The Epidemiology and Management of Liver Fluke in Dairy Cattle in Irrigated Regions of Victoria

Submitted by

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Abstract

Fasciola hepatica (*F. hepatica*) is a parasite more commonly known as liver fluke. Since colonisation in Australia, *F. hepatica* has been a problem in South-Eastern Australia (New South Wales, Victoria and Tasmania). The South-Eastern region of Australia has a temperate climate and ample water for the primary, intermediate host in Australia *Austropeplea tomentosa* (*A. tomentosa*). Control of *F. hepatica* in these regions relies heavily on the use of preventative flukicides, particularly triclabendazole, to treat the definitive hosts and reduce egg contamination. Diagnostic tools including liver fluke faecal egg counts (LFEC), serum ELISA (sELISA), bulk tank milk ELISA (BTM ELISA), and coproantigen ELISA (cELISA) play a crucial role in better informing *F. hepatica* control strategies on-farm. However, there has been no recent investigation of the prevalence of *F hepatica* in Victorian dairy cattle, the incidence of triclabendazole resistance in cattle or the management practices used by farmers to control liver fluke.

This thesis's focus was to: (1) apply several of these diagnostic tools to determine the prevalence of *F. hepatica* in irrigated dairy regions of Victoria; (2) assess the variation in coproantigen and egg shedding within a day (morning and afternoon milking) and between days for five days to determine the impact on the sensitivity of the tests and correlation with total fluke count; (3) determine what farm management practices dairy farmers use to control *F. hepatica* and identify what information needs to be communicated to farmers.

We found that *F. hepatica* was endemic in several Victorian dairy regions (mean prevalence 39%), with the highest prevalence (75-80%) observed in the Macalister Irrigation District. The state-wide estimated annual economic loss in milk production was $AUD 129 million. Triclabendazole-resistant *F. hepatica* was identified on three dairy farms.

During the morning and afternoon milkings, we observed a 2.6-8.8 fold range in coproantigen OD values and 5-16 fold range in liver fluke egg counts over the five days the animals were sampled; correlations for both tests with total fluke counts ranged from R= 0.58-0.78 and differences were observed between morning and afternoon samples. Sampling in the morning yielded higher positive correlations between the cELISA and LFEC tests and total fluke counts, suggesting that faecal sampling for liver fluke testing should

be collected in the morning. Our findings suggest that the cELISA and the LFEC (FlukeFinder kit®) are robust tests that can accurately detect cattle with burdens >10 flukes under Australian farm management practices.

The survey results identified four knowledge gaps where communication needs to be enhanced: diagnostic testing to inform flukicide use, rotation of flukicide actives, flukicide administration, and increased testing of replacement animals.

These studies have identified regions in Victoria at higher risk of *F. hepatica*, validated the coproantigen ELISA for fluke diagnosis in cattle and identified management options that can be utilised on-farm to mitigate production losses resulting from infection with *F. hepatica*. The research has improved our understanding of where *F. hepatica* is in Victoria, identified regions at higher risk and identified several management options that can be utilised on-farm to mitigated associated productions losses resulting from infection of dairy cattle with *F. hepatica*.

Publications

Elliott, T., Kelley, J., Rawlin, G., Spithill, T., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Veterinary Parasitology 209, 117-124.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends in Parasitology 32, 458-469.

Rathinasamy, V., Hosking, C., Tran, L., Kelley, J., Williamson, G., Swan, J., Elliott, T., Rawlin, G., Beddoe, T., Spithill, T.W., 2018. Development of a multiplex quantitative PCR assay for detection and quantification of DNA from *Fasciola hepatica* and the intermediate snail host, *Austropeplea tomentosa*, in water samples. Veterinary Parasitology 259, 17-24.

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Veterinary Parasitology 277, 109019.

Rathinasamy, V., Tran, L., Swan, J., Kelley, J., Hosking, C., Williamson, G., Knowles, M., Elliott, T., Rawlin, G., Spithill, T.W., Beddoe, T., 2021. Towards understanding the liver fluke transmission dynamics on farms: Detection of liver fluke transmitting snail and liver fluke-specific environmental DNA in water samples from an irrigated dairy farm in Southeast Australia. Veterinary Parasitology 291, 109373.

Conference presentations

JM Kelley, TP Elliott, G Rawlin and TW Spithill. Estimation of the liver fluke prevalence and economic losses from fasciolosis in dairy farms in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Australian Society of Parasitology Conference, ANU, Canberra 2014.

JM Kelley, TP Elliott, G Rawlin and TW Spithill. Establishing the prevalence of liver fluke infections in dairy cattle in the Macalister, Goulburn Valley and Upper Murray Irrigation Districts in Victoria. New Zealand and Australian Society of Parasitology Conference, Auckland,2015.

JM Kelley, TP Elliott, G Rawlin and TW Spithill. The resurgence of liver fluke in dairy cattle in irrigated regions of Victoria. Australian & New Zealand College of Veterinary Scientists Cattle Chapter, July 2015

JM Kelley, TP Elliott, G Rawlin and TW Spithill. Determining the prevalence of *Fasciola hepatica* in the Murray dairy region of Victoria, Australia. AgriBio Science Conference, September 2016.

JM Kelley, G Rawlin and TW Spithill. Determining the prevalence of *Fasciola hepatica* in the Murray dairy region of Victoria, Australia. International Congress for Tropical Medicine and Malaria, September 2016.

J.M. Kelley, V. Rathinsamay, T.P. Elliott, G. Rawlin, T. Beddoe, T.W. Spithill and M. Stevenson. The prevalence of *Fasciola hepatica* in irrigated dairy regions of Victoria, Australia. International Symposium of Veterinary Epidemiology and Economics, Chiang Mai, Thailand, 2018

Media and other

La Trobe University research assistant Jane Kelley to put liver fluke under microscope on Victorian dairy farms. ***The Weekly Times, March 2014***

Hidden cost of liver fluke. ***Vic Country Hour with Libby Price, March 2014***

Liver Fluke in Victorian Dairy Farms. ***La Trobe University Podcast interviews, May 2014***

Two new liver fluke tests developed for use on Vic dairy and beef farms. ***The Weekly Times, March 2015***

Keeping on top of liver fluke. ***Macalister Demonstration Farm Newsletter April 2015***

Healthier stock for dairy farmers. ***Science Meets Business, April 2015***

Aiming to break the cycle. ***Country News, January 2016***

Liver fluke research bid to save millions of dollars for farmers. ***The Devondaler, July 2016***

Einstein A Go-Go with Dr Shane, & Colleagues. ***Triple RRR, October 2016***

Liver fluke season is here – and it's a growing problem for beef farmers. ***Virbac, March 2018***

Finding new strategies to tackle liver fluke. ***Country News, April 2018***

Abbreviations

ABI	Abattoir inspection
ACT	Australian Capital Territory
ALA	Atlas of Living Australia
APVMA	The Australian Pesticides and Veterinary Medicines Authority
AUS	Australia
BJD	Bovine Johne's disease
BTM	Bulk tank milk
cELISA	Coproantigen ELISA
CG	Central Goulburn Irrigation District
CLOR	Clorsulon
CRT	Coproantigen reduction test
eDNA	Environmental DNA
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory-secretory antigens
FECRT	Faecal egg count reduction test
GM	Goulburn-Murray
IPM	Integrated parasite management
JDCAP	Johne’s disease Calf Accreditation Program
LFEC	Liver fluke faecal egg count
LGA	Local government area
LV	Loddon Valley Irrigation District
MC	Metacercariae
MID	Macalister Irrigation District
MV	Murray Valley irrigation district
N/A	Not applicable
NE	Nepal
NEJ	Newly excysted juvenile
No.	Number
NSW	New South Wales
NT	Northern Territory

NZ	New Zealand
OD	Optical density
ODR	Optical density ratio
OXY	Oxyclozanide
P/N	Positive and negative herds or animals
PC	Positive control
PCR	Polymerase chain reaction
PFEC	Paramphistome faecal egg count
PI	Post-infection
PNG	Papua New Guinea
PP	Percent positivity
QLD	Queensland
qPCR	Quantitative polymerase chain reaction
ROI	Republic of Ireland
SA	South Australia
SE	Sensitivity
sELISA	Serum ELISA
SP ratio	Sample positive ratio
SP	Specificity
TAS	Tasmania
TCBZ	Triclabendazole
Temp.	Temperature
TFC	Total fluke count
TIA	Torrumbarry Irrigation District
UK	United Kingdom
UM	Upper Murray Irrigation District
Unk.	Unknown
USA	The United States of America
VIC	Victoria
WA	Western Australia

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Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution."

No other person's work has been used without due acknowledgment in the main text of the thesis or below:

Chapter 2: Jane Kelley compiled the data shown in Tables 1 and 2 and Figures 1 and 2: all authors contributed to the drafting of the text. Jane and Terry Spithill were responsible for the final editing of the manuscript and the reply to the reviewers' comments.

Chapter 3: Jane Kelley was responsible for the majority of the analysis and interpretation of results and, with Terry Spithill, had a significant involvement in the drafting of the work and critical revisions that contributed to the interpretation of the work.

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Chapter 1 – Literature Review

The lifecycle, ecology and distribution of *Fasciola hepatica* in Australian cattle

1.1 Introduction

1.1.1 *Fasciola hepatica* and *Fasciola gigantica*

Infection with either *Fasciola gigantica* (*F. gigantica*) or *Fasciola hepatica* (*F. hepatica*) causes fasciolosis. *F. gigantica* is found in the tropics, whereas *F. hepatica* is found in temperate and sub-tropical regions (Mas-Coma et al., 2009). These two parasites have been found to co-exist in Vietnam, Japan and Korea, producing hybrids (Cwiklinski et al., 2016). *F. hepatica* is the most widely distributed vector born parasite in the world, infecting a wide range of unrelated hosts shown in Table 4 (Mas-Coma, 2005). The widespread distribution of *F. hepatica* is primarily due to its capacity to infect local Lymnaeidae snails (Correa et al., 2010). Globally more than 30 Lymnaeidae have been described as vectors for the parasites and are found on every continent except Antarctica (Correa et al., 2010). The global distribution of *F. hepatica*, the lack of host specificity and its ability to infect local Lymnaeidae makes the parasite a significant threat to livestock productivity and public health.

1.1.2 Global problems with fasciolosis

The global cost of fasciolosis has been estimated to be over $3 billion US per annum, and an estimated 17 million people and 300 million cattle are infected with *F. hepatica* (Spithill et al., 1999; Copeman and Copland, 2008; Cwiklinski et al., 2016). Compounding these problems are reports of triclabendazole (TCBZ) resistant *F. hepatica* in sheep, cattle and, most alarmingly, in humans, reviewed in Kelley et al. (2016) (Chapter 2). TCBZ is the preferred drug for the treatment of *F. hepatica* because it targets both the immature and mature stages of the parasite and has a high safety margin. Modelling by Haydock et al. (2016) in New Zealand (NZ) suggests that climate change is likely to expand the distribution of *F. hepatica* and increase the severity of the disease. Given that there is only a finite number of alternative drug classes at present, the loss of triclabendazole could compromise the future control of the parasite in livestock and humans.

1.1.3 *F. hepatica* in Australia

In Australia, *F. hepatica* has been a problem since the early settlement of the country, with outbreaks of fasciolosis occurring in New South Wales (NSW), Victoria (VIC) and

Tasmania (TAS) soon after the regions were first stocked (Seddon, 1950). The severity of the outbreaks led to the 1869 Royal Commission into Fluke, declaring that the parasite was a significant threat to the country's national wealth and prosperity (Penny, 1869). In the preceding year's severe losses continued in Victoria, and one property lost 25,000 sheep in a single year (Seddon, 1950). More recent work in Australia has estimated that *F. hepatica* costs the sheep industry $25 M per year and the Victorian dairy industry $129 M per year (Lane et al., 2015; Kelley et al., 2020). However, little research has investigated the distribution of *F. hepatica* in Australia.

1.1.4 PhD thesis context

This literature review explores the ecology and lifecycle of *F. hepatica* in Australia to inform control strategies, identify gaps in our knowledge and direct future research in Australia.

1.2 The life cycle of *F. hepatica*

F. hepatica is heteroxenous, relying on an intermediate snail from the family Lymnaeidae as a vector to transmit the infection to the definitive host. The life cycle of *F. hepatica* is shown in Figure 1.1.

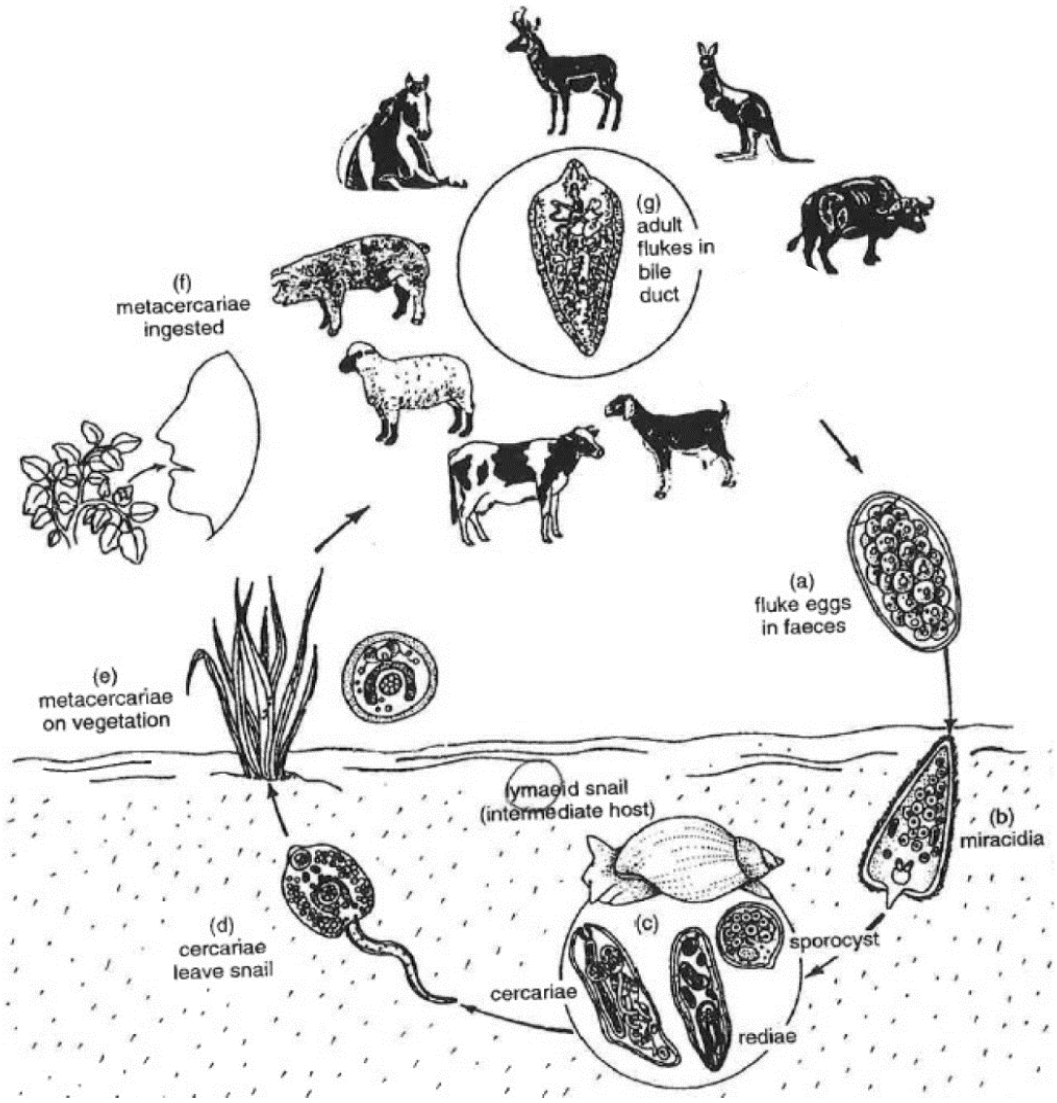

Figure 1.1. The *F. hepatica* life cycle in cattle sourced from Love et al. (2017)

1.2.1 Eggs

Adult *F. hepatica* residing within the definitive host bile ducts shed eggs that pass into the digestive system and are excreted from the host in faecal matter ((a) in Figure 1.1). The eggs remain viable in the faecal pat while it is moist, but once it dries out, death occurs rapidly (Andrews et al., 1999). Egg development occurs once the egg is liberated from the faecal matter via rainfall, irrigation, livestock or farm machinery as it has an inhibitory effect on the egg development (Boray, 1969; Andrews et al., 1999). Embryonation of the egg requires the presence of water and warm temperatures (Table 1). Egg development takes a few weeks at 23°C to 26°C, whereas development takes months if the temperature is below 15°C and days if the temperature is greater than 26°C (Boray, 1969; Andrews et al., 1999).

