix V. The confirmation of SLD infection was based on the diagnosis of the consulting or company veterinarian to each flock/farm, which was an increase in daily mortality with or without a concurrent decline in daily egg production, and the associated postmortem appearance of miliary focal hepatitis in any of the

affected birds.

Table 10 A summary of factors examined in the survey. The full survey is available in Appendix V

Farm/shed and environment factors	Bird factors
- Farm location - Prescence of slats, scratch areas, platform, perches - Nest space, water space, feed space, usable area, range density - Nest, platform, slat cleaning procedure - Range design and access - Shed ventilation and climate control - Light types	- Bird number and breed - Age of transfer - Nutrition and the use of feed additives - Production parameters (egg production, feed intake, mortality, bird weight) at different time points e.g. 5-10% HD, 60% HD, peak lay, 30wk, 40wk - Other known causes of death or disease e.g. smothering, feather pecking

Statistical Analyses

Case and control designation was used as a dichotomous dependent variable, and values of the independent variables recorded in the survey were assessed for association with the occurrence of SLD. A Case was a flock that experienced clinical SLD, whereas a flock that didn't was classified as a Control. Continuous variables were initially compared using Student's t-test and categorical variables using contingency tables and Fisher's exact test (two-tailed) as some expected values were <5. Initial screening to select variables for further analysis used a criterion of the probability value of the test being <0.20 to include all possible contributing factors (Hosmer*, et al.*, 2013)

Selected variables were examined for confounding by two approaches: (1) by observing substantial changes in logistic regression estimates when variables were included in

multivariate analyses (Dohoo, *et al.,* 2003), and (2) by assessing the level of agreement between variables using Kappa analysis. Variables were evaluated for both confounding and autocorrelation (collinearity), following the approach recommended by Dohoo *et al.*(2003).

A confounder s defined as a variable that is either positively or negatively corelated with both the outcome (disease) and the exposure (hypothesized causal factor), and potentially distorting the true association between them (Thrusfield, 2018). According to Elwood (2017), a variable needs to meet the following criteria to be considered a confounder:

(a) it must be a cause of the outcome;

(b) it must be either associated with, or a cause of, the exposure; and

(c) it must lie on a separate causal pathway from the exposure to the outcome.

These qualitative criteria for confounding were assessed prior to any quantitative assessment, ensuring that only variables met above criteria as confounders were carried forward for further statistical analysis.

Selected variables were analysed using an initial univariate logistic regression, followed by forward stepwise multiple logistic regression to evaluate variables' association with SLD occurrence in the presence of the other variables. The final analysis was based on a final logistic regression model with $P < 0.05$. The computerised statistical packages STATISTICA® v6.1 (Statsoft Inc., 2003) and JMP® 18.0.0 (SAS, 2022) were used for analyses.

6.3 Results

Out of the forty-eight flocks enrolled in the survey (n=48), thirty-two of these flocks (67%) experienced clinical SLD outbreaks (Case flocks), while 16 (33%) did not (Control flocks). Continuous variables were initially grouped by mean values for SLD Case and Control flocks and compared by Student's t-tests. Full data set for the continuous variable comparison is shown in Table 11. Categorical variables were stratified by a number of flocks with each level of the variables in the Case and Control categories and compared in contingency tables using Fisher's exact 2-tailed test. Comparisons for all categorical variables assessed are shown in Table 12. Due to the variability between survey participants on data recording and their ability to provide certain data, there was missing data for several variables. As a result, a further selection of variables was restricted to only those continuous variables with degrees of freedom exceeding 24. Furthermore, only variables showed associations with the occurrence of SLD that had P values <0.20 were selected.

There were 6 continuous variables met the criterion, which were 'age at transfer', range size', 'the age at which a second ration was given to the birds', 'the age of the flock when it reached 60% HenDay production', 'stocking density inside the house' and the 'size of the range area', see Table 13. Twelve categorical variables met the selection requirements above, including house design, ventilation style, internal mechanisms associated with ventilation, type of floor surface (concrete or other), type of lights (Cool white or Warm white lights), number of rations up to 40 weeks of age, the occurrence of feather pecking and presence of other diseases, see Table 14. The analyses aimed to determine the most parsimonious model that statistically significant to represent the data. Consequently, the variables for the final analyses were refined to avoid overfitting of the model and excessive standard errors, which

could render the coefficient estimated unstable.

Each of the selected variables was evaluated for confounding with each other selected variable. This is done by examining changes in logistic regression coefficients with the variable alone related to the severity category and with a second variable fitted to the model. Many categorical variables involving ventilation facilities (natural versus tunnel ventilation, circulating fans, foggers, extraction fans, roof chimneys, cool cells and extraction fans) were considered confounded. Table 15 shows an assessment of the measurement of agreement between these factors (Kappa analysis). Following the methodology suggested by Dohoo*, et al.* (2003) and Hosmer*, et al.* (2013), where several variables were correlated, one was selected as representative of the group of factors. The presence of Cool Cells was selected for purposes of further analysis.

Dohoo*, et al.* (2003) suggests that any change in coefficient over 30% could represent significant confounding between the two variables, and the inclusion of confounded variables can cause instability in estimating the effect of variables on the outcome. Table 16 shows univariate logistic regression analysis for the selected variables. The variable 'Age of transfer' returned a significant lack of fit for the model (χ^2 P= 0.010), indicating the predicted values did not adequately reflect the relationship between the independent and dependent variables. The variable for the presence of other diseases did not give a significant association using logistic regression with the occurrence of SLD.

After these two variables were eliminated, four variables remained for evaluation in a model for SLD occurrence: absence of cool cells, concrete flooring, cool white lighting and absence of feather pecking. Each variable was integrated into a logistic regression model with every

other variable and analysed for changes in the coefficient estimates. The floor construction (concrete floor) variable showed significant confounding with the absence of cool cells and the presence of cool white lighting. The coefficient for cool white lighting became unstable in combination with the absence of the feather pecking variable. Consequently, only the variables of the absence of cool cells and the absence of feather pecking were retained for subsequent analysis in the model.

When the two remaining variables were fitted into a multiple logistic regression analysis (Table 17), only the absence of cool cells was identified as a significant ($P < 0.05$) risk factor for the occurrence of SLD. The absence of cool cells from the house correlated with an increased risk of SLD. Cage-free layer houses, including a scratch area but no cool cells, had a 5.0 (95% CI 1.3 to 23) times increase in the odds of SLD compared to houses equipped with cool cells.

An earlier study showed that higher nest stocking density is a risk factor for SLD in fully slatted flooring houses (Gao*, et al.*, 2023a). This variable did not statistically correlate with SLD occurrence in the present study ($P = 0.24$). Consideration was given to evaluating this further in the present study. Nest density analysed using a factorial ANOVA analysis for Case and Control flocks with or without cool cells in the house. The interaction approached significance ($P = 0.080$), revealing no difference in the effect of nest density where cool cells were absent but a lower value (102 birds per m^2) in nest density for control flocks where a cool cell was present.

Table 11 Student's t-tests for continuous variables for SLD Case or Control flocks – full data

Variable	Mean CASE flocks	Mean CONTROL flocks	t-value	df	Student's t-test P=	CASE flocks valid n=	CONTROL flocks valid n=
Age at transfer (weeks)	15.63	14.77	2.889	41	0.006	30	13
No of birds transferred	22162	18013	1.176	46	0.246	32	16
Total floor area (m^2)	1444	1293	0.999	44	0.323	30	16
Total usable space (m^2)	1483	1555	-0.356	35	0.724	22	15
Stocking density (birds/m^2)	12.31	10.45	1.721	31	0.095	22	11
Perch space in lay (cm/ bird)	106.90	118.70	-0.617	39	0.541	29	12
Platform space (birds/m^2)	26.50	185.75	-3.011	6	0.024	4	4
Scratch area coverage of shed (%)	0.63	0.66	-0.267	38	0.791	29	11
Total nest space (m^2)	207.5	188.3	0.475	43	0.637	29	16
Nest density (birds/ m^2)	107.8	96.6	1.170	43	0.249	29	16
Age of first access to nest boxes (wk)	15.82	15.60	0.837	30	0.409	17	15
Feed space - Chain (birds/ m)	15.00	16.00	-0.421	12	0.681	12	2
Feed space - Pan (birds/ pan)	38.81	57.63	-5.218	24	0.000	16	10
Drinker space - nipple (birds per nipple)	11.20	11.88	-0.403	33	0.690	26	9
First let out age (weeks)	21.04	21.73	-1.290	35	0.206	26	11
Range size (ha)	7.536	2.579	1.424	27	0.166	23	6
Range density (birds/ha)	4418	7671	-2.736	27	0.011	23	6
Number of rations from arrival to 40 weeks of age	1.77	2.55	-2.903	35	0.006	26	11
Ration 2 start age (weeks)	30.86	20.46	2.429	25	0.023	14	13
Ration 3 start age (weeks)	47.67	35.00	1.664	12	0.122	9	5
Age at 5-10%HD[B] (weeks)	19.37	19.27	0.318	36	0.752	27	11
Weight at 5-10%HD[B] (kg)	1.61	1.59	0.419	29	0.678	21	10
Feed intake at 5-10%HD[B] (g/b/d)[A]	75.59	86.16	-2.981	24	0.006	16	10
Age at 60%HD[B] (weeks)	22.74	19.87	1.847	35	0.073	27	10
Weight at 60%HD (kg)	1.755	1.755	-0.011	27	0.991	20	9
Feed intake at 60%HD (g/b/d)	92.09	85.37	0.809	23	0.427	15	10

Variable	Mean CASE flocks	Mean CONTROL flocks	t-value	df	Student's t-test P=	CASE flocks valid n=	CONTROL flocks valid n=
HD% (approx. 60)	0.68	0.60	1.366	24	0.185	16	10
Age at peak lay (weeks)	32.25	33.22	-0.227	27	0.822	20	9
Weight at peak lay (kg)	1.85	1.85	0.005	24	0.996	18	8
Feed intake at peak lay (g/b/d)	107.71	111.02	-0.694	21	0.495	14	9
HD% (at peak)	90.7%	92.1%	-0.617	27	0.543	20	9
Weight at 30 weeks (kg)	1.86	1.85	0.102	16	0.920	13	5
Feed intake at 30 weeks (g/b/d)	108.75	118.17	-2.594	23	0.016	18	7
HD% (at 30 weeks)	0.81	0.89	-1.158	18	0.262	14	6
Weight at 40wk (kg)	1.94	1.90	0.420	12	0.682	11	3
Feed intake at 40wk (g/b/d)	117.52	117.56	-0.015	16	0.988	13	5
HD% (at 40 weeks)	0.84	0.85	-0.139	16	0.891	13	5