Table 1.1. Climatic tolerances of *F. hepatica*

	Temperature	Humidity	Duration of Survival	Reference
Embryonation	<5°C		2-2.5 years	(Boray, 1969; Andrews et al., 1999)
	23-26°C		21 days	
	30°C		8 days	
	>37°C		No development, dead within 24 days	
Miracidium lifespan	6°C		36hrs	(Boray, 1969)
	10°C		24hrs	
	25°C		6hrs	
Duration of MC[a] infectivity	-2°C		92 days	(Boray and Enigk, 1964; Boray, 1969)
	10°C		130 days	
	25°C		36 days, 50% survived 60 days	
	30°C		14 days, 20% survived 36 days	
	35°C		Died after 14 days	
MC[a] survival humidity	10°C	75%-80%	31 days	(Boray and Enigk, 1964)
	10°C	90%	122 days	
	20°C	75%-80%	3 days	
	20°C	90%	14 days, none after 27 days	
MC[a] survival on pasture	10°C	76%	70 days during winter	(Ono et al., 1954; Boray and Enigk, 1964; Meek and Morris, 1979a)
	20°C	67%	7 days during summer	
	25°C		42 days	
	25-32°C		Died in 10 days	

[a]MC – metacercariae

1.2.2 Miracidia

Sunlight or cold water triggers the release of miracidia from *F. hepatica* eggs ((b) in Figure 1.1) (Jepps, 1933; Andrews et al., 1999). After hatching, the miracidia only have a few hours to find an intermediate snail host (see Table 1). Miracidia move at a rate of 4-12 sec/cm and can travel a distance of 50m to find a snail (Boray, 1969; Andrews et al., 1999). Miracidia seek out and infect snails, relying on chance or a positive chemotactic reaction which occurs when a snail is within 15 cm of the miracidia (Neuhaus, 1953). The miracidia attack the snail's foot and mantel, which causes swelling and irritation (Boray, 1964a). Snails have varying degrees of susceptibility to *F. hepatica* infection shown in Table 2 and will often aggregate in the most susceptible snail (Anderson, 1978).

1.2.3 Intermediate hosts in Australia

The main intermediate host in Australia is the native snail *Austropeplea tomentosa* (*A. tomentosa*), which was formally known as *Lymnaea tomentosa* (shown as (c) in Figure 1.1). *A. tomentosa* is found in Australia and NZ; however, research by Puslednik et al. (2009) found that the snails had distinctly different lineages suggesting that the snails are different species. In addition, Puslednik et al. (2009) found evidence to suggest that *A. tomentosa* in Australia is three distinct species of snail. The snails have been regrouped and renamed by Ponder (2016a, 2016b, 2016c). The snails are now tentatively known as: *Austropeplea brazier* (*A. brazier*) found in NSW, Australian Capital Territory (ACT), Queensland (QLD) and VIC; *Austropeplea papyracea* (*A. papyracea*) found only in South Australia (SA); and *Austropeplea huonensis* (*A. huonensis*) found only in TAS (Puslednik et al., 2009; Ponder, 2016a, b, c). As much of the intermediate host research in Australia has focused on *A. brazier,* further research needs to assess the role *A. papyracea* and *A. huonensis* play in the epidemiology of *F. hepatica* in SA and TAS. For the remainder of the literature review, *A. tomentosa* will continue to be used to describe the main intermediate host snail in Australia, as all research to this point has considered it to be one snail. There are two other secondary intermediate hosts in Australia: *Pseudosuccinea columella* (*P. columella*) *and Radix viridis* (*P. columella*) that were both introduced to Australia in the 1970s and play a lesser role in the transmission of *F. hepatica*.

Table 1.2. Intermediate snail hosts in Australia susceptibility to *F. hepatica* infection and metacercariae production

Species	Source	Country	No. of snails infected with miracidia	Age of snail	No. of snails which produced cercariae	Average no. of MC [a] produced	Average development time of *F. hepatica* in snails	Reference
A. tomentosa	NSW [b]	AUS [c]	1250		40%	470	59 days	(Boray, 1978)
P. columella	NSW [b]	AUS [c]	1150		1%	412	114 days	(Boray, 1978)
P. columella	NSW [b]	AUS [c]	400	Young (2-4mm)	40%	151	70 days	(Boray, 1985)
P. columella	NSW [b]	AUS [c]	25	Adult (12-18mm)	0%	0	Unk [d]	(Boray, 1985)
P. columella	Unk [d]	NZ [e]	1000		18%	790	Unk [d]	(Boray, 1978)
P. columella	Unk [d]	USA [f]	21	6-11.5mm	38%	278	73 days	(Krull, 1941)
R. viridis	QLD [g]	AUS [c]	200		21%	155	59 days	(Boray, 1978)
R. viridis	Highlands	PNG [i]	125		36%	449	Unk [d]	(Boray, 1978)
R. viridis	Kathmandu	NE [j]	100		27%	214	Unk [d]	(Boray, 1978)

a MC – metacercariae

b NSW – New South Wales

c AUS – Australia

d Unk – Unknown

e NZ – New Zealand

f USA – United States of America

g QLD – Queensland

h NE – Nepal

i PNG – Papua New Guinea

j NE – Nepal

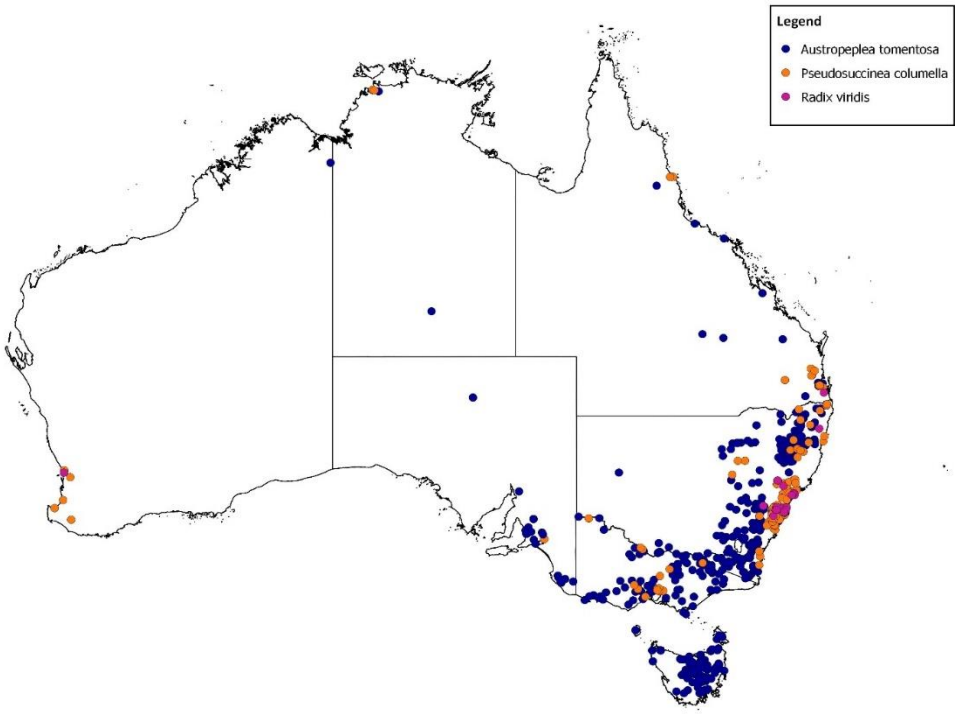

Figure 1.1. Occurrence records from 1864 to 2015 of *A. tomentosa, P. columella* and *R. viridis* in Australia. Data sourced from the Atlas of Living Australia (2020), see Appendix 1 for a complete list of contributing organisations.

1.2.3.1 Austropeplea tomentosa

A. tomentosa is distributed widely across Australia (Figure 1.2). The snail has a high susceptibility to miracidia and is the most efficient intermediate host in Australia at producing metacercaria (see Table 2). *A. tomentosa* has a high reproductive capacity, and one individual snail can produce 1265 eggs in 30 days (Boray, 1969). However, copulating does not occur below 16°C or above 30°C (see Table 3). Copulation ceases in large snail colonies and when there is a lack of aeration leading to stagnating water (Boray, 1964b). Copulation is triggered by a reduction in colony size and sudden physical changes in the environment, including increasing temperatures, rainfall and flooding. These environmental changes significantly reduce the size of *A. tomentosa* colony and trigger high copulation rates so that *A. tomentosa* can colonise new habitats (Boray, 1964b). Boray (1964a) and Lynch (1965) observed that *A. tomentosa* could expand rapidly after floods and colonise new areas leading to outbreaks of fasciolosis in livestock. Copulation does not occur during winter; it commences in spring and, depending on environmental factors, copulation can continue through summer till autumn (Boray, 1964b). The high reproductive capacity of *A. tomentosa,* its ability to rapidly colonise new habitats and migrate large distances make it an ideal host for *F. hepatica* in Australia.

Table 1.3. Climatic and environmental tolerances of *A. tomentosa*

		Temperature	Days viable	Hatching	Other	Reference
A. tomentosa		2-5°C	1095			(Boray, 1964a)
		26°C	Optimum temp[a]			(Boray, 1964a)
		>35°C	42			(Boray, 1964a)
	pH				5.4 to 7.3	(Boray, 1964a)
	Salinity ppm				25-160 ppm	(Boray, 1964a)
	Silicia suspension				<2-5 ppm	(Boray, 1964a)
Copulation		<16°C	No egg laying			(Boray, 1964b)
		>30°C	No egg laying			(Boray, 1964b)
Egg hatch		<2°C		60 days		(Boray, 1969)
		<5°C		56-60 days		(Boray, 1963)
		25-26°C		5-8 days		(Boray, 1963)
		30-31°C		9-10 days		(Boray, 1963)
Infected *A. tomentosa* (Sporocysts, rediae & cercariae)		< 10°C (Little development)	3 years			(Boray, 1969)

a Temp – Temperature

1.2.3.2 Pseudosuccinea columella

P. columella is native to North America but was discovered in Australian metropolitan creeks in Brisbane, Melbourne, Sydney and Perth in the late 1970s (Ponder, 1975; Boray, 1978). The current distribution of *P. columella* in Australia is shown in Figure 1 2. In NZ, the introduction of *P. columella* led to a rapid expansion of *F. hepatica* (Harris and Charleston, 1980). However, the NZ strain of *P. columella* was found to grow larger, produce more metacercariae and had a lower mortality rate during *F. hepatica* infection than the Australian strain shown in Table 2. Based on these observations, Boray (1978) concluded that *P. columella* would have a negligible impact on the epidemiology *F. hepatica* in Australia unless adaption occurred. Still, by 1985 *P. columella* had expanded into grazing regions in NSW and was acting as the intermediate host for *F. hepatica* (Boray et al., 1985). Ponder (1975) and Boray (1978) identified the most significant areas of risk if *P. columella* adapted and became an intermediate host for *F. hepatica* were in irrigated regions, warmer areas like QLD and in locations where *A. tomentosa* was not present (Western Australia (WA)) and Northern Territory (NT) because the snail has a higher temperature tolerance. Follow-up work was conducted in QLD by Baldock and Arthur (1985) and then by Molloy and Anderson (2006), who found that *P. columella* had not expanded the distribution of *F. hepatica,* but no investigations have been conducted in irrigation regions, in the NT or in WA where the snail is now present (Figure 1 2).

1.2.3.3 Radix viridis

R. viridis is an exotic snail from the Pacific region (Papua New Guinea, Philippines, China, and Japan) that was discovered in Brisbane creeks in 1974 (Boray, 1978). *R. viridis* is now found in QLD, NSW and WA (Figure 1 2). *R. viridis* has a low susceptibility to *F. hepatica* but can still produce a high number of infective metacercariae (Table 2). There has been no investigation into the role *R. viridis* plays in the epidemiology *F. hepatica* in Australia, other than it can transmit *F. hepatica* under laboratory conditions and produce viable metacercariae (Boray, 1978). Further research needs to be undertaken.

1.2.4 Sporocyst, redia and cercariae

There are three developmental stages within the intermediate host: sporocyst, redia and cercaria (see (c) and (d) in Figure 1 1). If miracidia successfully infect an intermediate host snail, it takes between 59-114 days for cercariae to be shed from the snail (Table 2). However, rediae that contain hundreds of cercariae cause considerable damage to snail tissues and organs, often killing the snail before shedding occurs (see Table 2). Shedding of cercariae occurs intermittently over many weeks and only ceases when the snail dies (Krull, 1941; Hodasi, 1972). Shedding of cercariae can be triggered by environmental stimuli, including changes in water temperature, rainfall, flushing of water through a snail habitat or water returning to dry habitats (Boray et al., 1969; Boray, 1969). These stimuli can trigger the mass release of cercariae into the environment, resulting in outbreaks of acute and sub-acute fasciolosis in grazing livestock (see section 3.1). Cercariae typically encyst on pasture, soil and in 2.2-10% of cases, the water surface within 2 hours of being shed from the snail (Krull, 1941; Boray, 1969; Andrews et al., 1999). During the encystment process, the cercaria sheds its epithelium, forms an outer layer and, finally, separates from its tail, forming a metacercaria (see (e) in Figure 1 1).

1.2.5 Metacercariae

In Australia, the highest output of metacercariae occurs during late spring, summer and autumn, with little to no metacercariae released during late winter and early spring as the *F. hepatica* lifecycle slows due to cold temperatures (Table 3) (Meek and Morris, 1979b). Metacercariae are made up of four layers; layers one and two form the outer cyst, and layers three and four form the inner cyst (Dixon, 1965). All layers play a role in protecting against desiccation, mechanical injury, toxic substances, bacteria and fungi (Dixon, 1965). Ninety-nine per cent of metacercariae are found at the water level and are more likely to be found on the underside of vegetation, which protects against direct sunlight and heat, prolonging the viability of the cyst (Hodasi, 1972). As the pasture grows, it increases the likelihood of ingestion by a definitive host (see (f) in Figure 1 1). Depending on the temperature and humidity, metacercariae can remain infective on pasture from a minimum of 10 days at higher temperatures but up to a year at low temperatures (Table 1). Not all ingested metacercariae will become adult *F. hepatica*: in artificial infections, between 14 to 46% of metacercaria reach sexual maturity within cattle (Ross et al., 1966).

1.2.6 Host

2.6.1 Newly excysted juvenile

The metacercaria's outer layers protect against enzymic action in the digestive system of the host till the metacercariae reach the site of excystment in the duodenum, where activation occurs, and the newly excysted juvenile (NEJ) emerges from the ventral plug (Dixon, 1965). Within the first two hours of ingestion, the NEJ emerges and begins to penetrate the duodenum wall by boring through the tissue. The NEJs migrate to the peritoneal cavity, where they feed on any available tissue and then randomly make their way to the liver. NEJs are sometimes found in other organs (lungs, spleen, pancreas) and occasionally prenatally infect calves (Boray, 1969; Rees et al., 1975). In cattle, NEJs reach the liver two to seven days post-infection (PI), the NEJs then penetrate the liver capsule and become immature *F. hepatica* (Doy et al., 1984; Andrews et al., 1999).

1.2.6.2 Immature F. hepatica

Immature *F. hepatica* migrate or traverse the liver for five to six weeks feeding on tissue, causing haemorrhagic tracts and fibrosis to form (Dawes, 1963; Boray, 1969). In light infections, the damage is isolated to the left lobe of the liver. In heavier infections, the damage will be found in other lobes, and the liver may also be enlarged, pale, deformed and hard due to the fibrosis (Boray, 1969). Definitive hosts have varying levels of resistance to *F. hepatica,* defined by Boray (1969) as early, delayed, and low resistance (Table 4). Cattle have delayed resistance to *F. hepatica* which is due to acquired resistance after exposure to the parasite and pathological changes to the liver parenchyma and bile ducts which act as mechanical barriers that prevent the maturation of *F. hepatica* (Boray, 1969; Sánchez-Andrade et al., 2002). It takes approximately seven weeks for immature *F. hepatica* to reach the bile ducts in cattle, where they reach sexual maturity and begin to release eggs (Andrews et al., 1999).

1.2.6.3 Mature F. hepatica and egg release

The mature *F. hepatica* resides and reproduces within the bile ducts causing fibrosis, anaemia and thickened bile duct walls (Boray, 1969). A systematic review by Machicado et al. (2016) was unable to determine whether liver fibrosis was caused by *F. hepatica* or the host immune response; however, the study did conclude that the intensity of *F. hepatica* infection played a role in the development of fibrosis in the liver. *F. hepatica* can

reproduce via self-fertilisation, but the parasite mainly reproduces via cross-fertilisation (Andrews et al., 1999; Beesley et al., 2017). Eggs produced by mature *F. hepatica* move into the bile ducts, pass into the gall bladder and then into the duodenum and, finally, are excreted from the host in faecal matter. Research has shown that egg output and the viability of the eggs vary widely between infected hosts (Table 4). Happich and Boray (1969a) found that an individual *F. hepatica* residing within the liver of a sheep had a consistent daily egg output between 21,000 and 24,000 eggs, whereas several studies have observed a variable release of eggs in cattle peaking 18-19 weeks after infection and then declining (Dorsman, 1956, 1960; Boray, 1969; Brockwell et al., 2013). The role each host plays in the lifecycle and the epidemiology of *F. hepatica* will depend on the grazing behaviour of the host, the habitat in which they exist, the *F. hepatica* burden, egg output and the viability of eggs (Ménard et al., 2000).

Table 1.4. Definitive and reservoir hosts of *F. hepatica* in Australia

Host	Species	Resistance to *F. hepatica*	Definitive or reservoir host	TFC	Reference
Bennett's wallaby	*Macropus rufogriseus**	Early	Definitive	80	(Boray, 1969; Obendorf, 1983)
Brushtail possum	*Trichosurus vulpecula**	Low	Reservoir	1-9	(Boray, 1969; Spratt and Presidente, 1981; Whittington, 1982)
Bush rat	*Rattus fuscipes**	Low	Reservoir	6-10	(Boray, 1969; Spratt and Presidente, 1981)
Emus	*Dromaius novaehollandiae**	Unk	Unk	1	(Vaughan et al., 1997)
Grey kangaroo	*Macropus giganteus**	Delayed	Definitive	1-95	(Boray, 1969; Spratt and Presidente, 1981; Obendorf, 1983)
Red-bellied pademelon	*Thylogale billardierii**	Unk	Definitive	Unk	(Boray, 1969; Obendorf, 1983)
Red-neck wallaby	*Macropus rufogriseus banksianus**	Early	Definitive	1-95	(Boray, 1969; Spratt and Presidente, 1981)
Wallaby	*Wallabia bicolor**	Early	Definitive	1-5	(Boray, 1969; Spratt and Presidente, 1981)
Wombat	*Vombatus ursinus**	Early	Reservoir	1-5	(Boray, 1969; Spratt and Presidente, 1981; Obendorf, 1983)
Cow	*Bos taurus*	Delayed	Definitive		(Boray, 1969)
Donkeys	*Eguus asinus*	Delayed	Unk		(Boray, 1969; Pandey, 1983)
European rabbit	*Oryctolagus cuniculus*	Low	Reservoir		(Boray, 1969; Ménard et al., 2000)
Fallow deer	*Darna dama*	Delayed	Reservoir		(Boray, 1969; Jenkins et al., 2020)
Goats	*Capra hircus*	Low	Unk		(Boray, 1969; Chartier and Reche, 1992)
Hares	*Lepus europeanus*	Low	Reservoir		(Boray, 1969; Walker et al., 2011)
Horse	*Eguus caballus*	Delayed	Unk		(Boray, 1969)
Pigs	*Sus spp.*	Early	Unk		(Boray, 1969; Valero et al., 2001)
Roe Deer's	*Capreolus capreolus*	Delayed	Reservoir		(Boray, 1969; Mezo et al., 2008)
Sheep	*Ovis aries*	Low	Definitive		(Brydone, 1960; Boray, 1969)
Water buffalo	*Bubalus bubalis*	Delayed	Unk		(Boray, 1969; Cringoli et al., 2009)
Wild boars	*Sus scrofa*	Delayed	Unk		(Mezo et al., 2008)

a TFC – Total number of flukes within the liver of the host

b * – Animal native to Australian

c Unk – Unknown

1.2.6.4 Definitive and reservoir hosts

F. hepatica infects a wide range of unrelated hosts (Table 4.) Hosts can be categorised as either a reservoir (an accidental host) for *F. hepatica* or a definitive host that actively contributes to pasture contamination and transmission of the parasite. Hares and rabbits are considered reservoir hosts as the *F. hepatica* eggs they shed have low viability and cannot infect intermediate host snails (Boray, 1969; Ménard et al., 2000; Walker et al., 2011). In contrast, kangaroos, pademelons and wallabies are definitive hosts in Australia because they release many viable *F. hepatica* eggs that contaminate pasture grazed by other definitive hosts (Boray, 1969; Spratt and Presidente, 1981; Obendorf, 1983). A study conducted in Tasmania identified kangaroos as the source of *F. hepatica* infection in cattle (Obendorf, 1983). Other native Australian animals, like the brushtail possum, are highly susceptible to *F. hepatica* and produce large numbers of viable eggs but are considered to be a reservoir for *F. hepatica* as they do not share the same habitats as the intermediate host snail or other definitive hosts (Boray, 1969; Spratt and Presidente, 1981). Research into the role introduced animals such as deer, horses, goats, and pigs play in the transmission of *F. hepatica* in Australia is lacking, but Jenkins et al. (2020) recently found that 35.5% of deer in alpine regions of NSW were infected with *F. hepatica*. Further work needs to be undertaken in Australia to understand better the role that native species and introduced animals play in the epidemiology of *F. hepatica*.

1.3 Production losses

Each year livestock producers in Australia spend $10 million (equivalent to AUD 16.6 million in 2018) on flukicides to mitigate production losses caused by *F. hepatica* (Love et al., 2017). The production losses due to fasciolosis depend on whether the disease is acute, sub-acute, clinical, or sub-clinical, the burden within the animal and the prevalence within the herd.

1.3.1 Acute and sub-acute disease

In livestock, acute conditions occur when large numbers of metacercariae (>2,000 in sheep) are ingested during a small window of time (Boray, 1969). In acute cases, death occurs from two weeks PI when NEJs penetrate the liver capsule, whereas sub-acute death occurs from eight weeks PI when immature *F. hepatica* begin to migrate to the bile ducts (Table 5). The penetrating NEJs destroy the liver tissue, cause anaemia and blood loss,

resulting in liver failure and death (Gordon, 1955). Acute fasciolosis has a sudden onset but is a rare occurrence in cattle, usually only occurring in young animals, or animals with other diseases, poor nutritional status, or forced to graze *A. tomentosa* habitat during dry conditions (Gordon, 1955; Boray, 1969). Acute and sub-acute fasciolosis result in significant production losses and high mortality rates but can be mitigated by avoiding the grazing of high-risk pasture and using an effective immature flukicide (i.e. TCBZ) (see Chapter 2).

1.3.2 Clinical and sub-clinical disease

Production losses in cattle with a clinical or sub-clinical *F. hepatica* infection are often missed because clinical signs are not always obvious (Table 5). Clinical conditions occur when cattle ingest more than >200 metacercariae; signs will present 12 weeks PI and may include pale mucous membranes (anaemia), bottle jaw, weight loss and reduced production (Boray, 1969; Vercruysse and Claerebout, 2001). The most significant infection in cattle is sub-clinical (<200 metacercariae) as there are no signs of infection, often delaying the detection and treatment of *F. hepatica* (McCann et al., 2010). The lack of intervention leads to heavily contaminated pastures, increased liver condemnations and decreases in milk production, weight gain and fertility (reviewed by Schweizer et al.,2005) (Boray, 1969). Routine monitoring of *F. hepatica* should occur in cattle at the herd-level and in vulnerable individuals to reduce the production impacts of fasciolosis and inform flukicide use on-farm (Kuerpick et al., 2013).

1.3.3 *F. hepatica* burden

Production losses resulting from *F. hepatica* infection are heavily influenced by the burden of *F. hepatica* residing within the liver. Research by Cawdery et al. (1977) and Dargie (1987) found that 54 and 30 flukes indicated the threshold for definitive loss in the cattle, respectively. However, more recent work by Charlier et al. (2008) found evidence to suggest that as few as ten flukes in dairy cattle could decrease production. More work is needed to establish the burden that represents the true threshold for production loss in cattle.

Table 1.5. Life cycle of *F. hepatica* in cattle, the presentation of fasciolosis and diagnostics test detection limits

Week	**1 hr**	**2 hr**	**2-7 days**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**…**	**18**	**References**
Stage	MC[a] excyst	NEJ[b]	NEJ[b]	Immature *F. hepatica*							Mature *F. hepatica*								(Boray, 1969; Love et al., 2017)
Location in host	Small intestine	Peritoneal cavity	Penetrate liver capsule	Left lobe of the liver						Migration to bile ducts	Bile ducts								(Boray, 1969; Love et al., 2017)
Acute				Large numbers of NEJs penetrate the liver, destroying liver tissue causing profound anaemia, blood loss, haemorrhaging and ultimately liver failure. Signs are not always recognisable but can include jaundice and abdominal pain.															(Boray, 1969; Love et al., 2017)
Sub-acute										Late immature *F. hepatica* 8-10 weeks PI[c] damage liver tissue by burrowing, causing haemorrhaging, anaemia, liver damage and death. Signs include jaundice, ill-thrift and anaemia.									(Boray, 1969; Love et al., 2017)
Chronic - clinical														Adult *F. hepatica* in the bile ducts 12-week PI[c] ingest blood causing anaemia, chronic inflammation and enlargement of bile ducts. Signs include bottle jaw, anaemia, pale mucous membranes and oedema (bottle jaw).					(Boray, 1969; Love et al., 2017)
Chronic - sub-clinical														These animals contribute the most to pasture contamination. Signs are not always recognisable but can include reduced productivity and thriftiness.					(Boray, 1969; Love et al., 2017)

Week	1 hr	2 hr	2-7 days	2	3	4	5	6	7	8	9	10	11	12	13	14	…	18	References
Diagnostics																			
FEC												Eggs detected in faeces					…	Peak egg shedding	(Boray, 1969; Brockwell et al., 2013)
cELISA											Coproantigen detected in faeces						…		(Brockwell et al., 2013)
sELISA						IgG detected				Peak IgG							…		(Reichel, 2002; Brockwell et al., 2013)

a MC – Metacercariae

b NEJ – Newly excysted juvenile

c PI – Post-infection

1.3.4 Herd-level thresholds for production loss

The most frequently used threshold to identify herds incurring production losses is when the herd-level prevalence of *F. hepatica* exceeds 25%. The 25% threshold was first described by Hörchner et al. (1970), cited in Cawdery et al. (1977), who found a 20-25% prevalence of fasciolosis in a herd indicated reduced weight gain and milk production in cattle. The threshold has been more recently validated by Charlier et al. (2007) and Howell et al. (2015), who both found that when exposure to *F. hepatica* (as determined by bulk milk tank (BTM) ELISA) exceeded 25%, there was a decrease in annual milk yield by 3%, and 15%, respectively. In addition, the 25% threshold has also been applied to diagnostic outcomes from liver fluke faecal egg count (LFEC) (Malone and Craig, 1990; Kelley et al., 2020) and the coproantigen ELISA (cELISA) (Elliott et al., 2015; Kelley et al., 2020) to identify herds that are incurring production losses. The 25% threshold is a quick and straightforward way to identify herds incurring production losses in large-scale studies, identify higher risk regions and is an efficient method of directing resources to the most *F. hepatica* affected regions.

1.4 *F. hepatica* diagnostics

There is no gold standard field diagnostic test for *F. hepatica* in cattle. The most commonly used diagnostic tests are LFEC, serum ELISA (sELISA), BTM ELISA and cELISA. In cattle, *F. hepatica* often only infects a few susceptible individuals with less resistance to infection, which hampers the detection of the parasite in large herds (Vercruysse and Claerebout, 2001; Daniel et al., 2012). Detection of these highly infected individuals is key, as these individuals contribute the most to pasture contamination (Malone and Craig, 1990). Each diagnostic test has its advantages and disadvantages that influence the sensitivity, specificity and application on-farm, discussed below. The sensitivity reflects the likelihood of an *F. hepatica* infected cow testing positive, whereas the specificity is the probability of an *F. hepatica* negative cow testing negative.

1.4.1 LFEC

Historically field diagnosis of *F. hepatica* relied on LFEC, which is a labour-intensive but straightforward technique that does not require chemicals or specialised laboratory equipment. The sensitivity of LFEC depends on the technique and the volume of faecal matter used (Table 6). In cattle, Happich and Boray (1969b) and Kajugu et al. (2015) found

that sedimentation is superior to sedimentation-flotation. The sedimentation-flotation technique's sensitivity and specificity using 10g of faeces from naturally infected cattle was 64% and 93% (Charlier et al., 2008). In contrast, the sedimentation technique's sensitivity and specificity were 69%-89.6% and 97.7%-98.3% using 10 g faeces collected from naturally infected cattle (Rapsch et al., 2006). The sensitivity of the LFEC can be increased by repeat sampling or by increasing the volume of faecal matter used (Conceição et al., 2002; Rapsch et al., 2006). However, some studies have observed that increases in faecal volume decreased the sensitivity and specificity of the LFEC, as the higher volume of debris in the sample increased the difficultly in identify *F. hepatica* eggs (Conceição et al., 2002; Charlier et al., 2008; Daniel et al., 2012). The technician's experience counting the *F. hepatica* eggs and the ability to distinguish between *F. hepatica* and paramphistome eggs also influences the sensitivity and specificity of the LFEC (Charlier et al., 2008).

The accuracy of the LFEC is also influenced by the burden of *F. hepatica* within the host, egg shedding by adult *F. hepatica* and the host's faecal matter output. Cattle with burdens of less than ten flukes are challenging to detect as the low egg counts (1-4 eggs) are diluted in the faeces and can be easily missed (Martínez-Sernández et al., 2016). Similarly, in heavily infected cattle, reduced egg shedding may occur as damage and crowding in the liver may obstruct egg release from the animal (Happich and Boray, 1969b). Malone and Craig (1990) determined that the cut-off for cattle production loss is 5 ep2g using LFEC, as counts in cattle seldom exceed 20 ep2g. Any count exceeding 40 ep2g in cattle is considered clinical and indicates a severe level of infection (Malone and Craig, 1990; Smeal, 1995; Vercruysse and Claerebout, 2001). Many studies have tried to interpret how LFEC relates to the *F. hepatica* burden in the host, but only two studies have found a correlation between the two variables. In cattle, Brockwell et al. (2013) observed a correlation R^2 0.84, and in sheep, George et al. (2017) observed a correlation R^2 0.571. Several studies have found that LFEC does not consistently increase with *F. hepatica* burden, and the test can only detect sexually mature *F. hepatica* 7 to 8 weeks PI (Table 5) (Dorsman, 1956; Boray, 1969; Happich and Boray, 1969a; Malone and Craig, 1990; Brockwell et al., 2013). In addition, Brockwell et al. (2013) observed a 2-4 fold variation in LFEC when cattle were sample consecutively, suggesting considerable variability in cattle's daily egg output.