[A] gram feed / bird/ day
[B] Henday % egg production over a week

Table 12 Categorical variables across Case and Control flocks – full data

Variable	Level	No. case flocks	No. control flocks	Fisher's exact P= (2-tailed test)	Odds Ratio	95% Confidence Interval for Odds Ratio	
Breed	Hyline Brown	23	8	0.131	8.6[B]	0.79	95
	Isa Brown	8	5		4.8	0.38	60
	Lohmann Brown	1	3		Reference		
Breed consolidated	Hyline Brown	23	8	0.201	2.6	0.73	8.9
	Other	9	8				
Perches in lay	Exposed	17	11	0.752	0.77	0.21	2.9
	Unexposed	10	5				
Production system	Conventional free-range	23	5	0.012	5.62	1.5	20
	Aviary/ Barn	9	11				
Ventilation system	Natural/ Roof extraction	20	6	0.037	4.00	1.2	13
	Tunnel	12	10				
Walls of house	Curtain	17	11	0.735	0.66	0.17	2.6
	Solid	9	5				
Circulation fans	Exposed	26	5	0.004	7.86	2.0	30
	Unexposed	6	11				
Foggers	Exposed	23	6	0.025	5.00	1.4	18
	Unexposed	2	10				
Extraction fans	Exposed	9	11	0.055	0.241	0.06	0.91
	Unexposed	17	5				
Mini vents	Exposed	13	7	1.000	0.78	0.26	3.0
	Unexposed	19	9				
Roof vents	Exposed	4	2	1.000	1.37	0.22	8.6
	Unexposed	19	13				
Chimneys	Exposed	12	2	0.020	7.00	1.3	38
	Unexposed	12	14				
Cool Cells	Exposed	10	11	0.029	0.21	0.060	0.75
	Unexposed	17	5				
Floor construction	Concrete	22	6	0.062	3.67	1.0	13
	Other	10	10				
Slat type	Wire mesh	16	6	0.378	1.83	0.55	6.2
	Expanded Plastic	16	11				
Nest brand	Big Dutchman	10	6	0.690	1.67	0.34	8.3
	Roxell	15	5	0.231	3.00	0.61	15
	Other	5	5		Reference		

Variable	Level	No. case flocks	No. control flocks	Fisher's exact P= (2-tailed test)	Odds Ratio	95% Confidence Interval for Odds Ratio	
Lighting temperature	Cool white Warm white	13 19	2 14	0.057	4.79	0.93	25
Feeder type	Pan Chain	17 15	10 6	0.758	0.68	0.20	2.3
No. rations given to 40 weeks	1 >1	12 14	0 11	0.001	Undefined		
Feed additives[C]	Exposed Unexposed	8 19	3 10	1.000	1.40	0.30	6.5
Water additive[D]	Exposed Unexposed	11 14	6 5	0.720	0.65	0.16	2.7
Pecking	Marked Not observed	3 29	5 9	0.044	0.19	0.040	0.94
Other disease	Colibacillosis None	13 16	5 11	0.066	3.78	1.1	14
Antibiotic use for non SLD	Exposed Unexposed	16 16	5 11	0.355	2.2	0.62	7.8

[A] Odds Ratio for risk of SLD given exposure to the factor.
[B] significantly different from the reference level
[C] feed additives used as prevention for SLD included Solaminovit®, Fysal®, Balancius®,Selacid Green®, Oregano essential oil products, Herbal Weld®, Citristim® and various organic acids
[D] water additives used included vinegar, mushroom extract, and Shell Improver®

Table 13 Student's t-test outcomes for continuous variables (for degrees of freedom >26 and P<0.20) comparing SLD Case and Control flocks.

Variable	Mean Case flocks	Mean Control flocks	Valid n Cases	Valid n Control flocks	t-value	df	Student' t-test P=	Brown-Forsythe[A] P=	Mann-Whitney U test P=
Age at transfer (weeks)	15.63	14.77	30	13	2.889	41	0.006	0.700	
Range density (birds/ha)	4418	7671	23	6	-2.736	27	0.011	0.094	
Ration 2 start age (weeks)	30.86	20.46	14	13	2.429	25	0.023	0.046	0.190
Age at 60%HD (weeks)	22.74	19.87	27	10	1.847	35	0.073	0.392	
Stocking density (birds/m^2 usable space)	12.31	10.45	22	11	1.721	31	0.095	0.014	0.012
Range size (ha)	5.81	2.58	23	6	1.688	27	0.103	0.134	

[A] if Brown-Forsythe statistic is significant (P<0.05), the non-parametric Mann -Whitney U test was performed

Table 14 Contingency analysis for categorical variables across Spotty Liver Disease Case and Control flocks selected for further analyses (P<0.20)

Variable	Level	No. case flocks	No. control flocks	Total n=	Fisher's exact P= (2-tailed test)	Odds Ratio	95% Confidence Interval for Odds Ratio	
Production system	Conventional free-range	23	5	48	0.012	5.7	1.5	21
	Aviary/ Barn	9	11					
Ventilation system	Natural/ Roof extraction	20	6	48	0.131	2.8	0.80	9.6
	Tunnel	12	10					
Circulation fans	Present	25	5	48	0.004	7.9	2.0	30
	Absent	7	11					
Foggers	Present	24	6	48	0.025	5.0	1.4	18
	Absent	8	10					
Extraction fans	Present	9	11	42	0.055	0.24	0.064	0.91
	Absent	17	5					
Chimneys	Present	12	2	40	0.020	7.0	1.3	37
	Absent	12	14					
Cool Cells	Present	10	11	48	0.029	0.21	0.060	0.75
	Absent	22	5					
Floor construction	Concrete	22	6	48	0.062	3.7	1.0	13
	Other	10	10					
Lighting temperature	Cool white	13	2	48	0.057	4.8	0.93	25
	Warm white	19	14					
No. rations given to 40 weeks	1	12	0	37	0.001	Un-defined		
	>1	14	11					
Pecking	Present	3	5	46	0.044	0.19	0.040	0.94
	Absent	29	9					
Other disease	Colibacillosis	13	5	45	0.066	3.8	1.1	14
	None	16	11					

Table 15 Measure of agreement values (Kappa coefficient) between categorical house construction variables.

Variable	Level	Production system	Ventilation system	Circulation fans	Foggers	Extraction fans	Chimneys	Cool Cells	Floor construction	Lighting temperature
Production system	Flat deck free-range	-	0.400*	0.565*	0.652*	-0.311*	-0.144	-0.347*	-0.286*	0.412*
Ventilation system	Natural/ Roof extraction	0.400*	-	0.404*	0.829*	-0.922*	0.316*	-0.918*	-0.286*	0.098
Circulation fans	Present	0.565*	0.404*	-	0.556*	-0.434*	0.011	-0.414*	-0.217	0.353*
Foggers	Present	0.652*	0.829*	0.556*	-	-0.644*	0.069	-0.660*	-0.217	0.352*
Extraction fans	Present	-0.311*	-0.922*	-0.434*	-0.644*	-	-0.372*	1.00*	0.214	-0.073
Chimneys	Present	-0.144	0.316*	0.011	0.069	-0.372*	-	-0.39*	0.316*	-0.410*
Cool Cells	Present	-0.347*	-0.918*	-0.414*	-0.660*	1.00*	-0.39*	-	0.224	-0.049
Floor construction	Concrete	-0.286*	-0.286*	-0.217	-0.217	0.214	0.316*	0.224	-	-0.137
Lighting temperature	Cool white	0.412*	0.098	0.353*	0.352*	-0.073	-0.410*	-0.049	-0.137	-

* Significant agreement between variables in the matrix, $P<0.05$.

Table 16 Univariate logistic regression analysis of selected variables for the occurrence of SLD

Variable	Level	β[A]	Std Err[B]	Ω[C]	Wald's test value	95% Confidence Interval for Odds Ratio	P=	Comments
Age at transfer	weeks	1.249	0.483	3.5	6.67	1.5 to 10	0.010	Significant lack of fit for the model[D]
Cool cell	Absent	0.788	0.330	4.8	5.699	1.4 to 19	0.017	
Floor type	Concrete	-0.650	0.321	3.7	4.096	1.1 to 14	0.043	
Lighting temperature	Cool White	-0.783	0.419	4.8	3.500	1.1 to 34	0.061	
Feather pecking	Absent	0.840	0.412	5.4	4.161	1.1 31	0.041	
Other disease present	Colibacillosis	-0.290	0.328	1.8	0.784	0.51 to 6.9	0.376	Wald test P >0.20

[A] Logistic regression coefficient.
[B] Standard error of the estimate
[C] Odds ratio for presence of the variable level with occurrence of SLD
[D] lack of fit = model does not adequately capture the relationship between the independent and dependent variable

157

Table 17 Multivariate logistic regression analysis of remaining variables with occurrence of SLD

Variable	Level	β[A]	Standard error of β	Wald's test χ^2	P=	Ω[B]	95% confidence limits of Ω
Intercept		0.396	0.446				
Cool cells	Absent	0.806	0.366	4.858	0.028	5.0	1.3 to 23
Feather pecking	Absent	0.766	0.442	2.998	0.083	4.6	0.85 to 30

[A] Logistic regression coefficient
[B] Odds ratio for risk of SLD

6.4 Discussion

This study focused on identifying management factors that may further influence the expression of clinical SLD in cage-free laying flocks housed with a scratch area. Previous analytical epidemiological investigations have identified several factors associated with the occurrence of SLD in cage-free layer systems. Gao *et al.* (2023a) reported the presence of a scratch area as a major risk factor for SLD, likely mediated by increased hen exposure to fresh faeces. In a separate study, Gao *et al.* (2023b) examined poultry housing design in systems with fully slatted floors and identified higher nest density (birds per m^2 of total nest space) and the use of natural ventilation as additional risk factors. In contrast, tunnel ventilation systems, which incorporate evaporative cooling and exhaust fans, were found to be protective against SLD in fully slatted housing environments.

The present study builds on this work by investigating management factors that could further impact SLD expression, specifically in sheds that incorporate a scratch area. While several other authors have described aspects of SLD epidemiology (e.g. Courtice et al., 2018; Crawshaw, 2019; Moore et al., 2019; Phung et al., 2020; Groves et al., 2024), these studies have largely been descriptive in nature or focused on molecular epidemiology, such as characterisation of *Campylobacter hepaticus* strains. To the best of the author's knowledge, this series of investigations represents the only published analytical epidemiological studies to date aimed at identifying disease risk factors for SLD in commercial laying hens.

The analysis aimed to identify a parsimonious model that best predicted flock-case control status, more specifically, a model containing the fewest possible significant variables while maintaining explanatory power (Dohoo, *et al.*, 2003). Confounding was recognised as potential analytical challenge, as it arises when a variable is statistically associated with both

184 the outcome (i.e. occurrence of SLD) and one or more predictor variables. Efforts were made
185 to control confounding to ensure the validity of the final statistical model. Multiple logistic
186 regression was used to determine the final model.
187
188 After accounting for considerable confounding in the analysis, only one factor retained
189 statistical significance in predicting the occurrence of SLD in the surveyed flocks: the
190 presence of a **cool cell** system within the poultry house ($P = 0.028$, 95% CI 1.3 to 23).
191 Specifically, sheds with a scratch area but without cool cells were found to have a fivefold
192 higher risk of developing SLD compared to those equipped with cool cells.
193
194 Cool cells, which are typically associated with tunnel-ventilated sheds, enhance the cooling
195 capacity of the shed under heat-challenged weather conditions, subsequently reducing heat
196 stress in birds. For tunnel ventilation to operate at maximum efficiency, sheds must be well-
197 sealed to maintain negative air pressure. However, this level of environmental control is
198 difficult to achieve in free-range systems, where pop holes remain open to allow outdoor
199 access for birds for a duration of the day. Despite this limitation, the author speculates that
200 sheds with tunnel ventilation and cool cells are often newer builds or retrofitted systems and
201 are generally constructed with improved insulation and airflow capacity. In addition, tunnel
202 ventilated sheds will be more superior in cooling down at night when birds are housed indoor,
203 when compared to naturally ventilated sheds, improving the thermo-comfort of the birds via
204 multiple means.
205
206 These findings are consistent with those from the fully slatted shed survey, which also
207 identified tunnel-ventilated sheds as protective against SLD (Gao, *et al.* 2023a). They also
208 align with the observations of Garcia and Courtice (2024), who reported that reducing shed

temperature contributed to improved SLD management outcomes. Furthermore, Courtice *et al.* (2018) suggested that hot weather and environmental stress may act as triggers for SLD outbreaks. Even though open pop holes in free-range sheds compromise the full efficiency of tunnel ventilation, the presence of such system still appears to significantly reduce the risk of SLD, emphasising the potential role of heat stress in SLD pathogenesis, which warrants further investigation.