Hagens and Over (1966) determined that the highest egg output in cattle occurred between 12:00 pm to 12:00 am, whereas the highest volume of faecal matter output was between 12:00 am to 12:00 pm. Further work by Dorsman (1956) and Dorsman (1960) observed the same pattern of *F. hepatica* egg release, which increased during the morning, peaking at 1:30 pm and then declining throughout the afternoon. Based on these observations, Dorsman (1956) proposed that faecal sample collection should occur at 1:30 pm, assuming that the highest egg output most accurately reflected the true burden within the animal. Sample collection also needs to consider the season as Mazeri et al. (2016) found that the sensitivity was highest in summer (81%), whereas samples collected in autumn were the least accurate due to the presence of immature *F. hepatica* (58%) (Table 6). The LFEC is routinely used for testing cattle and sheep for drug-resistant *F. hepatica*, specifically TCBZ resistance, which has been reviewed in Kelley et al. (2016) (Chapter 2). Recognising the LFEC test's limitations, several immunological diagnostic tests have been developed for detecting *F. hepatica* in cattle.

Table 1.6. Sensitivity and specificity LFEC in cattle

LFEC technique	Faecal volume	Source of samples	SE % [a]	SP % [b]	Reference
Sedimentation-Flotation	4g	Abattoir	43	100	(Charlier et al., 2008)
Sedimentation-Flotation	10g	Abattoir	64	93	(Charlier et al., 2008)
Sedimentation	5g	Abattoir	67	100	(Anderson et al., 1999)
Sedimentation	5g	P/N Herd [c]	100	97.5	(Ibarra et al., 1998)
Sedimentation	5g	Abattoir – Autumn	58	99	(Mazeri et al., 2016)
Sedimentation	5g	Abattoir – Summer	81	99	(Mazeri et al., 2016)
Sedimentation	5g	Abattoir – Winter	77	99	(Mazeri et al., 2016)
Sedimentation	10g	Spiked – <15 eggs added	33	N/A	(Conceição et al., 2002)
Sedimentation	10g	Spiked – 120-1000 eggs	100	N/A	(Conceição et al., 2002)
Sedimentation	10g	Spiked – 20-80 egg	100	N/A	(Conceição et al., 2002)
Sedimentation	10g	Abattoir x 1 serial samples	69	98.3	(Rapsch et al., 2006)
Sedimentation	10g	Abattoir x 2 serial samples	86.1	97.7	(Rapsch et al., 2006)
Sedimentation	10g	Abattoir x 3 serial samples	89.6	97.8	(Rapsch et al., 2006)
Sedimentation	30g	Spiked	83.3	N/A	(Conceição et al., 2002

a SE – sensitivity

b SP – specificity

c P/N – F. hepatica positive and negative herds or animals were compared

d N/A – Specificity was not determined

1.4.2 Serum ELISA

One such technique is the sELISA which utilises excretory-secretory (ES) antigens from adult *F. hepatica* to detect anti-*F. hepatica* antibodies within serum samples. In-house ES sELISA tests are frequently used, but the methodology varies between laboratories making comparisons of diagnostic outputs challenging (Table 7). The sensitivity of the ES ELISA varies from 72%-100%, and specificity varies from 70%-98.8% (Ibarra et al., 1998; Anderson et al., 1999; Cornelissen et al., 1999; Sánchez-Andrade et al., 2002; Salimi-Bejestani et al., 2005a; Charlier et al., 2008; Mazeri et al., 2016). The sensitivity range reflects the differences in the cattle used, trial design, and variation in ES sELISA method used in each study. Whereas the specificity of the ES ELISA varies because of cross-reactivity with *Paramphistomum* spp. (rumen fluke) and *Dictyocaulus* spp. (lungworm) (Ibarra et al., 1998; Cornelissen et al., 1999). As cattle are commonly infected with all three parasites, it limits the test's on-farm application. The assay's advantage is that the ES ELISA can detect *F. hepatica* infection from two weeks PI (Cornelissen et al., 1999; Salimi-Bejestani et al., 2005a; Salimi-Bejestani et al., 2008). Charlier et al. (2008) observed a weak (R 0.3) correlation between *F. hepatica* burden and sELISA values, whereas Brockwell et al. (2013) observed a correlation of R^2 0.85. The most widely used commercial sELISA is by Institut Pourquier (Montpellier, France), which has a sensitivity ranging from 88%-98.2% and specificity ranging from 84%-100%, and there is no evidence that it cross-reacts with other parasites (Reichel, 2002; Molloy et al., 2005; Rapsch et al., 2006; Charlier et al., 2008). The Institut Pourquier sELISA can detect *F. hepatica* from two weeks in cattle, but there is no correlation with the burden of adult *F. hepatica* in the liver (Reichel, 2002; Charlier et al., 2008). Several other sELISA have been developed to test cattle for *F. hepatica*, and the assays are listed and described in Table 7.

The major drawback of the sELISA is that anti-*Fasciola* antibodies can persist for up to 6 months after effective treatment of *F. hepatica* with a flukicide (Ibarra et al., 1998; Cornelissen et al., 1999; Castro et al., 2000; Brockwell et al., 2013). In naturally infected cattle, a positive result could be a current or historical infection. Seasonal changes affect the sELISA sensitivity and specificity. In winter, it is 94% and 89%, whereas, in summer, it decreases to 72% and 87%, respectively (Charlier et al., 2008; Mazeri et al., 2016). Timing of blood collection must consider herd treatment history and the season. Blood collection is a time-consuming and invasive practice, requiring trained staff to collect samples from

several individuals within the herd. Given that the sELISA and the BTM ELISA have a correlation coefficient of R 0.83 (P<0.001) and Bloemhoff et al. (2015) found that both ELISAs accurately identified *F. hepatica* negative farms, the BTM ELISA could be used instead (Salimi-Bejestani et al., 2005b; Salimi-Bejestani et al., 2007).

Table 1.7. Sensitivity and specificity sELISA in cattle.

sELISA technique	Source of samples	SE % [a]	SP % [b]	Cut-off	Reference
Antigen of *Fasciola* (DIG ELISA)	P/N Herd [c]	98	80	Diameter > 7mm	(Ibarra et al., 1998)
Cathepsin-L ELISA	Natural	90	75	OD > 0.25 [d]	(Cornelissen et al., 2001)
Cathepsin-L ELISA	Artificial	99	99	OD > 0.25 [d]	(Cornelissen et al., 2001)
Cathepsin-L ELISA	Artificial and Natural	100	95	OD > 0.39 [d]	(Cornelissen et al., 1999)
ES (DOT-ELISA)	P/N Herd [c]	93	95	Visible blue spot	(Ibarra et al., 1998)
ES ELISA (in-house)	P/N Herd [c]	97	99	OD > 0.5 [d]	(Ibarra et al., 1998)
ES ELISA (in-house)	Artificial and Natural	100	83	OD > 0.95 [d]	(Cornelissen et al., 1999)
ES ELISA (in-house)	P/N Herd [c] and Artificial	98	96	PP > 15 [e]	(Salimi-Bejestani et al., 2005a)
ES ELISA (in-house)	Abattoir	86	70	OD > 0.36 [d]	(Anderson et al., 1999)
ES ELISA (in-house)	Natural	92	86	OD > 0.396 [d]	(Sánchez-Andrade et al., 2002)
ES ELISA (in-house)	Abattoir – Autumn	80	76	PP > 10 [e]	(Mazeri et al., 2016)
ES ELISA (in-house)	Abattoir – Summer	72	87	PP > 10 [e]	(Mazeri et al., 2016)
ES ELISA (in-house)	Abattoir – Winter	94	89	PP > 10 [e]	(Mazeri et al., 2016)
ES ELISA (in-house) IgG	Abattoir	87	90	ODR >0.09 [f]	(Charlier et al., 2008)
In-house IgG ELISA	Natural	94	100	OD > 0.304 [d]	(Sánchez-Andrade et al., 2002)
Institut Pourquier	Abattoir	88	84	S/P > 30% [g]	(Charlier et al., 2008)
Institut Pourquier	Abattoir	92	94	S/P > 30% [g]	(Rapsch et al., 2006)
Institut Pourquier	Natural and Artificial Infection	98	100	S/P > 54-77% [g]	(Reichel, 2002)
Institut Pourquier	P/N Herd [c]	98	98	S/P > 30% [g]	(Molloy et al., 2005)
Synthetic Peptide 7 ELISA	Artificial and Natural	99	100	OD > 0.25 [d]	(Cornelissen et al., 1999)

a SE – sensitivity

b SP – specificity

c P/N – *F. hepatica* positive and negative herds or animals were compared

d OD – Optical density cut-offs utilised in each study to distinguish between positive and negative cattle

e PP – Percent positivity

f ODR – Optical density ratio calculated by the optical density of the sample minus the negative control divided by positive control minus the negative control

g S/P ratio – S is the OD of the sample divided by the P, which is the OD of the positive control run on each plate

1.4.3 Milk ELISA

The use of an ELISA to detect anti-*F. hepatica* antibodies in BTM samples were first described by Salimi-Bejestani et al. (2005a). The BTM ELISA has been used in several studies to determine herd-level exposure to *F. hepatica,* production losses caused by *F. hepatica* and identify at-risk regions (Pritchard et al., 2005; Salimi-Bejestani et al., 2005b; Charlier et al., 2007; Bennema et al., 2009; McCann et al., 2010; Kuerpick et al., 2013; Selemetas et al., 2014; Bloemhoff et al., 2015; Howell et al., 2015; Novobilský et al., 2015). The BTM ELISA has been optimised to identify herds with a herd-level exposure greater than 25%, which is the threshold for production loss in cattle (Hörchner et al., 1970; Salimi-Bejestani et al., 2005b). The test sensitivity and specificity vary between the methods used (Table 8). The highest values were obtained by using the Institut Pourquier (Montpellier, France) BTM ELISA, followed by Euroclone (Pero, Italy) BTM ELISA, in-house ES BTM ELISA and, finally, an in-house Cathepsin-L BTM ELISA (Molloy et al., 2005; Salimi-Bejestani et al., 2005b; Charlier et al., 2007; Bennema et al., 2009; Duscher et al., 2011; Kuerpick et al., 2013; Selemetas et al., 2014). Like the sELISA, the BTM ELISA cannot differentiate between current and historic infection and anti-*F. hepatica* antibodies persist for months after effective treatment with a flukicide (Ibarra et al., 1998; Cornelissen et al., 1999; Castro et al., 2000; Brockwell et al., 2013). The BTM ELISA is also influenced by the stage of *F. hepatica* infection in individuals within a herd, individual milk yield, and individuals' seropositivity (Duscher et al., 2011; Selemetas et al., 2014). Earlier work by Reichel et al. (2005) calculated that an in-herd prevalence of 60% was needed to obtain a positive BTM ELISA result; however, more recent work by Duscher et al. (2011) established that the minimum in-herd prevalence was 20%. The BTM ELISA accurately detects herds that are incurring production losses but provides little insight into infected individuals.

Table 1.8. Sensitivity and specificity BTM ELISA in cattle.

sELISA technique	Source of samples	SE % [a]	SP % [b]	Cut-off	Reference
Euroclone	P/N Herds [c]	89	89	>15%	(Duscher et al., 2011)
In-house antibody detection ELISA	Natural	60	90	>15% PP [d]	(Selemetas et al., 2014)
In-house ES ELISA	Natural	96	80	>27 % of PC [e]	(Salimi-Bejestani et al., 2005b)
In-house ES ELISA	Natural	96	80	ODR >0.8 [f]	(Bennema et al., 2009)
In-house ES ELISA	Natural	96	80	ukn [g]	(Charlier et al., 2007)
In-house ES ELISA	Natural	96	80	ODR >0.8 [f]	(Kuerpick et al., 2013)
Institut Pourquier	P/N Herds [c]	95	95	>30%	(Duscher et al., 2011)
Institut Pourquier	P/N Herds [c]	98	99	>30%	(Molloy et al., 2005)

a SE – sensitivity

b SP – specificity

c P/N – F. hepatica positive and negative herds or animals were compared

d PP – per cent positivity

e PC – per cent positive value

f ODR – Optical density ratio calculated by the optical density of the sample minus the negative control divided by positive control minus the negative control

g Unk – The cut-off to distinguish between herds with a prevalence > 25% is unknown

1.4.4 cELISA

The cELISA by Bio-X Diagnostics Belgium has been used in several studies to detect coproantigen (cathepsins) in faecal samples collected from infected cattle and sheep (Mezo et al., 2004; Brockwell et al., 2013; Brockwell et al., 2014; Elliott et al., 2015; Martínez-Sernández et al., 2016; Kelley et al., 2020). The cELISA can detect immature *F. hepatica* from 6 weeks PI, can detect low burdens (<10) in cattle, is semi-quantitative, can be used on an individual or pooled samples and can be used to test for drug resistance (Brockwell et al., 2013). However, consecutive daily testing by Brockwell et al. (2013) revealed a 2-6 fold variation in the coproantigen level in cattle. Recognising the effect of variable coproantigen release on the sensitivity of the cELISA, Martínez-Sernández et al. (2016) modified the method and incorporated a commercial streptavidin-polymerised horseradish peroxidase conjugate. The modified method improved the detection limit from 0.6 ng/mL to 0.15 ng/mL and enabled the detection of single *F. hepatica* in cattle, but resulted in a 6-12 fold increase in the variation (Mezo et al., 2004; Brockwell et al., 2013; Martínez-Sernández et al., 2016). The modified method also reduced the correlation between the coproantigen level and *F. hepatica* burden from R^2 0.87 (Brockwell et al., 2013) and R 0.6 (Charlier et al., 2008) to R 0.2998 in cattle (Martínez-Sernández et al., 2016).

The other drawback of the cELISA is that four studies have found that the manufacturer cut-off is too high, resulting in a high incidence of false negatives in low burden cattle (< 10) (Charlier et al., 2008; Brockwell et al., 2013; Palmer et al., 2014; George et al., 2017). Consequently, several studies have used different cut-offs to improve the sensitivity and specificity of the cELISA, making it difficult to compare the findings (Table 9). In some instances, cut-offs established for cattle have been applied to sheep, even though Palmer et al. (2014) found that the cut-off must be specific to the host. The sensitivity of the cELISA ranges from 77%-100%, and Martínez-Sernández et al. (2016) observed some seasonal variation when utilising the Palmer et al. (2014) cut-off (Table 9). The specificity of the cELISA is 99% in all but one study, in which Charlier et al. (2008) found the specificity to be 93% (Table 9). This finding is likely a result of the cut-off used, as several studies have found no cross-reactivity between the cELISA and nematodes (*Trichuris* spp., *Nematodirus* spp., *Haemonchus* spp., *Ostertagia*, *Teladorsagia* spp., *Trichostrongylus* spp., *Oesophagostomum* spp., and *Dictyocaulus* spp.), cestodes (*Moniezia* spp. and

Echinococcus spp.), trematodes (*P. cervi, C. daubneyi* and *Dicrocoelium* spp.) and coccidia (Mezo et al., 2004; Brockwell et al., 2013; Kajugu et al., 2015; Mazeri et al., 2016). Even with the drawbacks, the cELISA is a highly sensitive and specific diagnostic tool that can detect low burden infection in cattle, immature *F. hepatica,* and it can be used to test for TCBZ resistance as coproantigens do not persist after treatment (reviewed by Kelley et al., 2016; Chapter 2).

Table 1.9. Sensitivity and specificity faecal BIO K 201 (Bio-X Diagnostics, Belgium) cELISA in cattle.