As mentioned earlier, tunnel-ventilated sheds may be constructed as new builds or retrofitted usually from older, naturally ventilated sheds, both requiring significant capital investment by layer farmers or companies. Despite these costs, there has been a notable increase in the adoption of tunnel ventilation within the Australian layer industry, largely due to its capacity to maintain thermo-comfort for birds. A survey by Scott *et al.* (2017) reported that cooling pads and tunnel ventilation were presented in only 16% (4 of 25) of NSW free-range layer farms interviewed, compared to 53% (8 of 15) of barn and 53% (8 of 15) of free-range broiler farms. However, by 2022-2023, where the current study was conducted, 22 out of 48 free-range layer farms interviewed (46%) had adopted tunnel ventilation. If this trend continues, the wider implementation of tunnel ventilation systems may offer a significant benefit for the control and prevention of SLD in free-range laying hens.

The assessment of nest density in sheds with scratch areas in relation to SLD occurrence has been interesting but within expectation. An almost statistically significant relationship between nest density and the occurrence of SLD outbreaks was established ($P = 0.08$), but mainly in sheds that had cool cell ventilation systems. A nest density of 102 birds per m^2 was found to have a protective effect in houses with scratch areas and cool cells. This is lower than 112 birds per m^2 established in the fully slatted survey (Gao*, et al.*, 2023a) and much

234 lower than the 120 birds per m^2 permitted by the *Australian Animal Welfare Standards and*
235 *Guidelines (S&G) for Poultry* (DAFF, 2022). Once again, nest box access is considered a
236 critical resource to the laying hens, especially in young laying hens, and competition over
237 nest boxes would elicit a stress response Cronin*, et al.* (2008).

CHAPTER SEVEN: General Discussion

7.1 Background

The Spotty Liver Disease (SLD) caused by *Campylobacter hepaticus* or *Campylobacter bilis* in cage-free adult chicken systems is considered the re-emergence of Avian Vibrionic Hepatitis (AVH) from the 1950-60s (Crawshaw, 2019; Phung*, et al.*, 2022b). The disease condition disappeared for decades when cage systems became the predominant housing type for laying hens, reappearing after the consumer-driven expansion of cage-free systems in recent years (Courtice, *et al.,* 2018; Crawshaw, 2019). Since the isolation of *Campylobacter hepaticus*, there has been significant advancement in understanding the pathogen characterisation and transmission and disease management using antimicrobial and non-antimicrobial treatments and/or vaccination (Courtice, *et al*., 2023; Phung, *et al.,* 2020; Quinteros, *et al.*, 2021; Willson*, et al*., 2019). Although *C. hepaticus* or *C. bilis* has been recognised as a necessary cause of SLD, i.e. the causative agent of the disease, little is known about other sufficient causes of the disease i.e. risk factors (Van, *et al.,* 2017; Van, *et al.,* 2023). This thesis aims to fill these knowledge gaps in the epidemiology of SLD by identifying risk factors and/or protective factors using analytical epidemiologic studies. Specifically, "key determinants", that is, risk factors that can be modified by management, were sought.

7.2 Scratch area as a risk factor of SLD

Based on the preliminary analytical epidemiological survey from **Chapter 3** (Gao*, et al.*, 2023b), the presence of a "**scratch area**" in the housing area of a cage-free operation, barn or free-range, was found to be significantly associated with an increased risk of SLD infection ($P = 0.003$). A "scratch area" is defined as an indoor space where hens have direct access to the floor or surface for the expression of natural behaviours such as scratching, foraging and dustbathing (DAFF, 2022; Singh and Groves, 2021). These areas typically consist of bare

flooring (e.g. concrete or clay), with or without bedding materials such as wood shavings or sawdust, where poultry faeces can readily accumulate. Inadvertently, scratch areas allow hens direct and prolonged contact with high concentrations of faecal matter, thereby enhancing the likelihood of faecal-oral transmission of pathogens, including *C. hepaticus* and *C. bilis*.

Faecal-oral transmission is fundamental to the infection and spread of SLD. this mode of transmission not only poses an ongoing risk to the current flock but also facilitates the re-infection of *C. hepaticus* in subsequent replacement flocks. PCR results from this study demonstrated that during clinical SLD outbreaks, C. *hepaticus* was detectable in high proportion of cloacal swabs from randomly selected birds, indicating active infection and transmission within the flock. Furthermore, Courtice *et al.* (2023) reported that flocks can remain infected for extended periods post-infection, continuing to shed the organism into the environment. This environmental contamination perpetuates a vicious cycle of re-infection, increasing the difficulty of eradicating the pathogen from affected farm and environments. This underscores the importance of long-term biosecurity measures and addressing other putative risk factors associated with SLD to effectively reduce disease persistence and recurrence.

This finding is particularly noteworthy considering that nearly all flocks in the study had access to outdoor ranges, which were also likely to be contaminated with *C. hepaticus*. Yet, SLD did not occur uniformly across these flocks. Additionally, the only flock without range access, a flock housed in conventional barn shed, still developed SLD; further highlighting that exposure to contaminated indoor scratch areas may play a more critical role in SLD development than range access alone.

The author speculates that outdoor range areas may be less conducive to the survival of *C. hepaticus* and *C. bilis* due to factors such as desiccation, ultraviolet (UV) exposure, and climatic variability. Furthermore, given the expansive nature of range areas, faecal contamination is likely to be more dispersed, reducing the bacterial load and consequently the likelihood of transmission. In contrast, indoor scratch areas may represent a more concentrated and persistent source of infection.

Table 18 Scratch area requirements and inclusions for cage-free housing systems according to *Australian Animal Welfare Standards and Guidelines (S&G) for Poultry*

Type of cage-free houses	Scratch area inside the poultry house
Conventional barn	Mandatory, must allow at least one third of the flock to forage and dust-bathe at the same time
Aviary barn	
Conventional (barn) free-range	Not required.
Aviary free-range	Not required, however exposed flooring (like scratch areas) exist indoors due to the flooring design of aviary systems.

Cage-free egg production has been steadily expanding in Australia, by 2023, 15.2% of Australian egg production was from barn systems and 56.5% from free-range layer flocks, calculated to a total of 71.7% (Australian Eggs, 2023). According to *Australian Animal Welfare Standards and Guidelines (S&G) for Poultry*, once legislated by the State and Territories governments, hens must have access to a scratch area, as well as appropriate substrate(s) for pecking, foraging and scratching, unless they can access an outdoor area (DAFF, 2022). In addition, the floor design must allow simultaneous scratch area access for at least one-third of the flock. This means that in a “barn” poultry house, where the birds are always housed indoors, a scratch area must exist and prevent the use of a “fully slatted” flooring design. Free-range housing systems, on the other hand, do not require the inclusion of scratch area(s) indoors (DAFF, 2022). The indoor housing area can be fully slatted in a conventional free-range set-up, greatly reducing hens’ access to poultry litter and faeces.

However, due to the flooring design of most aviary systems, although not required by the *Standards & Guidelines*, laying hens housed in aviary free-range sheds would have partial or full access to an open floor i.e. scratch areas (Frohlich*, et al.*, 2012). As a result, a great proportion of the Australian cage-free laying hens will be exposed to the most significant SLD risk factor, scratch area, either due to regulatory requirements or housing designs. This highlights the importance of identifying further putative risk factors or key determinants of SLD, especially to aid the management of SLD in flocks housed in sheds with scratch areas.

7.3 Risk factors identified in fully slatted sheds

Despite the absence of a scratch area, almost half of the laying flocks in fully slatted houses (barn and free-range) in the preliminary analytical epidemiological study developed clinical SLD, as described in Chapter 3 (Gao*, et al.*, 2023b). This led to the speculations that other putative risk factors could precipitate SLD occurrence, even when the faecal-oral transmission of the causative agents is greatly reduced. A retrospective case-control study on fully slatted sheds was designed to identify further factors, especially key determinants, that may significantly impact the expression of SLD in such sheds, as described in **Chapter 4** (Gao*, et al.*, 2023a).

Figure 3 An example of a fully slatted conventional (barn) free-range flock

It quickly became evident that, in layer houses with full slat coverage of the floor area, a **naturally ventilated** environmental control system is a further key determinant of the risk for SLD ($P = 0.002$). A natural ventilation system is defined as an open-sided shed with curtains or shutters and stirring fans and foggers inside the shed, as opposed to the tunnel ventilation system, which is typically an air-tight shed equipped with evaporative cooling using cool cells and extraction fans (Weaver Jr, 2002). Strictly speaking, when a free-range flock is ranging during daylight hours, tunnel-ventilated sheds are not in a 'true' tunnel, i.e. not achieving maximum efficiency due to air leakage through the pop-holes. Nevertheless, the study found that sheds with a tunnel ventilation system appeared to be protected against SLD. The author hypothesised that it is the overall ability of tunnel-ventilated sheds to maintain thermoregulation of the shed and thermos-comfort of the birds, as these sheds tend to be newly designed and built comparing to naturally ventilated sheds, with solid walls, better temperature and humidity control, and are thermoregulated overnight when the birds return to

100 the house to sleep. This finding agrees with the field observations that SLD has been mostly
101 associated with hot and/or humid environmental conditions or weather, where the laying hens
102 are most at risk for heat stress (Courtice, *et al*., 2018; Grimes and Reece, 2011). Furthermore,
103 mitigation strategies reducing heat stress, such as cooling the sheds before the onset of and/or
104 during the SLD outbreak, have been successfully used to manage SLD, as reported by Gracia
105 and Courtice (2024). Studies have shown that heat stresses can cause reduced appetite and
106 feed intake, lower body weight, decreased egg production and suppressed gastrointestinal or
107 immune function of laying hens, which all have the potential to worsen a disease outbreak
108 such as SLD (Fouad*, et al.*, 2016; Tilbrook and Fisher, 2021). Lastly, more efficient
109 ventilation in tunnel-ventilated sheds may contribute to reduced humidity levels and
110 improved litter conditions, creating environments that promote the desiccation of causative
111 agents *C. hepaticus* or *C. bilis*. Like other *Campylobacter* species, these pathogens are highly
112 sensitive to desiccation, and reduced moisture in the environment may therefore play a role in
113 limiting their survival and transmission (Zhang and Sahin 2020).
114
115 Further analysis of fully slatted sheds with natural ventilation systems identified an additional
116 statistically significant risk factor for SLD: **nest density**, defined as the number of birds per
117 square metre of next space (P =. 0.015). In the surveyed flocks, data showed that for every
118 additional bird per m^2 of nest space, the risk of SLD increased by a factor of 1.172, which is
119 the equivalence to a 17.2% increase in risk. Based on these study findings, the optimal
120 recommended nest space allocation for naturally ventilated, fully slatted systems would be no
121 more than 112 birds per m^2 of nest space.
122
123 This association is biologically plausible, as increased competition for key resources, such as
124 nest boxes around peak lay, can trigger a physiological stress response in laying hens. This

125 phenomenon was demonstrated by Cronin *et al.* (2008) by measuring plasma corticosterone
126 levels as an indicator of stress in laying hens. Their study found that competition for nest
127 boxes in 23-week-old laying hens resulted in a significant, though transient, increase in the
128 stress indicator plasma corticosterone concentration ($P < 0.001$), highlighting the importance
129 of nest boxes as an important resource for young laying hens.
130
131 The transient nature of this stress response in young birds could explain the almost
132 prescriptive age of onset of SLD, which commonly occurs around the start and peak of lay. In
133 contrast, although older hens could be carriers for *C. hepaticus*, they are usually
134 asymptomatic (Courtice, et al. 2023). Supporting this, Cronin *et al.* (2012) found that older
135 hens exhibit greater flexibility in their selection of laying sites and using a less preferred or
136 unfamiliar nest box may be less stressful as birds mature. Importantly, all flocks included in
137 the survey complied with *Australian Animal Welfare Standards and Guidelines (S&G) for*
138 *Poultry* (DAFF, 2022), which require a minimum of $1m^2$ of nest area per 120 birds from the
139 point of lay. This highlights the gap in literatures and industry knowledge with regards to
140 SLD and potential risk factors such as nest density.
141
142 Shed stocking density was not included in the final multivariate logistic regression analysis of
143 after accounting for confounding (with nest space) and model fit. Interestingly, prior to that,
144 stocking density was found to be protective in the multivariate logistic regression model,
145 suggesting that stocking density cannot be entirely discounted as a potential risk factor for
146 SLD. It had an odds ratio of less than 1.0, suggesting that flocks with higher shed stocking
147 densities were less likely to experience SLD. In this study, stocking density was calculated as
148 the number of birds per m^2 of usable floor space, including slatted and scratch areas but
149 excluding nest areas (DAFF, 2022). There was no significant correlation between nest density

and shed stocking density (correlation coefficient, r = -0.05, P = 0.766). However, nest density (birds/m^2) was strongly negatively correlated with the ratio of total nest space to the total usable floor space (r = -0.92, P < 0.0001). A plausible explanation was that flocks with higher shed stocking densities in this study also had more nest space relative to usable floor area, which may explain the apparent protective effect of stocking density. This further emphasises the critical role of adequate nest space access, rather than overall floor space, in reducing the risk of SLD.

7.4 Risk factors important in sheds with scratch areas

Chapters 5 and 6 of this thesis documented findings from a retrospective case-control study of cage-free houses with scratch areas, where Chapter 5 presented the findings from descriptive epidemiological analyses (Groves*, et al.*, 2024), and Chapter 6 discussed the analytical epidemiological investigation of the survey.

The main statistically significant risk factor of SLD occurrence in this survey group was the **absence of a cool cell** in sheds with a scratch area, which has a five times higher risk of SLD than those with a cool cell present (P = 0.0275). Cool cells are a key feature of tunnel-ventilated sheds, which are required for evaporative cooling (Weaver Jr, 2002). This finding is in agreeance with the finding from the fully slatted survey (Chapter 4), where naturally ventilated sheds (no cool cells) have a much higher risk of laying hens developing SLD, most likely due to the higher chance of hens suffering from heat stress in those sheds (Tilbrook and Fisher, 2021; Weaver Jr, 2002). Once again, this emphasises the importance of heat stress in the induction of SLD outbreaks, and the pathogenesis of this effect needs to be further studied.

Nest density was also assessed in this survey, although not statistically significant ($P = 0.08$), it did appear to support the finding of higher nest density as a contributory factor to the occurrence of SLD outbreaks, as reported in the fully slatted survey (Gao*, et al.*, 2023a). It was found that lowering the nest density in sheds with scratch areas and no cool cells, i.e. naturally ventilated, show no beneficial effect on reducing SLD occurrence. Notably, based on data available in this survey, a nest density of 102 birds per m^2 was found to have a protective effect in houses with scratch areas and cool cells, much lower than the 112 birds per m^2 established in the fully slatted survey (Gao*, et al.*, 2023a). Therefore, it is possible to elude the hierarchy between the effects of scratch areas, ventilation systems and nest density as risk factors of SLD, where the presence of scratch area has the strongest impact on the likelihood of SLD, followed by naturally ventilated sheds, then nest density.

Figure 4 An example of a conventional (barn) free-range flock with scratch areas

Figure 5 An example of an aviary free-range shed, with birds having access to the open floor (i.e. scratch area)

7.5 Other findings

Across the three observational epidemiological studies conducted in this thesis (Chapters 3, 4, 5 and 6), an extensive collection of potential risk factors was examined. They were enlisted to test the author's hypotheses and the opinions of the Australian veterinarian community on SLD and risk factors (Chapter 2). A subset of variables was repeated and studied across all three epidemiological investigations due to their likely associations with the incidence of SLD, but did not yield a statistically significant correlation, apart from those previously noted.

Analyses of factors concerning resource availabilities (except nest boxes), such as access to feeders, drinkers, perches, platforms and floor space (density), did not show any statistically significant findings. Similarly, despite the extensive rearing survey as part of the preliminary

study (Gao*, et al.*, 2023b), the author was unable to identify any significant rearing risk factors of SLD. In addition, smothering and concurrent diseases These factors were considered due to the potential risk of causing physiological and behavioural stress, where the laying hens have to compete for resources, acclimate from the rearing set-up to a laying farm post-transfer or combat health issues (Campbell*, et al.*, 2018; Cronin*, et al.*, 2012; Tilbrook and Fisher, 2021). Despite the lack of statistically significant causal effects on SLD, stressors to the laying hens must be minimised in principle for better welfare, health and production outcomes, including for SLD.

The author could not draw any link or statistically significant relationship between SLD occurrence and feed or nutrition-related factors such as feeding pattern and ration changes. However, according to survey responses, the use of feed additives (irrespective of class) was observed to aid the management of SLD outbreaks anecdotally. Although not statistically significant, feed additives mainly reduced the severity of the disease but were unable to prevent the onset of the disease. It is plausible that observational epidemiological studies (descriptive and analytical) are not suited to investigate such factors due to the high likelihood of confounding factors, where experimental studies should be used instead (Scott, 2018).

7.6 Limitations and further considerations

As with any field-based epidemiological investigation, the complexity of organising and executing field studies can significantly influence both the validity and interpretation of findings. Over the three-year period during which the author conducted farm visits, phone interviews and follow-ups, data collection was consistently challenged, especially by the unprecedented impact of the COVID-19 pandemic. In addition to pandemic-related travel

restrictions and resource limitations, logistical and budgetary constraints also limited the number of farms and flocks that could be included in the studies. As a result, smaller than anticipated sample sizes and incomplete data for certain variables were unavoidable, which may have reduced the statistical power of the analyses.

As a result, several aspects of these epidemiological studies were carefully considered when interpretating the results. A proportion of the studies were conducted retrospectively, with data collected after the outcome (SLD outbreaks) had occurred. To minimise recall bias, participants were asked to maintain logs of relevant events as well as their routine production data at the time of enrolment, which is prior to the outbreaks. Another variability is the quality and consistency of production records between different operations. Some layer farms maintained highly detailed and digitalised datasets, while smaller or independent operations tend to rely on manual and often incomplete records. This inter-participant (farm) variability resulted in missing or unusable data for several variables, further limiting the analytic power and preventing uniform variable inclusion across models.

As the studies progressed, the author adapted and refined the questionnaires to prioritise variables that were both biologically plausible and consistently recorded across participants. Variables that were difficult to measure, inconsistently recorded, or commercially sensitive were excluded to maximise response rates and data quality. However, this necessary adjustment means that certain potential risk factors for SLD may have gone unmeasured, and therefore unexamined in these studies. While analytical epidemiology is a powerful tool for risk factor identification, it is limited by the quality and completeness of field data. Variables not easily captured through observation or survey may be better suited for investigation via controlled experimental trials or mathematical modelling approaches.

Another important consideration was the independence of observations. While each flock was treated as an independent unit of analysis, the potential for clustering effects within farms needed to be acknowledged. Company-level differences in housing design, nutrition management, health management and husbandry management could influence both exposure and outcome variables, introducing potential confounding or selection bias, which were also considered when running statistical analyses.

Despite these limitations, the series of studies presented in this thesis has yielded valuable insights in the design and execution of field-based analytical epidemiological research. The research series followed a systematic and logical approach, beginning with an initial brainstorming and conceptual mapping of plausible risk factors (Chapter 2), followed by an extensive scoping survey (Chapter 3) to guide hypotheses generation. These findings enabled the design of more focused surveys (Chapter 4-6), each of which contributed to building a clearer picture of the multifactorial nature of SLD.

7.7 Conclusion

As demonstrated through multiple descriptive and analytical epidemiological studies, Spotty Liver Disease, like many other animal diseases, is multifactorial, which requires the presence of one or more other factors for the disease to be clinically expressed. In the presence of the causative agent *C. hepaticus* (and most likely *C. bilis*), the author believes that a scratch area has the strongest impact on the likelihood of SLD, followed by naturally ventilated sheds and then high nest density. A flowchart summarising the proportion of SLD-positive flocks found over three surveys was included, based on different combinations of scratch areas, natural ventilation systems, and high nest density (Figure 6). These figures also provide estimates of the likely reduction in SLD outbreaks that could be achieved by removing one of the factors.

A total of 121 cage-free flocks were examined across three epidemiological surveys (Chapters 3, 4, 5 and 6), with Survey 1 being the preliminary cross-sectional study, Survey 2 the fully slatted shed study, and Survey 3 the scratch area shed study. Seventy-one had clinical SLD, which equates to 59% of the total flocks. A total of 50 fully slatted flocks were studied across Survey 1 (n=11) and Survey 2 (n=49), of which 25 cases of SLD were observed, account for 42% of flocks. A total of 61 flocks examined had scratch areas between Survey 1 (n=13) and Survey 3 (n=48), and 45 out of the 61 flocks experienced SLD, which is 74%. Within the fully slatted survey, Survey 2 (n=49), 0% of the tunnel-ventilated sheds (n=8) experienced SLD, whereas 20 or 49% of naturally ventilated sheds had SLD (n=41). From a total of 61 flocks with scratch areas, Survey 1 (n=13) and Survey 3 (n=48), 25 flocks were housed in tunnel-ventilated sheds, from which 15 were positive for SLD, hence 60%. On the other hand, there were 36 flocks from naturally ventilated sheds, out of which 30 experienced SLD outbreaks, hence 83% positive.

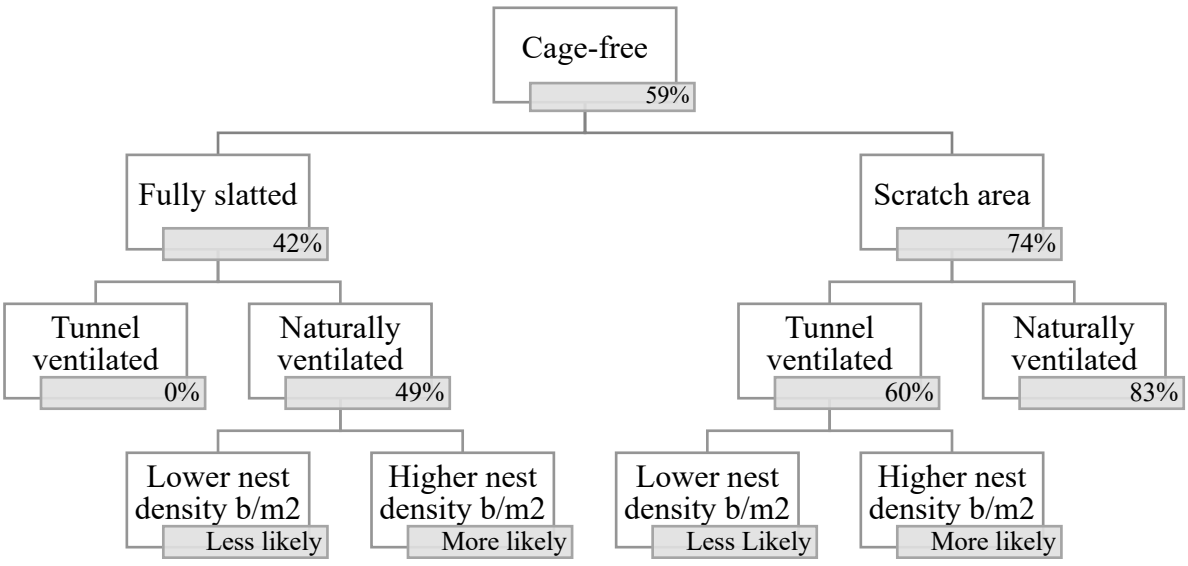

Figure 6 Percentage of the flocks experienced SLD across the three epidemiological surveys

In conclusion, this thesis contributes new insights into the epidemiology of Spotty Liver Disease in commercial cage-free laying systems in Australia, through the application of analytical epidemiological methods. Despite the inherent challenges of conducting large-scale field surveys, the studies presented here identified several key determinants that can inform future disease prevention strategies. These findings lay the foundation for more targeted experimental investigations and highlight the importance of integrating robust analytical epidemiology into poultry health research. The experience gained through this work provides a practical framework for designing future epidemiological studies in complex production systems and reinforces the value of evidence-based approaches to improve poultry health and welfare.