Diagnostic test	Source of samples	SE % [a]	SP % [b]	Cut-off	Reference
BIO K 201 cELISA	P/N Herd [c]	87	99	Kit cut-off * 0.67	(Palmer et al., 2014)
BIO K 201 cELISA	Abattoir	94	93	0.030 OD [d]	(Charlier et al., 2008)
BIO K 201 cELISA	Abattoir	96		0.084 OD [d]	(Martínez-Sernández et al., 2016)
BIO K 201 cELISA	Abattoir	100		0.114 OD [d]	(Mezo et al., 2004)
BIO K 201 cELISA	Abattoir	77	99	>7% of kit PC [e]	(Mazeri et al., 2016)
BIO K 201 cELISA	Abattoir - Autumn	87	99	Kit cut-off * 0.67	(Mazeri et al., 2016)
BIO K 201 cELISA	Abattoir - Summer	80	99	Kit cut-off * 0.67	(Mazeri et al., 2016)
BIO K 201 cELISA	Abattoir - Winter	85	99	Kit cut-off * 0.67	(Mazeri et al., 2016)

a SE – sensitivity

b SP – specificity

c P/N – F. hepatica positive and negative herds or animals were compared

d OD – Optical density cut-offs utilised in each study to distinguish between positive and negative cattle

e PC – Manufacture cut-off supplied with cELISA kit, approximately 7% of kit positive control

1.5 Spatial distribution of *F. hepatica* in Australia

In Australia, *F. hepatica* is found in all states and territories except for WA and the NT (Seddon, 1950; Palmer et al., 2014). The following section describes the environmental and farm management factors that influence the distribution of *F. hepatica* and the main intermediate host, *A. tomentosa,* in Australia.

1.5.1 *F. hepatica* prevalence studies in Australia

In Australia, seven *F. hepatica* prevalence studies have been conducted in NSW and Victoria (VIC), five in Queensland (QLD), two in Tasmania (TAS) and one in SA (Table 10). Thirteen of the 22 studies were conducted before 1990 and mainly used abattoir inspection data with a sensitivity 63.2%-68%, i.e. missing approximately 29% of infected livers (Rapsch et al., 2006; Charlier et al., 2008; Mazeri et al., 2016). The lack of current data is detrimental to the management and ongoing monitoring of the distribution of *F. hepatica* in Australia. Based on the literature, the estimated prevalence of *F. hepatica* in cattle ranges from <6% to 88% in NSW, 0%-23% in QLD, 41-80% TAS, 20%-98% in VIC and 12% in SA (Table 10). Further research is needed in TAS and SA because few studies have been conducted, and in the NT and WA, where introduced snails *P. columella* and *R. viridis* could now act as intermediate hosts for *F. hepatica* (Figure 1 2). The size of Australia is a limiting factor in accurately assessing the prevalence of *F. hepatica,* and presently abattoir studies are underutilised in identifying where *F. hepatica* is a problem. However, a new method developed by Innocent et al. (2017) in the United Kingdom (UK) could overcome this hurdle. The method utilises abattoir data, animal movement and climatic data to identify regions with a high *F. hepatica* prevalence, allowing targeted follow-up investigations with more sensitive diagnostic tools (Innocent et al., 2017). This approach could generate the evidence base to improve our understanding of the epidemiology of *F. hepatica* in Australia.

Table 1.10. Prevalence of *Fasciola hepatica* in Australian cattle from 1928-2014

State	Year	Livestock	Test	Prevalence	No. of Herds [a]	No. of Animals [a]	Reference
New South Wales	1960	Cattle	ABI [b]	50%		46,771	(Brydon et al., 1960)
		Dairy	ABI [b]	88%		46,771	(Brydon et al., 1960)
	1960	Cattle	ABI [b]	16%		5,000	(Brydon, 1960)
	1961	Cattle	LFEC	40%	9	315	(Pearson and Boray, 1961)
	1968	Cattle	ABI [b]	<6%-66%		57,700	(NSW Dept Ag Survey cited in Smeal, 1995)
	1977	Cattle	ABI [b]	44%		1,555,513	(NSW Dept Ag Survey cited in Watt, 1979)
	1998	Cattle	LFEC	39%		259	(Hort, 1998)
	2004	Cattle	Unk [c]	44%		Unk [c]	(Virbac, 2016)
Queensland	1963	Dairy	LFEC	23%	21	303	(Dixon, 1963)
	1980	Cattle [d]	ABI [b]	0.4%		5,647	(Roberts, 1982)
		Cattle [e]	ABI [b]	5%		621	(Roberts, 1982)
	1981	Beef	ABI [b]	1%		22,916	(Baldock and Arthur, 1985)
	1997	Dairy	BTM ELISA	8%	523		(Molloy and Anderson, 2006)
		Beef	sELISA	1%	142	5,103	(Molloy and Anderson, 2006)
	2004	Cattle	Unk [c]	17%		Unk [c]	(Virbac, 2016)
South Australia	2004	Cattle	Unk [c]	12%		Unk [c]	(Virbac, 2016)
Tasmania	1983	Cattle	Unk [c]	80%		Unk [c]	(Obendorf, 1983)
	2004	Cattle	Unk [c]	41%		Unk [c]	(Virbac, 2016)
Victoria	1928	Cattle	ABI [b]	29%		4,922	(Kellaway et al., 1929; p.24. in Seddon, 1950)
	1947	Cows	ABI [b]	29%		153	(Tinney, 1948; p., 25. in Seddon 1950)
		Heifers	ABI [b]	20%		179	(Tinney, 1948; p., 25. in Seddon 1950)
		Bulls	ABI [b]	50%		354	(Tinney, 1948; p., 25. in Seddon 1950)
	1977	Cattle	ABI [b]	41%		39,499	(Watt, 1979)
	1980	Dairy	ABI [b]	98%	36	87	(McCausland et al., 1980)
	2004	Cattle	Unk [c]	70%		Unk [c]	(Virbac, 2016)

State	Year	Livestock	Test	Prevalence	No. of Herds [a]	No. of Animals [a]	Reference
	2013	Dairy	cELISA	81%	15	450	(Elliott et al., 2015)
	2014	Dairy	cELISA and LFEC	39%	81	1669	(Kelley et al., 2020)

a No. – Total number of herds or animals sampled to determine the prevalence of *F. hepatica*

b ABI – Abattoir inspection of cattle livers assessing condemnations and scarring caused by *F. hepatica* infection

c Unk – The test used to determine the *F. hepatica* prevalence and/or the number of cattle tested is unknown

d Grain-fed cattle

e Grass-fed cattle

1.5.2 Environmental factors

1.5.2.1 Temperature and rainfall

Two key epidemiological factors influence the life cycle of *F. hepatica*: rainfall (water) which initiates the life cycle, and temperature, which regulates the progress of *F. hepatica* development in snails (Section 2.1, 2.3, 2.4 and 2.5). Rainfall provides the moisture needed for embryonation of *F. hepatica* eggs, creates the habitat for *A. tomentosa* and aids in the dispersal of *F. hepatica* eggs, miracidia, cercariae and metacercariae. However, the life cycle of *F. hepatica* only proceeds when there are favourable temperatures, typically between 10°C and 25°C (Table 1 and 3). It is generally assumed in Australia that the highest metacercarial challenge occurs in spring and autumn. Research has found that increased moisture due to high monthly rainfall coupled with decreasing evapotranspiration has led to increased transmission of *F. hepatica* to livestock (Meek and Morris, 1979b; Selemetas et al., 2014). In Australia, regions at greatest risk have annual rainfall greater than 600mm or greater than 430mm if supplemented by irrigation; however, it is unclear how summer irrigation changes the metacercarial challenge (Boray, 1969). Irrigated shires in VIC were found to have a much higher incidence of fasciolosis (Figure 1 3) (Watt, 1979; Durr et al., 2005). Further analysis utilising current *F. hepatica* data is needed to assess how temperature, rainfall and irrigation have affected the distribution of *F. hepatica* and the seasonality of metacercarial challenge in VIC since the last study conducted by Watt (1979).

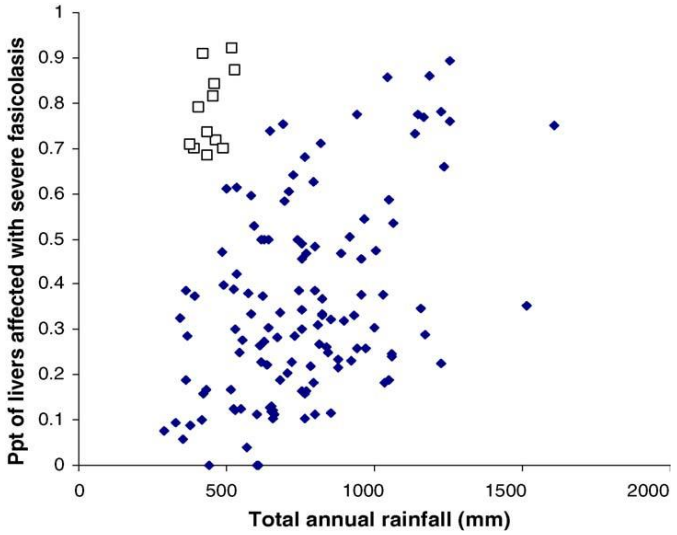

Figure 1.3. Scatter plot of mean annual rainfall per local government area (LGA) against the proportion of livers infected with fasciolosis from those areas. Non-irrigated LGA (◊); irrigated LGA (□). Sourced from Durr et al. (2005).

Irrigation is used in every state and territory in Australia. According to the ABS (2019), the largest user of irrigation is NSW, followed by VIC, QLD, SA, TAS, WA, NT and finally, the ACT (Table 11). All studies investigating the impact of irrigation on the epidemiology of *F. hepatica*, except for Boray et al. (1969), have been conducted in VIC (Meek and Morris, 1979b; Watt, 1979; McCausland et al., 1980; Durr et al., 2005; Elliott et al., 2015; Kelley et al., 2020). Given the high proportion of irrigated areas grazed by livestock in the NT, TAS, SA and NSW and the presence of suitable intermediate hosts shown in Figure 1 2, further research is needed in these states and territories (Table 11). Recently, a study found evidence to suggest that there has been a reduction in the prevalence of *F. hepatica* in the Goulburn-Murray irrigation region in VIC since irrigation upgrades occurred in the region (Kelley et al., 2020). However, it is not clear whether this is because of irrigation upgrades or that salinity is now present in the irrigation regions, shown in Figure 1 4. *A. tomentosa* tolerance for salt is between 25-160 ppm and the salinity in the Goulburn-Murray area often exceeds this range (Boray, 1964a; Hart et al., 2020). Given that irrigated farms have a higher incidence of *F. hepatica,* shown in Figure 1 3 and that 59% of Australian dairy farms are irrigated, further investigation is warranted to confirm this observation (Boray et al., 1969; Watson and Watson, 2015).

Border check irrigation is used on 50% of irrigated dairy farms (Watson and Watson, 2015). Border check irrigation temporarily submerges pasture, fills low lying spots with water (i.e. drains, cow tracks, holes) and often waterlogs the soil. Higher levels of *F. hepatica* exposure in livestock typically occur on high clay content soils that are more prone to waterlogging and poor drainage, as well as on alluvium soils that are associated with rivers and irrigation (Malone et al., 1992; Charlier et al., 2011; Selemetas et al., 2014; Selemetas et al., 2015). Waterlogged soils increase the duration in which snails can expand into new habitats, and high soil moisture increases the longevity of miracidia and metacercariae (Malone et al., 1984). On non-irrigated farms, typically 1-2% of *A. tomentosa* populations are infected with *F. hepatica,* whereas on irrigated farms, the proportion of infected snails can be as high as 43% (Table 12) (Boray et al., 1969). Boray et al. (1969) found that increases in snail infection above 2% led to outbreaks of fasciolosis in sheep. Monitoring the number of infected *A. tomentosa* using new tools like qPCR and eDNA could prove to be useful in forecasting high-risk periods for grazing livestock on irrigated farms (Rathinasamy et al., 2018).

Table 1.11. Irrigation use in each State and Territory in Australia. Data sourced from ABS (2019).

	Total irrigated area (ha) [a]	Irrigated area grazed by livestock (% ha) [a]
New South Wales	784,805	18%
Victoria	629,131	52%
Queensland	533,338	4%
South Australia	147,144	21%
Tasmania	100,105	57%
Western Australia	77,485	11%
Northern Territory	26,092	77%
Australian Capital Territory	74	N/A [b]

a ha - hectares

b N/A – No irrigated land in the ACT is grazed by livestock.

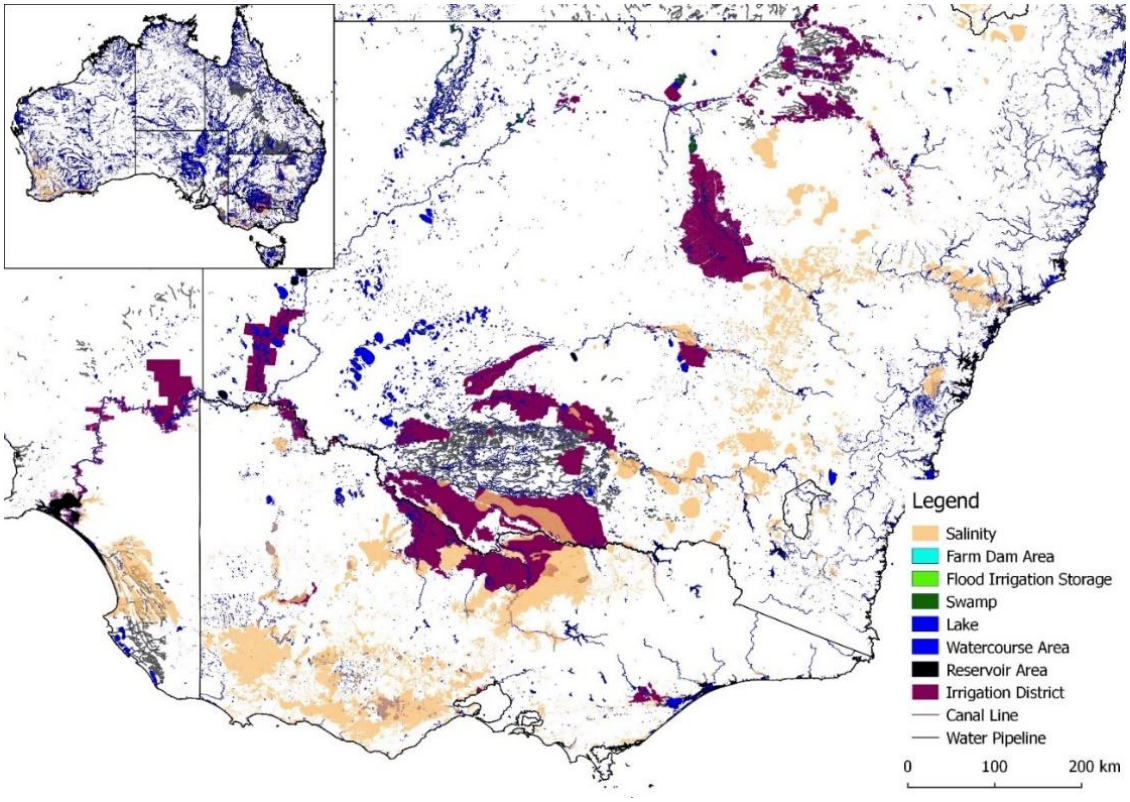

Figure 1.4. The hydrology of Australia, including human-made water bodies, irrigation infrastructure and areas of salinity. Data sourced from ABARES (2018a, 2018b), Crossman and Li (2015a, 2015b) and the Murray-Darling Basin Authority (2016).