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Appendices

Appendix I: Veterinary Opinion Survey used in Chapter 2

Appendix II: Preliminary study questionnaires – Rearing used in Chapter 3

Appendix III: Preliminary study questionnaires – Laying used in Chapter 3

Appendix IV: "Fully slatted" study survey used in Chapter 4

Appendix V: "Scratch area" study survey used in Chapters 5 and 6

Appendix I: Veterinary Opinion Survey used in Chapter 2

Spotty Liver Disease - Veterinarian Opinion Survey

Vet Survey ID _____

Digital Consent Form

This survey has been commissioned by the Australian Egg Limited and involves team of researchers from University of Sydney. You are invited to participate in this research project because it will give you an opportunity to directly impact the future of Spotty Liver Disease research in Australia. The survey results will underpin the commissioning of rational, evidence based research projects to address the important scientific and commercial questions pertaining to Spotty Liver Disease in laying hens.

Your participation in this research study is appreciated.

The procedure involves completing an online survey that will take approximately 10 minutes. Your identity will be kept confidential. The survey staff will be more than happy to contact you directly (phone at a time suitable to you) to ask the survey questions (as opposed to the online survey) if preferred.

If you have any questions about the research study, please contact:

Yuanshuo (Karen) Gao | Poultry Research Foundation
The University of Sydney | 425 Werombi Road, Camden, NSW 2570
E: yuanshuo.gao@sydney.edu.au | M: 0401 083 668

Peter Groves | Poultry Research Foundation
The University of Sydney | 425 Werombi Road, Camden, NSW 2570
E: peter.groves@sydney.edu.au | M: 0418 118 005

This research has been reviewed according to University of Sydney Human Ethics procedures for research involving human subjects.

ELECTRONIC CONSENT: Please select your choice below.

Clicking on the "yes" button below indicates that:
• you have read the above information
• you voluntarily agree to participate

If you do not wish to participate in the research study, please decline participation by clicking on the "no" button.

◯ Yes ◯ No

Spotty Liver Disease Risk Factors

Q1. Do you think there are risk factor(s) involved in the pathogenesis of Spotty Liver Disease, other than the presence of the pathogen Campylobacter hepaticus itself? ◯ Yes ◯ No

Q2. Do you consider feed formulation to be a risk factor? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q3. Do you consider feed quality to be a risk factor? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q4. Do you consider weather conditions to be a risk factor? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q5. Do you consider shed environmental condition to be a risk factor? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q6. Do you consider physiological stress to a risk factor e.g. onset of lay or peak lay? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q7. Do you consider water to be a risk factor for SLD? e.g. contamination ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q8. Do you consider bird health status to be a risk factor for SLD? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q9. Do you consider poor biosecurity to be a risk factor for SLD? ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q10. Do you consider behavioural issues to be a risk factor for SLD? e.g. cannibalism. ◯ Yes ◯ No

Please explain:

_____
(If no, why? If yes, how?)

Q11. In your opinion, are there any other risk factor(s) to SLD?

Thank you for your time!

Appendix II: Preliminary study questionnaire - Rearing used in Chapter 3

1. Personal Info

SLD Trial ID	_____
Date	_____
Participant name	_____
Contact number	_____
Email	_____
Farm owner	_____
Farm manager	_____

Other

Any comments

2. Property Info

Company name _____

Farm name _____

Farm address _____

Property Identification Number (PIC) _____

Farm size _____

Number of sheds _____

Is the site multi-aged? ◯ Yes ◯ No

If multi-age on site, how? E.g. age differences

Do you rear and lay on the same property? ◯ Yes ◯ No

Other domestic animals on property? ◯ Yes ◯ No

What domestic animals are they

Water Sources

Water source for drinking

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for cooling

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for washing

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for irrigation

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Any run off into surface water?

_____

Any water treatment(s)?

Any regular water testing(s)

_____
(What and frequency)

Other

Any comments

3A. Shed Interior

Trial Shed

Trial shed number

_____

Shed type

◯ Cage ◯ Barn ◯ Aviary ◯ Free Range ◯ Other

Other shed type (please specify and describe)

Number of bays

_____

Shed Dimensions

Shed width (m)

_____

Shed length (m)

_____

Shed Floor

Shed surface area (m2)

_____

Floor Type

◯ Concrete floor ◯ Clay floor ◯ Slats only ◯ Concrete with slats ◯ Clay with slats

Slats

Slat width (m)

_____

Slat length (m)

_____

Slatted area (m2)

_____

Slat coverage (%)

_____

Platforms

Platforms in the shed ◯ Yes ◯ No

Platform design
(e.g. where, material, shape)

Number of platforms in shed _____

Platform width (m) _____

Platform length (m) _____

Platform surface area (m2) _____

Total platform areas in shed _____

Total Useable Area

Total useable area (m2) _____

Perches

Perches in the shed ◯ Yes ◯ No

Perch design
(e.g. where, material, shape)

Perch length per perch (m) _____

Number of perches in shed _____

Total perch length in shed (m) _____

Aviary Shed

Number of aviary unit(s) _____

Aviary unit design

Litter

Litter in the shed ◯ Yes ◯ No (faeces only)

Litter material type _____

Litter depth (cm) _____

Litter quality ◯ Dry ◯ Wet

Other

Any comments

3B. Shed Feeding System

Feeding System	
Feeding sytem type	◯ Pan feeders ◯ Chain feeders ◯ Other
Pan brand	_____
Pan model	_____
Pan shape	◯ Round ◯ Eclipse
Pan diameter (cm)	_____
Feeding position size	_____ (Width (cm))
Feeding positions per pan	_____ (How many per pan)
Number of feeder lines in the shed	_____
Number of feeder pans per line	_____
Total number of feeder pans in the shed	_____
Total number of feeding positions in the shed	_____
Distance between pans (cm)	_____ (Rim to rim)
Pan height off floor (cm)	_____ (Height at rim)
Feeder line cable	◯ With cable ◯ Without cable ◯ Both (Prevent pans from swinging)

Chain Feeders Only	
Chain feeder brand	_____
Chain feeder model	_____
Number of chain feeders in the shed	_____
Length of each chain feeder line (m)	_____
Total length of chain feeder in the shed (m)	_____
Other feeder type (please specify and describe)	

3C. Shed Drinking System

Drinking System	
Drinking system type	◯ Nipple drinkers ◯ Other
Nipple Drinkers only	
Drinker model	_____
Drinker brand	_____
Number of drinker lines in the shed	_____
Number of drinker nipples per line	_____
Total number of drinker nipples per shed	_____
Distance between drinker nipples (cm)	_____
Drinker height off floor (cm)	_____ (Height at drinker nipple)
Drip cups	◯ With cups ◯ Without cups ◯ Both
Other drinker type (please specify and describe)	

3D. Range Info

Pop Hole

Position of pop holes

◯ One side only ◯ Both sides
◯ Other

Other pop hole positions (please specify and describe)

Total number of pop holes

_____

Size of pop holes

_____
(Width (cm))

Range Setup

Range design

Range size

_____
(Please specify unites)

Vegetation on range

☐ Grass ☐ Shrubs ☐ Trees ☐ No vegetation (mainly dirt floor)

Mowing frequency

_____

Shade on range

_____

Feed access on range

◯ Yes ◯ No

Drinking water on range

◯ Yes ◯ No

Water on Range	
Water body on range	◯ Yes ◯ No
Access to water body on range	◯ Yes ◯ No
Ditches/holes/drains when it rains	_____

Other Animals	
Predators on range	◯ Yes ◯ No
What predators are they	_____
Wildlife on range	◯ Yes ◯ No
What wildlife are they	_____
Other domestic animals on range	◯ Yes ◯ No
What domestic animals are they	_____

4A. Bird History

Bird Source	
Breed	_____
Hatchery	_____
Date of hatch	_____
Date of delivery to rearing farm	_____
Age of birds at visit (weeks)	_____
Number of birds delivered	_____
Number of birds on hand	_____
Intended laying site and shed	_____
Distance from laying farm (km)	_____
Intended delivery age to laying farm (weeks)	_____

Bird History	
Beak trimming	◯ None ◯ Once ◯ Twice
Beak trimming age(s)	_____
Rearing vaccination program	_____ (Age, strain, how, %)
Was there any known issues with vaccination this batch?	_____
Any monitoring tests available for this batch e.g. serology	_____

Standard worming program

_____

Attending vet and frequency

_____

Other

Any comments

4B. Bird Info

Bird Weight

Last body weight available (kg)

_____

How old was the flock at last weighing (weeks)

_____

Bird weight at end of rearing (kg)

_____
(Will this be available to us for this flock?)

Feeder Space - Pan

Feed space at delivery - Pan (birds per pan)

_____

Feed space at visit - Pan (birds per pan)

_____

Feeding position ratio at delivery - Pan (birds per feeding position)

_____

Feeding position ratio at visit - Pan (birds per feeding position)

_____

Feeder Space - Chain

Feed space at delivery - Chain (cm per bird)

_____

Feed space at visit - Chain (cm per bird)

_____

Drinker Space

Drinker space at delivery (birds per nipple)

_____

Drinker space at visit (birds per nipple)

_____

Perch Space

Perch space at delivery (cm per bird)

_____

Perch space at visit (cm per bird)

_____

Density - Floor

Stocking density at delivery - Floor (bird per m2)

_____

Stocking density at visit - Floor (bird per m2)

_____

Density - Useable Space

Stocking density at delivery - Useable Space (bird per m2)

_____

Stocking density at visit - Useable Space (bird per m2)

_____

Free Range Density

Free range density at delivery (m2 per 1000 bird)

_____

Free range density at visit (m2 per 1000 bird)

_____

Range Access

First let out age (wk)

_____

Range let out time

_____
(In 24hr format)

Range close time

_____
(In 24hr format)

Range access per day (hr)

_____

What percentage of flock uses the range (%)

_____

What percentage of the range is used by the birds (%)

_____

Does allowed range access vary with season and weather

◯ Yes ◯ No

How?

_____

4C. Bird Feed And Water

Feed Source

Feedmill

_____

Nutritionist

_____

Feed specifications available?

_____

Ration(s) since placement

_____

Age(s) of ration change (weeks)

_____

Feed type for each ration (e.g. crumble, mash, pellet)

_____

Feeding program

◯ Ad lib ◯ Restricted feeding

Feed management practice

◯ Varied feed run times
◯ Midnight runs ◯ N/A
◯ Other

Feed management practice - other

_____

Feed Additives

Feed additives?

☐ Probiotics ☐ Prebiotics ☐ Essential oils ☐ Organic acids ☐ Oregano ☐ Other
☐ N/A

Feed additives - other

_____

Feed additives - please specify

Feed Intake

Feed allocation per bird for each ration (g/bird/day)

_____

Total quantity of each ration ordered (T)

_____

Actual feed intake per bird for each ration at end of rearing (g/bird)

_____

(Will this info be made available?)

Change(s) in daily feed intake, when?

_____

(e.g. interruptions, sudden increase or decrease)

Water Intake

Water measurement available? ◯ Yes ◯ No

Water intake rate/ pattern

_____

Change(s) in daily water intake, when?

_____

(e.g. any interruptions, sudden increase or decrease)

Any water additives given this batch?

_____

Other

Any comments

4D. Bird Lighting

Light schedule (from day old)

_____

Change(s) in light schedule, when?

_____
(e.g. any interruptions)

Type of light units

_____

Dimmable light

_____

Colour of light

_____

Light intensity (lux)

_____

How many rows of lights in the shed

_____

Number of light units per row

_____

Total number of light units in the shed

_____

Other

Any comments

4E. Bird Environment Condition

Ventilation

Shed ventilation system	◯ Natural ventilation ◯ Tunnel ventilation ◯ Other
Shed ventilation system - other	_____
Any additional cooling system(s)	_____
Any known ventilation issues this batch?	_____

Environment Conditions

Any temperature and humidity monitoring system available?	_____
Targeted temperature range at time of visit (C)	_____
Maximum temperature reached in summer inside the shed (C)	_____
Maximum temperature reached in summer outside the shed (C)	_____
Targeted humidity range (%)	_____
Maximum humidity reached in summer inside the shed (%)	_____
Maximum humidity reached in summer outside the shed (%)	_____

Ammonia

Is ammonia an issue in the shed?	_____
Ammonia reading, if available	_____

Other

Any comments

4F. Bird Health

Bird Health

Total mortality at time of visit (%)

_____

Main cause(s) of mortality so far

_____

Disease history (for this flock, shed and farm)

_____

Antimicrobial history (for this flock, shed and farm)

_____

Any known antimicrobial resistance(s) on this farm?

_____

Any pecking in the flock at time of visit

_____

Pecking% (if applicable)

_____

Other medication history

_____
(E.g. worming, anti-coccidials)

Enteric Health

Has the flock experienced any diarrhoea event(s) (abnormal droppings)?

_____

Has the flock had coccidiosis? Were they treated?

_____

Has the flock had roundworm infestation? Were they treated?

_____

Has the flock had tapeworm infestation?

_____

Has the flock had bacterial enteritis? E.g. Gray gut? Were they treated?

_____

Other Issues

Has the flock experienced a peritonitis problem?

_____

Has the flock expereinced external parasite infestition? Was this treated? E.g. red mites, lice

_____

Any other diseases occurred in this flock? E.g. ILT, IB etc

_____

Has the flock experienced any wildlife invasion?

_____

Has the flock experience any smothering problems?

_____

Any other known cause(s) to mortality not mentioned above? E.g. Ventilation issues

_____

Other

Any comments

5. Biosecurity

Biosecurity	
Farm specific biosecurity plan available	◯ Yes ◯ No
Vehicle disinfection equipment available	◯ Yes ◯ No
Bird (pullet) crate disinfection	◯ Yes ◯ No
Any equipment sharing with another farm?	_____
Any personnel sharing with another farm?	_____
Any contractor sharing with another farm?	_____
Quarantine period between farms of same operation (days)	_____
Quarantine period required from another operation (days)	_____
Any known vector connection with other SLD positive farms?	_____ (E.g. Vehicle, personnel)
Shed manure disposal practice	_____
Dead bird disposal practice	_____
Shed resting time prior to this placement	_____
Shed cleaning and sanitisation at the end of last batch	_____
What was the rodent burden like in this shed (and range)?	_____

What is the rodent control program?

_____

What was the fly burden liken in this shed?

_____

What is the pest control program e.g. beetles and flies

_____

Other

Any comments

Appendix III: Preliminary study questionnaire - Laying used in Chapter 3

1. Company Info

SLD Trial ID	_____
Date	_____
Company name	_____
Farm name	_____
Farm owner	_____
Farm manager	_____
Participant name	_____
Farm address	_____
Postal address	_____
Contact number	_____
Email	_____
Company Info e.g. no. of rearing and/or laying farms; bird numbers; production types	_____

Other

Any comments

2. Property Info

Property Identification Number (PIC)

_____

Property size (production area)

_____

Total number of sheds on the farm

_____

Total number of birds on the farm

_____

Water Sources

Water source for drinking

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for cooling

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for washing

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Water source for irrigation

☐ Town water ☐ Bore water ☐ Dam water ☐ Rain water ☐ Other

Any run off into surface water?

_____

Any water treatment(s)?

Any regular water testing(s)

_____
(What and frequency)

Other

Any comments

3A. Shed Interior

Trial Shed

Trial shed number

_____

Shed type

◯ Free range ◯ Barn
◯ Other

Other shed type (please specify and describe)

Shed Dimensions

Number of bays

_____

Shed width (m)

_____

Shed length (m)

_____

Shed height (m)

_____

Shed Floor

Shed surface area (m2)

_____

Floor Type

◯ Concrete floor only ◯ Clay floor only ◯ Slats only ◯ Concrete with slats ◯ Clay with slats

Slat coverage (%)

_____

Platforms

Platforms in the shed

◯ Yes ◯ No

Platform design

Number of platforms in shed

_____

Platform width (m)

_____

Platform length (m)

_____

Platform surface area (m2)

_____

Total platform areas in shed

_____

Do birds use platform regularly? Any issue with getting birds onto platforms?

Perches

Perches in the shed ◯ Yes ◯ No

Perch design

Perch length per perch (m)

_____

Number of perches in shed

_____

Total perch length in shed (m)

_____

Perch space per bird (if known)

_____

Do birds use perches regularly? Any issue with getting birds onto perches?

Nest Boxes

Nest box type

_____

Nest box brand

_____

Nest box model

_____

Nest box design

_____

Rows of nest boxes in shed

_____

Number of nest boxes per row

_____

Total nest boxes in shed

_____

Nest box width (m)

_____

Nest box length (m)

_____

Nest box height (m)

_____

Nest box size (m2)

_____

Total nest box area in shed (m2)

_____

Nesting space per bird (if known)

_____

Any issue with getting birds to use nestboxes?

Total Useable Floor Area

Total useable floor area (m2)

_____

Useable floor area per bird (if known)

_____

Litter

Litter in the shed ◯ Yes ◯ No (faeces only)

Litter material type

_____

Litter depth (mm)

_____

Litter quality ◯ Dry ◯ Wet

3B. Shed Feeding System

Feeding System

Feeding sytem type	◯ Pan feeders ◯ Chain feeders ◯ Other
Pan brand	_____
Pan model	_____
Pan shape	◯ Round ◯ Eclipse
Pan diameter (cm)	_____
Feeding position size	_____ (Width (cm))
Feeding positions per pan	_____ (How many per pan)
Number of feeder lines in the shed	_____
Number of feeder pans per line	_____
Total number of feeder pans in the shed	_____
Distance between pans (cm)	_____ (Rim to rim)
Feeder line cable	◯ With cable (electrified) ◯ With cable (non-electrified) ◯ Without cable

Chain Feeders Only

Chain feeder brand	_____
Chain feeder model	_____
Chain feeder design	_____

Number of chain feeders in the shed _____

Length of each chain feeder line _____

Total length of chain feeder in the shed _____

Other Feeding System

Other feeder type (please specify and describe)

Feeder Accessibility

What percent of feeding spaces are accessible to birds at time of feeding? e.g. 100%, 75%, 50% or less. _____

What are the reason(s) if less than 100% feed accessibility?

Other

Any comments

3C. Shed Drinking System

Drinking System

Drinking system type ◯ Nipple drinkers ◯ Other

Nipple Drinkers only

Drinker model _____

Drinker brand _____

Number of drinker lines in the shed _____

Number of drinker nipples per line _____

Total number of drinker nipples per shed _____

Distance between drinker nipples (cm) _____

Drip cups ◯ With cups ◯ Without cups ◯ Both

Other Drinker Type

Other drinker type (please specify and describe)

Drinker Accessibility

What percent of drinkers are accessible to birds at time of drinking? e.g. 100%, 75%, 50% or less. _____

What are the reason(s) if less than 100% feed accessibility?

Other

Any comments

3D. Distance Between Structures

From Feed	
Feed line to Feed line (max)	_____ (Rim to rim)
Feed line to Feed line (min)	_____ (Rim to rim)
Feed line to drinker line (max)	_____
Feed line to drinker line (min)	_____
Feed line to nest boxes (max)	_____
Feed line to nest boxes (min)	_____
Feed line to pop holes (max)	_____
Feed line to pop holes (min)	_____
Feed line to perch (max)	_____
Feed line to perch (min)	_____
Feed line to platform (max)	_____
Feed line to platform (min)	_____

From Drinker	
Drinker line to drinker line (max)	_____
Drinker line to drinker line (min)	_____
Drinker line to nest boxes (max)	_____

Drinker line to nest boxes (min)

_____

Drinker line to pop holes (max)

_____

Drinker line to pop holes (min)

_____

Drinker line to perch (max)

_____

Drinker line to perch (min)

_____

Drinker line to platform (max)

_____

Drinker line to platform (min)

_____

From Nest Boxes

Nest boxes to pop holes (max)

_____

Nest boxes to pop holes (min)

_____

Nest boxes to perch (max)

_____

Nest boxes to perch (min)

_____

Nest boxes to platform (max)

_____

Nest boxes to platform (min)

_____

3E. Range Design and Access

Pop Hole

Position of pop holes

◯ One side only ◯ Both sides
◯ Other

Other pop hole positions (please specify and describe)

Pop hole design

_____

Total number of pop holes

_____

Height of pop holes (cm)

_____

Width of pop holes (cm)

_____

Range Boundary

Fencing type

_____

Can this flock access/mingle with other birds from a different flock/range?

_____

Range Setup

Range design

Range size

_____
(Please specify unites)

Vegetation on range

☐ Grass ☐ Shrubs ☐ Trees ☐ No vegetation (mainly dirt)

Other vegetations

Mowing frequency

_____

Irrigation frequency

_____

Shade on range

_____

Feed access on range ◯ Yes ◯ No

If feed on range, please describe.

_____

Drinking water on range ◯ Yes ◯ No

If drinking water in range, please describe.

_____

Other Water Body on Range

Are there any water bodies on range e.g. dam, pits normally, or when rains?

_____

Can birds access to these water bodies on range?

_____

Are there any ditches/holes/drains when it rains?

_____

Animals on Range

Predators on range ◯ Yes ◯ No

What predators are they

_____

Wildlife on range ◯ Yes ◯ No

What wildlife are they

_____

Domestic animals on range e.g. dogs ◯ Yes ◯ No

What domestic animals are they?

_____

Range Access

First let out age (wk)

_____

Range let out pattern (open and close)

_____
(In 24hr format)

Range access per day (hr)

_____

What percentage of flock uses the range (%)

_____

What percentage of the range is used by the birds (%)

_____

Does allowed range access vary with season and weather, and how?

_____

Any interruption(s) to range let out this batch? When and why?