Table 1.12. Preferred by *A. tomentosa* habitats in Australian

Waterbody type	Permanent vs. Temporary	Total no. snails [a]	No. infected snails	% infected	Reference
Creek	P [b]	22	6	27	(Bradley, 1926; Boray, 1969; Pullan et al., 1972)
Creek	P [b]	21	0	0	(Bradley, 1926)
Creek	P [b]	100	4	4	(Bradley, 1926)
Backwaters	P [b]	8	0	0	(Bradley, 1926; Boray, 1969; Pullan et al., 1972)
Stream	P [b]	1	0	0	(Bradley, 1926)
Stream feed by a spring	P [b]	22	8	36	(Bradley, 1926)
Spring	P [b]	10	0	0	(Bradley, 1926)
Spring	P [b]	60	1	2	(Bradley, 1926; Boray, 1969)
Spring (fenced)	P [b]	136	28	21	(Bradley, 1926)
Spring (Hill seepage)	P [b]	368	17	5	(Bradley, 1926; Pullan et al., 1972)
Spring (Hill seepage) with pugging	P [b]	218	11	5	(Bradley, 1926; Seddon, 1950; Pullan et al., 1972; Harris and Charleston, 1977)
Bog	P [b]	56	2	4	(Bradley, 1926; Seddon, 1950; Pullan et al., 1972)
Bog	P [b]	104	4	4	(Bradley, 1926)
Bog (fenced)	P [b]	32	3	9	(Bradley, 1926)

Waterbody type	Permanent vs. Temporary	Total no. snails [a]	No. infected snails	% infected	Reference
Bog with pugging	P [b]	31	5	16	(Bradley, 1926; Harris and Charleston, 1977)
Pond	P [b]	5	1	20	(Bradley, 1926)
Trough next to spring	P [b]	321	49	15	(Bradley, 1926; Pullan et al., 1972)
Irrigation	T [c]	500-2000	Unk [d]	6.8 [e] (Max 43)	(Boray et al., 1969; Pullan, 1972)
Surface collection of water	T [c]	66	11	17	(Bradley, 1926)

a No. – Total number of snails collected from each waterbody

b P – Permanent water bodies that act as a refuge for snails during adverse climatic conditions

c T – Temporary waterbody are created by rainfall, flood and irrigation, which allowing the snail to colonise new areas

d Unk – The total number of infected snails is unknown

e The average proportion of infected snails over the three year sampling period was 6.8. The highest proportion of snails over the three years was 43% which occurred in spring 1965

1.5.2.3 A. tomentosa habitat

A. tomentosa is a semi-aquatic snail found in various waterbodies in Australia (Table 12). *A. tomentosa* prefers waterbodies with slow-moving water, sparse vegetation, a muddy alluvial bottom, flat banks and uninterrupted sunlight (Boray, 1964a; Pullan et al., 1972). The population size of *A. tomentosa* within these habitats is limited by water availability and quality. The following factors can result in a reduction or elimination of *A. tomentosa* from the habitat: prolonged dry periods, a pH outside of 5.4-7.3, a salinity outside of 25-160ppm and if the water becomes nutrient deficient, stagnant, or turbid (Table 3) (Boray, 1964a; Lynch, 1965). Young *A. tomentosa* can survive the absence of water during prolonged dry periods by burrowing into mud and aestivating (Boray, 1969). Any environmental stimulus that alters the habitat where the snail is aestivating plays a crucial role in triggering the migration of snails to new waterbodies and the mass release of cercariae from snails (Boray, 1969). Environmental stimuli include sudden temperature changes, the return of water flow and the influx of water (i.e. rainfall, irrigation, or flood) into dry habitats.

Bradley (1926) surveyed 25 unique *A. tomentosa* habitats in NSW to determine the role different waterbodies play in the population dynamics of *A. tomentosa* and the transmission of *F. hepatica* to livestock (Table 12). Bradley (1926) classified the waterbodies as either permanent or temporary. Permanent water bodies include lakes, rivers, creeks, springs, swamps, bogs and on-farm dams fed by any previously listed sources (Smeal, 1995). Permanent water bodies act as a refuge to snails during adverse weather conditions (i.e. summer and drought), allowing snails to survive and proliferate when favourable conditions return (Boray, 1969). Environmental stimuli trigger snails to rapidly spread from the permanent habitats either by suspending their foot to the surface of the water or actively moving against the flow of water (100m in 2 weeks) to repopulate temporary waterbodies (Boray, 1964b; Boray, 1969).

Temporary water bodies include drains, leakages, outlets, irrigated channels, seepages and any surface collection of water created by rainfall, irrigation or flood (Boray, 1964a; Boray et al., 1969; Smeal, 1995). The water action that creates the temporary waterbodies also spreads faecal matter into low lying areas, increasing the likelihood of *F. hepatica* egg embryonation and infection of *A. tomentosa* (section 2.1 and 2.3.1). Temporary water

bodies pose a significant risk to grazing livestock as they are often unable to be fenced, and the *A. tomentosa* have moderate to high rates of *F. hepatica* infection (Table 9).

1.5.2.3 Waterbodies risk to livestock

In Australia, 60% of dairy farms have naturally occurring waterways on their property (Watson and Watson, 2015). Several studies have observed higher incidences of *F. hepatica* in cattle when waterbodies are present on farms or when the farms are prone to flooding (Alves et al., 2011; Kuerpick et al., 2013; Olsen et al., 2015). Similarly, higher incidences of *F. hepatica* in livestock have been observed on farms in the UK that have conserved native habitats as they had the unintended consequence of increasing snail habitat (Pritchard et al., 2005). In Australia, 4% of dairying land is conserved, but 15% of farmers intend to conserve more by planting trees (Watson and Watson, 2015). Pullan et al. (1972) found that a high abundance of aquatic vegetation and shade eliminated *A. tomentosa* from waterbodies. This finding should be explored to determine if conservation efforts by farmers in Australia will positively or negatively impact the abundance of *A. tomentosa* in waterbodies. Care will be needed during implementation to avoid the unintended consequences that arose in the UK.

1.5.3 Farm management

1.5.3.1 Dairy farms in Victoria Australia

In Australia, dairy cattle predominantly graze outside year-round. Victoria has the largest number of registered dairy farms, 3,516 of the 5,213 farms registered in Australia in 2018/2019 (Dairy Australia, 2019). There are three distinct dairy regions in Victoria; Western Victoria 1,171 farms, Gippsland 1,201 farms and Murray 1144 farms (Dairy Australia, 2019). There are slight regional differences in dairy production. The Western Victoria region relies on seasonal rainfall, Gippsland uses a combination of rainfall and irrigation, whereas the Murray relies on irrigation (Figure 1 4). The most commonly used irrigation systems on dairy farms are discussed in section 1.5.2.1. Each dairy region can support large dairy farms, large herd sizes, and high stocking rates, which increases the milk yield and, therefore, the profit. In 2018/2019, Victoria produced 5,574,000ML of milk, 63% of the total national milk volume (Dairy Australia, 2019). Moran et al. (2000) identified that the key driver of profit on dairy farms in Victoria is increasing the consumption of pasture per cow to increase milk yield per grazed hectare. Intensive grazing strategies are used across all regions to ensure high pasture consumption, and in

the last 15 years, some regions in Victoria now graze more than two cows per hectare (Watson and Watson, 2015). However, due to declining herd fertility over the last 20 years, dairy farmers have shifted away from seasonal calving in spring when there is high pasture availability to split calving in Autumn and Spring (Ooi et al., 2021). The advantage of the split calving system is that it provides additional opportunities for cows to conceive, but it is more reliant on supplementary feed (i.e. grains, concentrates, silage, hay) to combat low pasture availability, which can be costly (Ooi et al., 2021). The shift towards split calving and the greater use of supplementary feed could change the key drivers of dairy farm profitability in Victoria and potentially expose farms to more price volatility.

1.5.3.1 Pasture management

Dairy farms in Victoria are closed systems that have a limited number of paddocks for grazing. As a result, pasture management is highly ritualised with young stock (calves and heifers), dry cows, cropping, dry-land and the dispersal of effluent typically allocated to a few paddocks that are repeatedly used. Bullen et al. (2016) found that the limited pasture management options and the Victorian bovine Johne's disease Calf Accreditation Program (JDCAP) increased the frequency of anthelmintic treatments and accelerated the development of resistance in nematodes. No studies have been conducted in Australia assessing the impact of the JDCAP on the incidence of *F. hepatica* in calves and heifers and the development of *F. hepatica* drug resistance. Compounding calf, heifer and dry cow management is that these animals are typically grazed on more marginal pastures that have a lower pasture quality and are less maintained. In Denmark, these practices increased the likelihood of *F. hepatica* infection by four-fold (Takeuchi-Storm et al., 2017; Takeuchi-Storm et al., 2018). In contradiction to these findings, lower levels of infected snails were found in heifer and dry cow paddocks when compared to paddocks grazed by lactating animals (Schweizer et al., 2007). The implication of these findings needs to be explored to understand better the temporal patterns of *F. hepatica* transmission on dairy farms in Australia.

Research conducted by Olsen et al. (2015) found that the presence of dry-land and cropped-land negatively impacted F. hepatica incidence in cattle, which is in agreement with Schweizer et al. (2007), who found lower levels of infected snails on hay paddocks. On average, 43ha of land on dairy farms in Australia is cropped; however, the area of cropped-land is significantly less in Western VIC (15ha), Gippsland (VIC) (8ha), and TAS

(5ha) (Watson and Watson, 2015). Further research is needed in these regions to determine if they have a higher incidence of *F. hepatica.* A potentially compounding factor is the application of effluent (liquid manure) to cropped or grazed land. In Australia, it is common practice to apply diluted effluent to pasture to maximise nutrient retention for pasture growth and soil health. Effluent is applied to 21% of grazed land (approximately 34 hectares) on dairy farms using irrigation infrastructure, potentially contaminating the irrigation channels and drains with *F. hepatica* eggs (Watson and Watson, 2015). Research suggests that there is only a remote chance of transmission to cattle via effluent application so long as the effluent is heat-treated, aerated or in anaerobic conditions for at least 42 days (reviewed in John et al., 2019). However, these farms typically capture irrigation/effluent tailwater that has washed down paddocks and re-apply the water to improve on-farm water use efficiency (Birchall et al., 2008). Given that the irrigation tailwater recycling systems contain water, effluent and the remnants of fresh manure from paddocks, research needs to assess whether there is increased transmission of *F. hepatica* to livestock on pastures that are watered with tailwater.

Reactive pasture management in response to adverse weather sometimes increases the risk of *F. hepatica* transmission to cattle. During dry or drought conditions in Australia, pasture is only found in low lying areas and at the edges of waterbodies, forcing cattle to graze high-risk pasture resulting in outbreaks of fasciolosis (Osborne, 1958). In contrast, reactive management in wet conditions (i.e. heavy rainfall, flood) tries to avoid damage to pasture, particularly pugging, by putting animals on well-drained paddocks, previously damaged paddocks, or feed-pads. Pugging is a form of soil compaction caused by cattle hooves churning up the pasture and the soil profile. Pugging exacerbates waterlogging, resulting in water pooling within pastures due to poor drainage. Harris and Charleston (1977) in NZ found that pugged land was three times more likely to contain *P. columella* and six times more likely to contain *A. tomentosa.* Given that pugging occurs on 35% of dairy farms nationally, with a higher proportion of pugging occurring in Gippsland (VIC) (47%) and TAS (58%), farms should be encouraged to install and use feed-pads in order to avoid snail population increases within the pasture (Watson and Watson, 2015). No further research has been undertaken to determine whether the snail population increases due to pugging resulted in increased transmission of *F. hepatica* to cattle.

1.5.3.2 Herd management

Several studies in Europe have investigated the influence of herd management practices on the transmission of *F. hepatica* to livestock. The research identified several key variables that affect the transmission of *F. hepatica,* but it is difficult to assess whether these findings are relevant in Australia (Bennema et al., 2011; Charlier et al., 2011; Kuerpick et al., 2013; Novobilský et al., 2015; Olsen et al., 2015). There are substantial differences in-herd management practices between Europe and Australia, as cattle are not housed, they graze outside permanently, and herds and farms are substantially larger. It is likely that higher stocking rates, the proportion of grazed pasture in an animals diet and whether cattle were purchased from an infected farm are still relevant and increases the risk of transmission of *F. hepatica* (Bennema et al., 2011; Kuerpick et al., 2013; Novobilský et al., 2015; Olsen et al., 2015). In contrast, the length of the grazing period and month of turn out probably do not apply in Australia (Bennema et al., 2011; Kuerpick et al., 2013; Novobilský et al., 2015; Olsen et al., 2015). Research focusing on Australian herd management and *F. hepatica* transmission is needed to identify Australia's relevant risk factors. Any research undertaken must consider that several native and introduced animals often graze pasture intended for livestock found by Alves et al. (2011) to increase *F. hepatica* transmission risk. Little research has investigated the role native animals play in the *F. hepatica* transmission in Australia, but animals have been found to harbour considerable *F. hepatica* burdens (Section 2.6.4) (Table 4).

1.5.4 Climate change

In 2015, the Meat and Livestock industry in Australia listed *F. hepatica* as a priority endemic disease. It warned producers that the distribution of *F. hepatica* might increase due to climate change (Lane et al., 2015). The critical area of concern was the introduced exotic snails *P. columella* and *R. viridis,* which have a higher tolerance to warmer conditions and could lead to an expansion of *F. hepatica* in endemic regions, as well as into WA, which is currently *F. hepatica* free (Sections 2.3.2 and 2.3.3) (Figure 1 2). Modelling the impact of climate change on the distribution of *P. columella* and *R. viridis* in Australia may prove to be difficult as no *F. hepatica* model has yet accounted for the availability of the intermediate host (Haydock et al., 2016). Recently, Haydock et al. (2016) in NZ used a growing degree-day model validated against historical *F. hepatica* prevalence data to predict the future risk of *F. hepatica* infection in NZ. The predictions indicated

nationwide increases in *F. hepatica* infection risk; in some regions, the increases were as high as 186% and 184%, whereas, in other regions, there was little change (Haydock et al., 2016). It is evident that climate change will have a significant impact in NZ, and modelling should be undertaken in Australia to assess the impact of climate change on the incidence and distribution of *F. hepatica*.

1.6 Scope of the thesis

The literature review identified several areas where our understanding of *F. hepatica* in the Australian context is lacking and needs further investigation. The main objectives of this thesis are:

1. Chapter 1 found that little is known about the current prevalence of *F. hepatica* in Australia, and the last study conducted in Victoria was by Watt (1979) in the late 1970s. The aim of Chapter 3 is to determine the spatial distribution of *F. hepatica* on dairy farms in Victoria and identify regions where *F. hepatica* is endemic. The methodology described in Elliott et al. (2015) was used to determine the prevalence of *F. hepatica* within Victorian dairy regions. The research findings will identify endemic regions in Victoria, enabling the dairy industry to direct resources and prioritise extension with farmers in these regions.
2. Chapter 1 and Chapter 2 identified growing reports of TCBZ-resistant *F. hepatica* on cattle farms in Australia and globally. In Victoria, two dairy farms were found to have TCBZ-resistant *F. hepatica,* which is of great concern as TCBZ is the only drug registered for use in dairy cattle that kills immature *F. hepatica* (Brockwell et al., 2014; Elliott et al., 2015). The aim of Chapter 3 was to assess whether *F. hepatica* has developed resistance to TCBZ on dairy farms in other regions of Victoria. The TCBZ resistance testing method described in Brockwell et al. (2014) was used to test cattle and assess the efficacy of TCBZ on-farm. The research findings will raise awareness in the dairy industry about the need for regular TCBZ resistance testing and the use of non-chemical means of control in addition to chemical treatments.
3. Research published in Australia by Brockwell et al. (2013) found that when testing cattle daily using the cELISA, there was a 2-6 fold fluctuation in OD and that similar fluctuations were observed using LFEC (2-4 fold). The variability in the diagnostic results raised concerns about how the tests could be practically used on-farm to

inform treatment decisions. Chapter 4 assessed the sensitivity of the cELISA and the FlukeFinder® kit (LFEC) at AM and PM milking in naturally infected dairy cattle over five consecutive days. The faecal samples collected at AM and PM milking over the five-day period were tested using the methodology described in Kelley et al. (2020). The research outcomes should provide insight into how the industry can utilise the diagnostic tools on dairy farms in Victoria to inform *F. hepatica* control strategies.