_____

Other

Any Comments

_____

4A. Bird History

Bird Source	
Breed	_____
Hatchery	_____
Date of hatch	_____
Rearing site and shed	_____
Distance from rearing farm	_____
Date transferred	_____
Age transferred (wk)	_____
Number of birds transferred	_____
Number of birds on hand	_____

Bird History - General	
Laying vaccination program	_____ (Age, strain, how, %)
Any monitoring tests e.g. serology	_____
Any worming treatment in lay	_____

Other

Any comments

4B. Bird Space and Density

Free Range Density

Free range density (per bird)

_____

Feeder Space

Feeder space (per bird)

_____

Drinker Space

Drinker space (per bird)

_____

Perch Space

Perch space (per bird)

_____

Nest Box Space

Nest box space (per bird)

_____
(bird/m2 or bird/hole)

Stocking Density

Stocking density at delivery

_____

Stocking density at visit

_____

Other

Any Comments

_____

4C. Bird Feed and Water

Feed Source

Nutritionist

_____

Feedmill

_____

Feed specifications available?

_____

Ration(s) since arrival

☐ Prelay ☐ Layer ☐ Other

Please specify other ration type.

_____

Age(s) of ration change

_____

Feed type (e.g. mash, pellet)

_____

Feeding Practice

Feeding program

☐ Ad lib ☐ Restricted feeding

Please describe feeding program.

_____

Additional feed management practice

☐ Varied feed run times
☐ Midnight runs ☐ N/A
☐ Other

Please describe.

_____

Do the pans or chains ever run empty?

◯ Yes ◯ No

How long would the pans or chains be empty for?

_____

How often is this practice done?

_____

Feed Additives

Feed additives?

☐ Probiotics ☐ Prebiotics ☐ Eubiotics ☐ Organic acids ☐ Oregano ☐ Other ☐ N/A

Please specify:

_____

Feed additives specifically for Spotty Liver Disease?

☐ Probiotics ☐ Prebiotics ☐ Eubiotics ☐ Organic acids ☐ Oregano ☐ Other ☐ N/A

What are the feed additive(s) for SLD?

_____

Feed Intake

Feed allocation per bird for each ration (g/bird/day)

_____

Total quantity of each ration ordered (T)

_____

Actual feed intake per bird for each ration by the end of 35wk (g/bird)

_____

Feed intake at 5%HD

_____

Feed intake at 60%HD

_____

Feed intake at peak lay

_____

Did SLD affect feed intake (If applicable)? ◯ Yes ◯ No

Feed intake before SLD occurred (if applicable).

_____

Feed intake at time of SLD (if applicable).

_____

Any feed change(s) or interruption(s), when?

_____

(e.g. interruptions, sudden increase or decrease)

Water Intake

Water measurement	◯ Yes ◯ No
Water intake rate/pattern	_____
Any interruptions to water intake, when?	_____ (e.g. any interruptions, sudden increase or decrease)
Any water additive(s) given this batch? What and frequency.	_____
Any water additive(s) specifically for Spotty Liver Disease (if applicable)?	_____

Other

Any comments

4D. Bird Lighting

Light Program

Light schedule upon arrival (from rearing)

_____

Light schedule for laying (L:D)

_____

Changes in light schedule, when?

_____

Type of light units

_____

Are lights dimmable to mimic sunlight and sunset?

_____

Colour of light

_____

Light intensity (lux)

_____

Other

Any comments

4E. Bird Environment Condition

Ventilation System

Shed ventilation system

◯ Natural ventilation ◯ Tunnel ventilation ◯ Other

Please describe:
e.g. cooling, fans etc

_____

Any known ventilation issue(s) this batch?

_____

Shed Environment

Any temperature and humidity monitoring system available?

_____

Targeted temperature at time of visit (C)

_____

Maximum temperature reached in summer OUTSIDE the shed (C)

_____

Maximum temperature reached in summer INSIDE the shed (C)

_____

Targeted humidity range (%)

_____

Maximum humidity reached in summer OUTSIDE the shed (%)

_____

Maximum humidity reached in summer INSIDE the shed (%)

_____

Ammonia

Is ammonia an issue in the shed?

_____

Ammonia reading, if avaiable

_____

Other

Any comments

4F. Bird Weight and Egg Production

Weighing

How often are birds weighed? Method?

_____

Egg Production and Bird Weights

Can weekly WEIGHTS, UNIFORMITY and MORTALITY information be supplied?

_____

Body weight at arrival (kg)

_____

Age of first egg (week)

_____

Body weight at first egg (kg)

_____

Age of 5%HD (week)

_____

Body weight at 5%HD (kg)

_____

Age of 60%HD (week)

_____

Body weight at 60%HD (kg)

_____

Age of peak lay (week)

_____

What is the peak lay%

_____

Body weight at peak lay (kg)

_____

Body weight at onset of SLD (kg) (if applicable)

_____

Egg Collection

Over what period in the day are hens laying?

_____

What time(s) in the day are eggs collected?

_____

How are eggs collected? E.g. egg belt or manual collection?

_____

Any issues with egg collection so far? e.g. interruption, belt breakdown etc

_____

Laying Abnormality

Any issues with egg laying so far?

_____

Has the flock had a high level of prolapses? When?

_____

Has the flock had a high level of double yolk eggs? When?

_____

Has the flock had abnormal egg sizes for age e.g. small or large? When?

_____

Has the shell quality, albumen quality and yolk colour been satisfactory since early lay?

_____

Has there been any problem with floor eggs/ eggs outside?

_____

What's floor egg%?

_____

Other

Any comments

4G. Bird Health

Veterinarian

Attending veterinarian and frequency

Was veterinary intervention required for this batch?
Visit or over the phone?
What was the outcome?

Any diagnostic tests performed? What are the results?

Feather Pecking

Any pecking/unusual feather loss or cannibalism been noted so far? When?

Any pecking/unusual feather loss in the flock at time of visit?

Pecking% (if applicable)

Feather coverage score (50 birds)

Moulting

Has the flock gone through a partial or full moult since arrival? When?

Cause(s) of the moulting?

Diseases - General

Disease history (for this flock, shed and farm)

Total mortality at time of 35 weeks of age (%)

Total mortality at time of visit (%)

Main cause(s) of mortality so far

_____

Antimicrobial history (for this flock, shed and farm)

_____

Any known antimicrobial resistance(s)?

_____

Other medication history

_____
(E.g. worming, anti-coccidials)

Has the flock experienced any diarrhoea event(s) e.g. abnormal droppings?

_____

Has the flock had coccidiosis? Were they treated?

_____

Has the flock had roundworm or tapeworm infestation? Were they treated?

_____

Has the flock had bacterial enteritis e.g. gray gut? Were they treated?

_____

Other Health Issues

Has the flock experienced a peritonitis problem?

_____

Has the flock experienced external parasite infestation e.g. red mites, lice? Were they treated?

_____

Any other disease occurred during this batch? e.g. ILT, IB, Cholera, Mycoplasma etc?

_____

Has the flock experienced any wildlife invasion this batch?

_____

Has the flock experienced any smothering problems?

_____

Has the flock experienced any production problems?

_____

Has the flock experienced any unexplained mortality or production problems not mentioned above?

_____

Other

Any comments

5. Biosecurity

Biosecurity	
Farm specific biosecurity plan available	◯ Yes ◯ No
Vehicle disinfection equipment available	◯ Yes ◯ No
Bird (pullet) crate disinfection	_____
Any equipment sharing with another farm?	_____
Any personnel sharing with another farm?	_____
Any contractor sharing with another farm?	_____
Quarantine period between farms of the same operations (days)	_____
Quarantine period required from another operation e.g. visitors (days)	_____
Any known vector connect with other SLD positive farms?	_____ (e.g. contractors, vehicle)

Waste Disposal	
Shed manure disposal practice	_____
Dead bird disposal practice	_____

Cleaning and Disinfection

Shed/range resting time prior to this placement

_____

Shed/range cleaning and sanitisation at the end of last batch

_____

Rodent burden in shed and range e.g. light, moderate, heavy?
Rodent control program?

_____

Pest control e.g. beetles and flies?

_____

Other

Any comments

6. Spotty Liver Disease Questions

Spotty Liver Disease History

Does this company has a history of spotty liver disease?

_____

Does this farm has a history of spotty liver disease?

_____

Are there any changes to the disease experience since the first outbreak? e.g. the disease pattern, severity and/or response to treatment? Any possible reasons?

Did this flock experience any spotty liver disease outbreak(s)? ◯ Yes ◯ No

SLD Outbreak (if applicable)

How many times has SLD occurred so far?

_____

Date of outbreak(s)

_____

Age of outbreak(s)

_____

What were the signs that suggested that SLD was occurring? e.g. mortality rate, production loss, bird depression?

What was the mortality pattern like (before and) during the SLD outbreak? Bird numbers and/or %

How long did the SLD outbreak(s) last for?

_____

Estimated total mortality due to SLD (number of birds or %).

_____

Estimated total egg production losses due to SLD

_____

Did a poultry veterinary diagnose the SLD or confirmed the diagnosis (this batch)?

◯ Yes, by visit ◯ Yes, over the phone ◯ No

How was SLD diagnosed or confirmed by the veterinarian?

☐ Clinical signs ☐ Gross Pathology ☐ Histopathology ☐ PCR ☐ Culture ☐ By treatment
☐ Not sure

SLD Treatment and Management

Did this flock receive any preventative treatment(s) for SLD? E.g. any in feed or in water treatment, or vaccination specific for SLD

How effective were they?

Did this flock receive any antibiotic treatment(s) for SLD e.g. at the time of clinical outbreak?

How effective were they?

(What drug, for how long, dose rate.)

What were the signs/triggers that suggested that antibiotic treatment(s) was required? e.g. mortality rate, production loss, bird depression?

Did this flock receive any non-antibiotic treatment(s) for SLD e.g. at the time of clinical outbreak?

How effective were they?

(e.g. any in water or in feed treatment specifically for SLD)

Has SLD recurred after any of therapeutic treatment(s)?
If yes, how soon?

Any other management strategies not mentioned above applied to prevent the occurrence of SLD or to minimise SLD impact?

Rainfall

Were there any rain event(s) since arrival? ◯ Yes ◯ No

When and how often did the rain occur?

Did birds access the range during and/or after rain?