4. Chapter 2 discussed how non-chemical and chemical control strategies could be used to create an integrated parasite management plan to control *F. hepatica* on individual farms. However, little is known in Australia about how dairy farmers currently control *F. hepatica*. The aim of Chapter 5 is to determine the *F. hepatica* control methods currently used on dairy properties by surveying producers across Victoria's dairy regions. The survey will provide insight into how farmers currently control *F. hepatica* and identify control strategies that could be improved to manage *F. hepatica* better on farms. The industry will be able to use this information to create extension programs that focus on improving farmers understanding of *F. hepatica* control options.

The overall intention of the thesis was to provide the dairy industry, government, veterinarians, and producers with the evidence they will need to develop effective *F. hepatica* control strategies specific to the dairy industry and enable the delivery of tailored extension programs for dairy producers in Victoria.

1.7 References

ABARES 2018a. Australian Dryland Salinity Assessment Spatial Data, Department of Agriculture Fisheries and Forestry, ed. (https://data.gov.au/data/dataset/d86b702b-73ad-4a7e-96ef-4e17fd0240cd, data.gov.au).

ABARES 2018b. Australian irrigation areas Vector (National), Department of Agriculture and Water Resources, ed. (https://data.gov.au/data/dataset/australian-irrigation-areas-vector-version-1a-national-land-and-water-resources-audit, data.gov.au).

ABS 2019. Water use on Australian Farms 2017-18, Australian Bureau of Statistics, ed. (https://www.abs.gov.au/AUSSTATS/abs@.nsf/Lookup/4618.0Main+Features12017-18?OpenDocument).

Alves, D., Carneiro, M., Martins, I., Bernardo, C., Donatele, D., Pereira Júnior, O., Almeida, B., Avelar, B., 2011. Distribution and factors associated with *Fasciola hepatica* infection in cattle in the south of Espírito Santo State, Brazil. Journal of Venomous Animals and Toxins including Tropical Diseases 17, 271-276.

Anderson, N., Luong, T., Vo, N., Bui, K., Smooker, P.M., Spithill, T.W., 1999. The sensitivity and specificity of two methods for detecting *Fasciola* infection in cattle. Veterinary Parasitology 83, 15-24.

Anderson, R., 1978. Population dynamics of snail infection by miracidia. Parasitology 77, 201-224.

Andrews, S., Graczyk, T., Fried, B., Fairweather, I., Threadgold, L., Torgerson, P., 1999. Fasciolosis. The Life Cycle of *Fasciola hepatica*. CAB International, Oxon, UK, 1-29.

Atlas of Living Australia 2020. Lymnaeidae (https://biocache.ala.org.au/occurrences/search?q=qid:1589350354936).

Baldock, F., Arthur, R., 1985. A Survey of fasciolosis in beef cattle killed at abattoir in Southern Queensland. Australian Veterinary Journal 62, 320-326.

Beesley, N.J., Williams, D.J., Paterson, S., and Hodgkinson, J. 2017. *Fasciola hepatica* demonstrates high levels of genetic diversity, a lack of population structure and high gene flow: possible implications for drug resistance. International journal for parasitology 47(1), 11-20.

Bennema, S., Ducheyne, E., Vercruysse, J., Claerebout, E., Hendrickx, G., Charlier, J., 2011. Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. International Journal for Parasitology 41, 225-233.

Bennema, S., Vercruysse, J., Claerebout, E., Schnieder, T., Strube, C., Ducheyne, E., Hendrickx, G., Charlier, J., 2009. The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). Veterinary Parasitology 165, 51-57.

Birchall, S., Dillon, C., Wrigley, R. 2008. Effluent and Manure Management Database for the Australian Dairy Industry (Melbourne, Dairy Australia), pp. 1-236.

Bloemhoff, Y., Forbes, A., Danaher, M., Good, B., Morgan, E., Mulcahy, G., Sekiya, M., Sayers, R., 2015. Determining the Prevalence and Seasonality of *Fasciola hepatica* in Pasture-based Dairy herds in Ireland using a Bulk Tank Milk ELISA. Irish Veterinary Journal 68, 16.

Boray, J., 1963. The ecology of *Fasciola hepatica* with particular reference to its intermediate host in Australia. In: International Veterinary Congress, 17th.

Boray, J., 1964a. Studies on the ecology of *Lymnaea tomentosa*, the intermediate host of *Fasciola hepatica*. 1. History, geographical distribution, and environment. Australian Journal of Zoology 12, 217-230.

Boray, J., 1964b. Studies on the ecology of *Lymnaea tomentosa*, the intermediate host of *Fasciola hepatica*. 2. The sexual behaviour of *Lymnaea tomentosa*. Australian Journal of Zoology 12, 231-238.

Boray, J., 1978. The potential impact of exotic *Lymnaea spp*. on fascioliasis in Australasia. Veterinary Parasitology 4, 127-141.

Boray, J., Enigk, K., 1964. Laboratory studies on the survival and infectivity of *Fasciola hepatica*- and *F. gigantica* metacercariae. Zeitschrift fur Tropenmedizin und Parasitologie 15, 324-331.

Boray, J., Fraser, G., Williams, J., Wilson, J., 1985. The occurrence of the snail *Lymnaea columella* on grazing areas in New South Wales and studies on its susceptibility to *Fasciola hepatica.* Australian Veterinary Journal 62, 4-6.

Boray, J., Happich, F., Andrews, J., 1969. The epidemiology of fasciolosis in two representative endemic regions of Australia. Australian Veterinary Journal 45, 549-553.

Boray, J.C., 1969. Experimental fascioliasis in Australia, In: Advances in Parasitology. Elsevier, pp. 95-210.

Bradley, B., 1926. Observations on the Water Snails of Monaro and New England, New South Wales, with Especial Reference to their Cercaria Carrying Capacity. Medical Journal of Australia 1.(6).

Brockwell, Y., Spithill, T., Anderson, G., Grillo, V., Sangster, N., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Veterinary Parasitology 196, 417-426.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. International Journal for Parasitology: Drugs and Drug Resistance 4, 48-54.

Brydon, P., 1960. The major causes for condemnation of meat in NSW. Australian Veterinary Journal 36, 113-117.

Brydon, P., Fewster, G.E., Whittem, J.H., Charles, D.C., 1960. Discussion: The major causes for condemnation of meat in NSW. Australian Veterinary Journal 36, 296-297.

Bullen, S., Beggs, D., Mansell, P., Runciman, D., Malmo, J., Playford, M., Pyman, M., 2016. Anthelmintic resistance in gastrointestinal nematodes of dairy cattle in the Macalister Irrigation District of Victoria. Australian Veterinary Journal 94, 35-41.

Castro, E., Freyre, A., Hernandez, Z., 2000. Serological responses of cattle after treatment and during natural re-infection with *Fasciola hepatica*, as measured with a dot-ELISA system. Veterinary Parasitology 90, 201-208.

Cawdery, M.H., Strickland, K., Conway, A., Crowe, P., 1977. Production effects of liver fluke in cattle I. The effects of infection on liveweight gain, feed intake and food conversion efficiency in beef cattle. British Veterinary Journal 133, 145-159.

Charlier, J., Bennema, S.C., Caron, Y., Counotte, M., Ducheyne, E., Hendrickx, G., Vercruysse, J., 2011. Towards assessing fine-scale indicators for the spatial transmission risk of *Fasciola hepatica* in cattle. Geospatial Health, 239-245.

Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Veterinary Parasitology 153, 44-51.

Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. Preventive Veterinary Medicine 78, 57-66.

Chartier, C., Reche, B., 1992. Gastrointestinal helminths and lungworms of French dairy goats: prevalence and geographical distribution in Poitou-Charentes. Veterinary Research Communications 16, 327-335.

Conceição, M.A.P., Durão, R.M., Costa, I.H., da Costa, J.M.C., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Veterinary Parasitology 105, 337-343.

Copeman, D., Copland, R., 2008. Importance and potential impact of liver fluke in cattle and buffalo. ACIAR Monograph Series 133, 21.

Cornelissen, J.B., Gaasenbeek, C.P., Boersma, W., Borgsteede, F.H., van Milligen, F.J., 1999. Use of a pre-selected epitope of cathepsin-L1 in a highly specific peptide-based immunoassay for the diagnosis of *Fasciola hepatica* infection in cattle. International Journal for Parasitology 29, 685-696.

Cornelissen, J.B., Gaasenbeek, C.P., Borgsteede, F.H., Holland, W.G., Harmsen, M.M., Boersma, W.J., 2001. Early immunodiagnosis of fasciolosis in ruminants using recombinant *Fasciola hepatica* cathepsin L-like protease. International Journal for Parasitology 31, 728-737.

Correa, A.C., Escobar, J.S., Durand, P., Renaud, F., David, P., Jarne, P., Pointier, J.-P., Hurtrez-Boussès, S., 2010. Bridging gaps in the molecular phylogeny of the Lymnaeidae (Gastropoda: Pulmonata), vectors of Fascioliasis. BMC Evolutionary Biology 10, 381.

Cringoli, G., Musella, V., Maurelli, M., Morgoglione, M., Santaniello, A., Condoleo, R., Guariglia, I., Rinaldi, L., 2009. Helminths and arthropoda in buffalo farms from the Lazio region (Italy). Veterinary Research Communications 33, 129-131.

Crossman, S., Li, O. 2015a. Surface Hydrology Lines (National), Geoscience Australia, C., ed. (http://pid.geoscience.gov.au/dataset/ga/83130).

Crossman, S., Li, O. 2015b. Surface Hydrology Polygons (National), Geoscience Australia, C., ed. (http://pid.geoscience.gov.au/dataset/ga/83135).

Cwiklinski, K., O'neill, S., Donnelly, S., Dalton, J., 2016. A prospective view of animal and human Fasciolosis. Parasite Immunology 38, 558-568.

Dairy Australia 2019. The Australian Dairy Industry - In Focus 2019 (https://www.dairyaustralia.com.au/resource-repository/2020/07/09/australian-dairy-industry-in-focus-2019#.YSBj_ogzbcs, Dairy Australia), pp. 1-52.

Daniel, R., Van Dijk, J., Jenkins, T., Akca, A., Mearns, R., Williams, D., 2012. Composite faecal egg count reduction test to detect resistance to triclabendazole in *Fasciola hepatica*. Veterinary Record 171, 153-153.

Dargie, J., 1987. The impact on production and mechanisms of pathogenesis of trematode infection in cattle and sheep. International Journal for Parasitology 17, 453-463.

Dawes, B. 1963. The migration of juvenile forms of *Fasciola hepatica* L. through the wall of the intestines in the mouse, with some observations on food and feeding. Parasitology 53(1-2), 109-122.

Dixon, K., 1963. Incidence of liver fluke (*Fasciola hepatica*) in Queensland cattle. Australian Veterinary Journal 39, 53-56.

Dixon, K., 1965. The structure and histochemistry of the cyst wall of the metacercaria of *Fasciola hepatica* L. Parasitology 55, 215-226.

Dorsman, W., 1956. Fluctuation within a day in the liver fluke egg count of the rectal contents of cattle. Veterinary Record 68, 571-574.

Dorsman, W., 1960. The diagnosis of subclinical fascioliasis by means of faecal examination, and the control of liver flukes (*Fasciola hepatica*). Bulletin de l'Office International des Epizooties 54, 502-508.

Doy, T., Hughes, D., 1984. Early migration of immature *Fasciola hepatica* and associated liver pathology in cattle. Research in Veterinary Science 37, 219-222.

Durr, P., Tait, N., Lawson, A., 2005. Bayesian hierarchical modelling to enhance the epidemiological value of abattoir surveys for bovine fasciolosis. Preventive Veterinary Medicine 71, 157-172.

Duscher, R., Duscher, G., Hofer, J., Tichy, A., Prosl, H., Joachim, A., 2011. *Fasciola hepatica*– Monitoring the milky way? The use of tank milk for liver fluke monitoring in dairy herds as base for treatment strategies. Veterinary Parasitology 178, 273-278.

Elliott, T., Kelley, J., Rawlin, G., Spithill, T., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Veterinary Parasitology 209, 117-124.

George, S., Vanhoff, K., Baker, K., Lake, L., Rolfe, P., Seewald, W., Emery, D., 2017. Application of a coproantigen ELISA as an indicator of efficacy against multiple life stages of *Fasciola hepatica* infection in sheep. Veterinary Parasitology 246, 60-69.

Gordon, H.M., 1955. Some aspects of fascioliasis. Australian Veterinary Journal 31, 182-189.

Hagens, F., Over, H., 1966. Egg-Output and Egg-Count of *Fasciola hepática* in Cattle. Tijdschrift voor Diergeneeskunde 91, 523-531.

Happich, F., Boray, J., 1969a. Quantitative diagnosis of chronic fasciolosis. 2. The estimation of daily total egg production of *Fasciola hepatica* and the number of adult flukes in sheep by faecal egg counts. Australian Veterinary Journal 45, 329-331.

Happich, F., Boray, J., 1969b. Quantitative diagnosis of chronic fasciolosis. 1. Comparative Studies on Quantitative Faecal Examinations for Chronic *Fasciola hepatica* Infection in Sheep. Australian Veterinary Journal 45, 326-328.

Harris, R., Charleston, W., 1977. An examination of the marsh microhabitats of *Lymnaea tomentosa* and *L. columella* (Mollusca: Gastropoda) by path analysis. New Zealand Journal of Zoology 4, 395-399.

Harris, R., Charleston, W., 1980. Fascioliasis in New Zealand: a review. Veterinary Parasitology 7, 39-49.

Hart, B., Walker, G., Katupitiya, A., Doolan, J., 2020. Salinity Management in the Murray–Darling Basin, Australia. Water 12, 1829.

Haydock, L., Pomroy, W., Stevenson, M., Lawrence, K., 2016. A growing degree-day model for determination of *Fasciola hepatica* infection risk in New Zealand with future predictions using climate change models. Veterinary Parasitology 228, 52-59.

Hodasi, J., 1972. The output of cercariae of *Fasciola hepatica* by *Lymnaea truncatula* and the distribution of metacercariae on grass. Parasitology 64, 53-60.

Hörchner, F., Hennings, R., Verspohl, F., Averbeck, W., Boch, J., 1970. Anthelmintic control of bovine fascioliasis in the Steinfurt area. II. Results of three years of treatment. Berliner und Münchener Tierärztliche Wochenschrift 83, 21-26.

Hort, C., 1998. A survey of *Fasciola hepatica* infection of sheep and cattle in NSW and Victoria. In: Proceedings of the Australian Sheep Veterinary Society Conference, Sydney, pp. 61-64.

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. Preventive Veterinary Medicine 121, 41-48.

Ibarra, F., Montenegro, N., Vera, Y., Boulard, C., Quiroz, H., Flores, J., Ochoa, P., 1998. Comparison of three ELISA tests for seroepidemiology of bovine fascioliosis. Veterinary Parasitology 77, 229-236.

Innocent, G.T., Gilbert, L., Jones, E.O., McLeod, J.E., Gunn, G., McKendrick, I.J., Albon, S.D., 2017. Combining slaughterhouse surveillance data with cattle tracing scheme and environmental data to quantify environmental risk factors for liver fluke in cattle. Frontiers in Veterinary Science 4, 65.

Jenkins, D., Porter, M., Baker, A., Shamsi, S., Barton, D., 2020. Investigation into fallow deer (*Dama dama*) as definitive hosts of *Fasciola hepatica* (liver fluke) in alpine New South Wales. Australian Veterinary Journal 98, 546-549.

Jepps, M.W., 1933. Miracidia of the liver fluke for laboratory work. Nature 132, 171-171.

John, B.C., Davies, D.R., Williams, D.J., Hodgkinson, J.E., 2019. A review of our current understanding of parasite survival in silage and stored forages, with a focus on *Fasciola hepatica* metacercariae. Grass and Forage Science 74, 211-217.