Did birds drink from puddle water from the range? ◯ Yes ◯ No

Were the birds restricted from range access for any period of time during rainfall?	◯ Yes ◯ No
If restricted access, for how long and how did the birds respond to it, were they stressed?	_____
Did SLD follow any rain event(s)? E.g. within 1-2 weeks (if applicable)	_____
How soon did SLD follow the rain? (if applicable)	_____

Heat Stress

Has there been any ventilation breakdown or interruption during this batch that may have led to SLD?	_____
Were there any extreme heat event(s) since arrival? E.g. >35C	◯ Yes ◯ No
When and how often did the extreme heat occur?	_____
How were the bird kept cool during extreme heat?	_____
Did SLD follow any extreme heat event(s)? E.g. within 1-2 weeks (if applicable)	_____
How soon did SLD follow the extreme heat? (if applicable)	_____

Other environmental Stress

Any other unusual climate/weather event(s) since arrival?	◯ Yes ◯ No
If yes, what unusual climate/weather events occurred?	_____
Did SLD follow any of these unusual weather event(s)? E.g. within 1-2 weeks (if applicable)	_____
How soon did SLD follow the unusual climate/weather event? (if applicable)	_____

Any Interruption(s) to Normal Routine and SLD

Were there any feed change(s) or interruption(s) linked to SLD?	◯ Yes ◯ No
If yes, how and when did the feed change(s) or interruption(s) occur?	_____
Were there any water interruption(s) linked to SLD?	◯ Yes ◯ No
If yes, how and when did the water interruption(s) occur?	_____
Were there any malfunction(s) to next boxes or egg belt linked to SLD?	◯ Yes ◯ No
If yes, how and when did the egg collection malfunction(s) occur?	_____
Were there any unintended interruptions to range access linked to SLD?	◯ Yes ◯ No
If yes, how and when did the range access interruption(s) occur?	_____
Did SLD follow any of the above interruptions (feed, water, egg collection, range access)? E.g. within 1-2 weeks (if applicable)	_____

Behaviour and Health

Did SLD follow any pecking or moulting? E.g. within 1-2 weeks (if applicable)	_____
Did SLD follow any health issue or disease? E.g. within 1-2 weeks (if applicable)	_____

Other

Any other possible trigger(s) to SLD not mentioned above (if applicable)?	_____
Any comments	_____

Appendix IV: “Fully slatted” study survey used in Chapter 4

Fully slatted survey

Date:	
Company Name:	

	Company name				
	Layer farm name				
	Shed number				
	SLD occurance (Y/N)				
REARING FARM					
	Date hatched				
	Breed				
	Hatchery				
	Perches in rearing? (Y/N)				
	Ventilation in rearing shed: tunnel or natural				
LAYING FARM					
	Location of laying farm (Suburb)				
	Date transferred				
	Age transferred (wk)				
	Number of birds transferred				
Shed set up	Shed Type (conventional vs aviary free-range)				
	Total usable space (m2)				
	Stocking density (birds/m2)				
Slat	Slat brand				
	Slat material (e.g. plastic)				
	Is the slat cleaned during the batch? (Y/N)				
	If yes, how?				
	Is the slat cleaned between batches? (Y/N)				
	If yes, how?				
Perch	Perches in lay (Y/N)				
	Total perch length available (m)				
	Perch space in lay (cm/bird)				
Platform	Any platform(s) in shed? (Y/N)				
	Total platform area available (m2)				
	Platform space (birds/m2)				
	Is the platform cleaned during batch (Y/N)?				
	If yes, how?				
	Is the platform cleaned between batches (Y/N)?				
	If yes, how?				
Feeding System	Feeder type (chain or pan)				
	Total feed chain length (m)				
	Feed space - Chain (birds per m)				
	Total number of pans (if applicable)				
	Pan brand				
	Feed space - Pan (birds per pan)				
Drinking system	Drinker type				
	Total nipples in shed				
	Drinker space - nipple (birds per nipple)				
	Drinker space - bell (birds per bell)				
Ventilation	Ventilation in laying farm: tunnel or natural				
Lighting	Light colour (warm or cool white)				
Nesting	Nest box brand				
	Total nest space avaialble (m2)				
	Nest space (birds/m2)				
	Age of first access to nest boxes (wk)				
	Night closure (to prevent bird access) (Y/N)				
	Nest box cleaning during batch (Y/N)				
	If yes, how?				
	Nest box cleaning between batches (Y/N)				
	If yes, how?				
Range	First let out age (wk)				
	Range size (ha)				
	Range density (birds/ha)				
	Soil type				
Feed and Water	Nutritionist				
	Feedmill				
	Number of rations from arrival to 40 weeks of age				

	Company name				
	Layer farm name				
	Shed number				
	SLD occurance (Y/N)				
	Ration 1 start age (wk)				
	Ration 2 start age (wk)				
	Ration 3 start age (wk)				
	Ration 4 start age (wk)				
	Ration 5 start age (wk)				
	Any in feed additives in lay till 40 weeks of age (Y/N)				
	Feed Additive 1 (if applicable)				
	Feed Additive 2 (if applicable)				
	Feed Additive 3 (if applicable)				
	Feed Additive 4 (if applicable)				
	Any water additives in lay till 40 weeks of age (Y/N)				
	Water Additive 1 (if applicable)				
	Water Additive 2 (if applicable)				
	Water Additive 3 (if applicable)				
	Water Additive 4 (if applicable)				
Arrival	Body weight at arrival (kg)				
First Egg	Age at first egg (wk)				
5% HD	Age at 5% HD (wk)				
	Feed intake at 5%HD (g/bird/day)				
	Body weight at 5%HD				
60%HD	Age at 60% HD				
	Feed intake at 60%HD (g/bird/day)				
	Body weight at 60%HD				
Peak lay	Age at peak lay (wk)				
	Peak lay %HD				
	Feed intake at peak lay (g/bird/day)				
	Body weight at peak lay (kg)				
SLD outbreak (if applicable)	Age at SLD outbreak (wk)				
	Feed intake at SLD (g/bird/day)				
	Body weight at SLD (kg)				
	Lowest %HD during SLD				
	How long did				
	Highest daily mort% during SLD				
40 weeks	%HD at 40 weeks				
	Feed intake at 40wks (g/bird/day)				
	Body weight at 40wks (kg)				
	Total mortality at 40wks				
Egg production	Any problem with floor eggs? (Y/N)				
	Floor egg% at peak lay				
	Age occurred				
	Duration of floor egg issue (>2%) (days)				
Disease	Other disease other than SLD (Y/N)				
	Age of disease occurance (wk)				
	Any antibiotic treatment not for SLD?				
SLD SPECIFIC	Did SLD occur this batch (Y/N)				
	Has SLD reoccured so far?				
	Age of outbreak 1 (wk)				
	How long did the first outbreak last?				
	Age of outbreak 2 (wk, if applicable)				
	How long did the second outbreak last?				
	SLD diagnosis based on (select all that apply):				
	Clinical signs (depression)				
	Gross pathology (white spots)				
	Histopathology				
	PCR				
	Culture				
	By treatment				
	Not sure				
	Was this flock treated with antibiotics for SLD (Y/N)				
	What antibiotic was used?				
	Other antibiotic used, please specify:				
	Range access prior to SLD outbreak?				
	Any comments on SLD outbreak of this flock not covered above?				

Appendix V: “Scratch area” study survey used in Chapters 5 and 6

SPOTTY LIVER DISEASE SURVEY - SCRATCH AREA

	Date:				
	Company Name:				
	Layer farm name:				
	Shed number:				
	Age at visit (wk):				
	No of birds transferred:				
BIRD INFO					
	Breed				
	Hatchery				
	Rearing site				
	Perches in rearing? (Y/N)				
	Ventilation in rearing shed: tunnel or natural				
LAYING FARM					
Shed set up	Shed Type (conventional or aviary freerange)				
	Shed length (m)				
	Shed width (m)				
	Total floor area (m2)				
	Total usable space (m2)				
	What's included and/or excluded for total usable space (e.g. platform, nest space)				
	Stocking density (birds/m2)				
Perch	Perches in lay (Y/N)				
	Total perch space (m)				
	Perch space in lay (m/bird)				
Platform	Any platform(s) in shed? (Y/N)				
	Total platform (m2)				
	Platform space (birds/m2)				
	Shed number:				
Ventilation in laying farm	Shed type by ventilation (e.g. natural, roof extraction or tunnel)				
	Circulation fans (Y/N)				
	Foggers (Y/N)				
	Extraction fans (Y/N)				
	Mini-vents (Y/N)				
	Roof vents (Y/N)				
	Roof extraction/chimneys (Y/N)				
	Cool cells (Y/N)				
	Temperature set point(s)				
	Please describe ventilation methods up to 40 wks of age (or by season) e.g. how often would you put sheds in tunnel or use extraction fans if applicable				
	Comments for ventilation:				
Slat	Presence of slats (Y/N)				
	Slat area (m2)				
	Slat coverage of shed (%)				
	Slat brand				
	Slat material (e.g. plastic)				
Scratch Area	Presence of scratch area (Y/N)				
	Total scratch area (m2)				
	Scratch area coverage of shed (%)				
	Floor type (clay or concrete)				
	Any bedding material used? And what?				
	Any litter/manure amendment product used? And what?				
	Cleaning practice of scratch area (Y/N and how)				
	Comments for scratch area:				
	Shed number:				
Nest	Nest box brand				
	Total nest space (m2)				
	Nest density (birds/m2)				
	Age of first access to nest boxes (wk)				
	Night closure (to prevent bird access) (Y/N)				
Feeding System	Feeder type (chain or pan)				
	Total chain length (m)				
	Feed space - Chain (birds per m)				
	Total pans in shed				
	Feed space - Pan (birds per pan)				
	Brand of pan				
	Shape of pan (round or oval)				

Drinking system	Drinker type (nipples or bell)				
	Total no. of nipples				
	Nipple brand				
	Drinker space - nipple (birds per nipple)				
	Drinker space - bell (birds per bell)				
Lighting	Light colour (cool or warm white)				
Range	First let out age (wk)				
	Range size (ha)				
	Range density (birds/ha)				
	Range on one side or both				
Verandah	Does the shed have verandahs (Y/N)				
	Verandah on one side or both				
	Verandah width				
	Total veranda area (m2)				
	Verandah floor type (clay or concrete)				
	Weather protection				
	Cleaning practice of verandah (Y/N and how)				
	Comments for verandah:				
	Shed number:				
Egg production	Any problem with floor eggs? (Y/N)				
	Age occurred				
	Duration of floor egg issue (>2%) (days)				
	Floor egg% at peak lay				
	Comments for floor eggs:				
Feed and Water	Feedmill				
	Nutritionist				
	Number of rations from arrival to 40 weeks of age				
	Ration 1 start age (wk)				
	Ration 2 start age (wk)				
	Ration 3 start age (wk)				
	Ration 4 start age (wk)				
	Ration 5 start age (wk)				
	Any in feed additives in lay till 40 weeks of age (Y/N)				
	Feed Additive 1 (if applicable)				
	Feed Additive 2 (if applicable)				
	Feed Additive 3 (if applicable)				
	Feed Additive 4 (if applicable)				
	Any water additives in lay till 40 weeks of age (Y/N)				
	Water Additive 1 (if applicable)				
	Water Additive 2 (if applicable)				
	Water Additive 3 (if applicable)				
	Water Additive 4 (if applicable)				
	Shed number:				
Disease and mortality	**By 40wks of age:**				
	Any smothering (Y/N) and how many birds lost				
	Any pecking (Y/N) and severity				
	Any non-SLD diseases (Y/N) and how many birds lost				
	Any antibiotic used for non-SLD diseases? And what was used?				
SLD SPECIFIC	Was this flock vaccinated for SLD? (Y/N)				
	Which vaccine used?				
	Did SLD occur this batch (Y/N)				
	Range access prior to SLD outbreak? (Y/N)				
	Date of 1st outbreak				
	Age of 1st outbreak (wk)				
	How was SLD diagnosed?				
	Highest daily mort during SLD, prior to treatment				
	How long did the mortality last?				
	%HD prior to SLD outbreak				
	Lowest %HD during SLD outbreak				
	How long did production drop last for 1st outbreak?				
	Did egg production recover, to what extent?				

	Was this flock treated with antibiotics for SLD (Y/N)				
	What antibiotic was used?				
	Duration and dose rate of antibiotics				
	Did the flock respond to antibiotics				
	Shed number:				
	Was this flock treated with non-antibiotic therapy for SLD (Y/N)				
	What non-antibiotics used?				
	Duration of non-antibiotics used				
	Did SLD reoccur? (Y/N)				
	Age of outbreak 2 (wk, if applicable)				
	How long did the second outbreak last?				
	Was this flock treated with antibiotics for 2nd SLD outbreak (Y/N)				
	Any comments on SLD outbreak of this flock not covered above?				