Kajugu, P.-E., Hanna, R., Edgar, H., McMahon, C., Cooper, M., Gordon, A., Barley, J., Malone, F., Brennan, G., Fairweather, I., 2015. *Fasciola hepatica*: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Veterinary Parasitology 212, 181-187.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends in Parasitology 32, 458-469.

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Veterinary Parasitology 277, 109019.

Krull, W., 1941. The number of cercariae of *Fasciola hepatica* developing in snails infected with a single miracidium. Proceedings of the Helminthological Society of Washington 8, 55-58.

Kuerpick, B., Conraths, F.J., Staubach, C., Froehlich, A., Schnieder, T., Strube, C., 2013. Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infection in dairy herds in Germany. Parasitology 140, 1051-1060.

Lane, J., Jubb, T., Shephard, R., Webb-Ware, J., Fordyce, G. 2015. MLA Final Report: Priority List of Endemic Diseases for the Red Meat Industries. MLA. Available from https://www.mla.com.au/research-and-development/search-rd-reports/final-report-details/Animal-Health-and-Biosecurity/Priority-list-of-endemic-diseases-for-the-red-meat-industries/2895.

Love, S., Hutchinson, G., Boray, J.C. 2017. Liver fluke disease in sheep and cattle, Department of Primary Industries, ed. (https://www.dpi.nsw.gov.au/about-us/publications, NSW Government), pp. 1-14.

Lynch, J., 1965. The ecology of *Lymnaea tomentosa* (Pfeiffer 1855) in South Australia. Australian Journal of Zoology 13, 461-474.

Machicado, C., Machicado, J.D., Maco, V., Terashima, A., Marcos, L.A., 2016. Association of Fasciola hepatica Infection with Liver Fibrosis, Cirrhosis, and Cancer: A Systematic Review. PLOS Neglected Tropical Diseases 10, e0004962.

Malone, J., Craig, T., 1990. Cattle liver flukes: risk assessment and control. Compendium on Continuing Education for the Practicing Veterinarian 12, 747-754.

Malone, J., Loyacano, A., Hugh-Jones, M., Corkum, K., 1984. A three-year study on seasonal transmission and control of *Fasciola hepatica* of cattle in Louisiana. Preventive Veterinary Medicine 3, 131-141.

Malone, J.B., Fehler, D.P., Loyacano, A.F., Zukowski, S.H., 1992. Use of LANDSAT MSS Imagery and Soil Type in a Geographic Information System to Assess Site-Specific Risk of Fascioliasis on Red River Basin Farms in Louisiana. Annals of the New York Academy of Sciences 653, 389-397.

Martínez-Sernández, V., Orbegozo-Medina, R.A., González-Warleta, M., Mezo, M., Ubeira, F.M., 2016. Rapid enhanced MM3-COPRO ELISA for detection of *Fasciola* coproantigens. PLoS Neglected Tropical Diseases 10, e0004872.

Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. Journal of Helminthology 79, 207-216.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Advances in Parasitology 69, 41-146.

Mazeri, S., Sargison, N., Kelly, R.F., Bronsvoort, B.M.d., Handel, I., 2016. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. PloS one 11.

McCann, C., Baylis, M., Williams, D., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. The Veterinary Record 166, 612-617.

McCausland, I., Vandegraaff, R., Nugent, L., 1980. Fascioliasis in dairy cows on irrigated pasture. Australian Veterinary Journal 56, 324-326.

Meek, A., Morris, R., 1979a. The longevity of *Fasciola hepatica* metacercariae encysted on herbage. Australian Veterinary Journal 55, 58-60.

Meek, A., Morris, R., 1979b. An epidemiological investigation of ovine fascioliasis (*Fasciola hepatica*) on both irrigated and non-irrigated pastures in Nothern Victoria. Australian Veterinary Journal 55, 365-369.

Ménard, A., L'Hostis, M., Leray, G., Marchandeau, S., Pascal, M., Roudot, N., Michel, V., Chauvin, A., 2000. Inventory of wild rodents and lagomorphs as natural hosts of *Fasciola hepatica* on a farm located in a humid area in Loire Atlantique (France). Parasite 7, 77-82.

Mezo, M., González-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). Journal of Parasitology 90, 845-852.

Mezo, M., González-Warleta, M., Castro-Hermida, J.A., Ubeira, F.M., 2008. Evaluation of the flukicide treatment policy for dairy cattle in Galicia (NW Spain). Veterinary Parasitology 157, 235-243.

Molloy, J., Anderson, G., 2006. The distribution of *Fasciola hepatica* in Queensland, Australia, and the potential impact of introduced snail intermediate hosts. Veterinary Parasitology 137, 62-66.

Molloy, J., Anderson, G., Fletcher, T., Landmann, J., Knight, B., 2005. Evaluation of a commercially available enzyme-linked immunosorbent assay for detecting antibodies to *Fasciola hepatica* and *Fasciola gigantica* in cattle, sheep and buffaloes in Australia. Veterinary Parasitology 130, 207-212.

Murray-Darling Basin Authority 2016. Basin Irrigation and Salinity Mapping Murray-Darling Basin Authority, ed. (https://data.gov.au/data/dataset/3ae82857-c929-4007-8e55-240de9d704ed, data.gov.au).

Neuhaus, W., 1953. Über den chemischen Sinn der Miracidien von *Fasciola hepatica*. Zeitschrift für Parasitenkunde 15, 476-490.

Novobilský, A., Sollenberg, S., Höglund, J., 2015. Distribution of *Fasciola hepatica* in Swedish dairy cattle and associations with pasture management factors. Geospatial Health 9, 293-300.

Obendorf, D., 1983. Liver fluke infection in northwestern Tasmania. In: Papers and Proceedings of the Royal Society of Tasmania, pp. 1-3.

Olsen, A., Frankena, K., Toft, N., Thamsborg, S.M., Enemark, H.L., Halasa, T., 2015. Prevalence, risk factors and spatial analysis of liver fluke infection in Danish cattle herds. Parasites & Vectors 8, 160.

Osborne, H., 1958. Observations on the seasonal incidence of acute fascioliasis and infectious necrotic hepatitis (black disease) in sheep. Australian Veterinary Journal 34, 301-304.

Ono, Y., Isoda, M., Matsumura, S., 1954. I. Preventive study of *Fasciola hepatica* infection. II. Effects on metacercariae of various environmental conditions and drugs. Journal of the Japanese Veterinary Medicine Association 7, 153-155.

Ooi, E.C., Stevenson, M.A., Beggs, D.S., Mansell, P.D., Pryce, J.E., Murray, A., Pyman, M.F., 2021. The impact of declining dairy fertility on calving patterns and farm systems: A case study from northern Victoria, Australia. Agricultural Systems 193, 103228.

Palmer, D., Lyon, J., Palmer, M., Forshaw, D., 2014. Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses. Australian Veterinary Journal 92, 357-361.

Pandey, V., 1983. Observations on *Fasciola hepatica* in donkeys from Morocco. Annals of Tropical Medicine & Parasitology 77, 159-162.

Pearson, I., Boray, J., 1961. The anthelmintíc effect of the intramuscular injection of carbon tetrachloride on *Fasciola hepatica* in cattle. Australian Veterinary Journal 37, 73-78.

Penny, J. 1869. Report of Royal Commission on Fluke. In Launceston Examiner (Tas. :1842 - 1899) (Tas.), p. 2.

Ponder, W., 1975. The occurrence of *Lymnaea (Pseudosuccinea) columella*, an intermediate host of *Fasciola hepatica*, in Australia. Australian Veterinary Journal 51, 494-495.

Ponder, W.F., Hallan, A., Shea, M. and Clark, S. A. 2016a. Australian Freshwater Molluscs. In *Austropeplea (Austropeplea) huonensis* (The Australian Museum).

Ponder, W.F., Hallan, A., Shea, M. and Clark, S. A. 2016b. Australian Freshwater Molluscs. In *Austropeplea (Austropeplea) brazieri* (The Australian Museum).

Ponder, W.F., Hallan, A., Shea, M. and Clark, S. A. 2016c. Australian Freshwater Molluscs. In *Austropeplea (Austropeplea) papyracea* (The Australian Museum).

Pritchard, G., Forbes, A., Williams, D., Salimi-Bejestani, M., Daniel, R., 2005. Emergence of fasciolosis in cattle in East Anglia. The Veterinary Record 157, 578-582.

Pullan, N., Climo, F., Mansfield, C.B., 1972. Studies on the distribution and ecology of the family lymnaeidae (Mollusca: Gastropoda) in New Zealand. Journal of the Royal Society of New Zealand 2, 393-405.

Puslednik, L., Ponder, W.F., Dowton, M., Davis, A.R., 2009. Examining the phylogeny of the Australasian Lymnaeidae (Heterobranchia: Pulmonata: Gastropoda) using mitochondrial, nuclear and morphological markers. Molecular Phylogenetics and Evolution 52, 643-659.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International Journal for Parasitology 36, 1153-1158.

Rathinasamy, V., Hosking, C., Tran, L., Kelley, J., Williamson, G., Swan, J., Elliott, T., Rawlin, G., Beddoe, T., Spithill, T.W., 2018. Development of a multiplex quantitative PCR assay for detection and quantification of DNA from *Fasciola hepatica* and the intermediate snail host, Austropeplea tomentosa, in water samples. Veterinary Parasitology 259, 17-24.

Rees, J., Sykes, W., Rickard, M., 1975. Prenatal infection with *Fasciola hepatica* in calves. Australian Veterinary Journal 51, 497-499.

Reichel, M., 2002. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke (*Fasciola hepatica*) infection in sheep and cattle. Veterinary Parasitology 107, 65-72.

Reichel, M., Vanhoff, K., Baxter, B., 2005. Performance characteristics of an enzyme-linked immunosorbent assay performed in milk for the detection of liver fluke (*Fasciola hepatica*) infection in cattle. Veterinary Parasitology 129, 61-66.

Roberts, J., 1982. The prevalence and economic significance of liver disorders and contamination in grain-fed and grass-fed cattle. Australian Veterinary Journal 59, 129-132.

Ross, J., Todd, J., Dow, C., 1966. Single experimental infection of calves with the liver fluke *Fasciola hepatica* (Linnaeus 1758). Journal of Comparative Pathology 76, 67-81.

Salimi-Bejestani, M., Cripps, P., Williams, D.J., 2008. Evaluation of an ELISA to assess the intensity of *Fasciola hepatica* infection in cattle. Veterinary Record 162, 109-111.

Salimi-Bejestani, M., Daniel, R., Cripps, P., Felstead, S., Williams, D.J., 2007. Evaluation of an enzyme-linked immunosorbent assay for detection of antibodies to *Fasciola hepatica* in milk. Veterinary Parasitology 149, 290-293.

Salimi-Bejestani, M., McGarry, J., Felstead, S., Ortiz, P., Akca, A., Williams, D.J., 2005a. Development of an antibody-detection ELISA for *Fasciola hepatica* and its evaluation against a commercially available test. Research in Veterinary Science 78, 177-181.

Salimi-Bejestani, M., Daniel, R., Felstead, S., Cripps, P., Mahmoody, H., Williams, D., 2005b. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Veterinary Record-English Edition 156, 729-731.

Sánchez-Andrade, R., Paz-Silva, A., Suárez, J., Panadero, R., Pedreira, J., López, C., Díez-Baños, P., Morrondo, P., 2002. Influence of age and breed on natural bovine fasciolosis in an endemic area (Galicia, NW Spain). Veterinary Research Communications 26, 361-370.

Schweizer, G., Braun, U., Deplazes, P., Torgerson, P., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Veterinary Record 157, 188-193.

Schweizer, G., Meli, M.L., Torgerson, P.R., Lutz, H., Deplazes, P., Braun, U., 2007. Prevalence of *Fasciola hepatica* in the intermediate host *Lymnaea truncatula* detected by real time TaqMan PCR in populations from 70 Swiss farms with cattle husbandry. Veterinary Parasitology 150, 164-169.

Seddon, H.R., 1950. Diseases of domestic animals in Australia. Part I. Helminth infestations. Diseases of domestic animals in Australia. Part I. Helminth infestations.

Selemetas, N., Ducheyne, E., Phelan, P., O'Kiely, P., Hendrickx, G., de Waal, T., 2015. Spatial analysis and risk mapping of *Fasciola hepatica* infection in dairy herds in Ireland. Geospatial Health 9, 281-291.

Selemetas, N., Phelan, P., O'kiely, P., Waal, T., 2014. Weather and soil type affect incidence of fasciolosis in dairy cow herds. The Veterinary Record 175, 371-371.

Smeal, M.G., 1995. Parasites of cattle. In. University of Sydney, Post-Graduate Foundation in Veterinary Science.

Spithill, T., Smooker, P., Copeman, D., 1999. *Fasciola gigantica*: epidemiology, control, immunology and molecular biology., In: Fasciolosis. Commonwealth Agricultural Bureau International, Oxworth, pp. 465–525.

Spratt, D.M., Presidente, P.J., 1981. Prevalence of *Fasciola hepatica* infection in native mammals in southeastern Australia. Australian Journal of Experimental Biology and Medical Science 59, 713-721.

Takeuchi-Storm, N., Denwood, M., Hansen, T.V.A., Halasa, T., Rattenborg, E., Boes, J., Enemark, H.L., Thamsborg, S.M., 2017. Farm-level risk factors for *Fasciola hepatica* infection in Danish dairy cattle as evaluated by two diagnostic methods. Parasites & Vectors 10, 555.

Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A.-S., Sengupta, M.E., Beesley, N.J., Hodgkinson, J., Williams, D., Thamsborg, S.M., 2018. Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasites & Vectors 11, 674.

Vaughan, J., Charles, J., Boray, J., 1997. *Fasciola hepatica* infection in farmed emus (*Dromaius novaehollandiae).* Australian Veterinary Journal 75, 811-813.

Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infection in cattle: defining the threshold. Veterinary Parasitology 98, 195-214.

Virbac 2016. FlukeKill™ Liver Fluke Control Manual, Australia, V., ed. (virbac.com.au, Virbac Australia Pty Ltd.), p. 4.

Walker, S., Johnston, C., Hoey, E., Fairweather, I., Borgsteede, F., Gaasenbeek, C., Prodohl, P., Trudgett, A., 2011. Potential role of hares in the spread of liver fluke in the Netherlands. Veterinary Parasitology 177, 179-181.

Watson, P., Watson, D. 2015. Sustainability Framework NRM Survey (http://www.dairyingfortomorrow.com.au/wp-content/uploads/Australian-Dairy-Sustainability-Framework-NRM-Survey-2015.pdf, Dairy Australia), pp. 1-64.

Watt, G., 1979. An approach to determining the prevalence of liver-fluke in a large region. In: Proceedings of the 2nd International Symposium on Veterinary Epidemiology and Economics, 7-11 May 1979, Canberra, Australia., pp. 152-155.

Whittington, R., 1982. Chronic fascioliasis in a brushtail possum (*Trichosurus vulpecula*). Australian Veterinary Journal 59, 192-192.

1.8 Appendix

Appendix 1. The *A. tomentosa*, *P. columella* and *R. viridis* record data was sourced from the following organisations

Name	Citation	No. of records
Atlas of Life in the Coastal Wilderness	Records provided by Atlas of Life in the Coastal Wilderness accessed through ALA website	1
Australian Museum	Records provided by Australian Museum accessed through ALA website	1738
Australian River Assessment System	Mapping Western Australia's Biodiversity Department of Environment and Conservation URL: http://naturemapdecwagovau/	205
Carnarvon Basin (Aquatic Projects) - Invertebrates	Mapping Western Australia's Biodiversity Department of Environment and Conservation URL: http://naturemapdecwagovau/	8
Citizen Science - ALA Website	Records provided by Citizen Science - ALA Website, accessed through ALA website	1
Global Biodiversity	Records provided by the Global Biodiversity Information Facility accessed through ALA website	35
Healthy Waterways	Records provided by Healthy Waterways Waterwatch Program accessed through ALA website	16
iNaturalist	iNaturalistorg: iNaturalist Research-grade Observations doi:1015468/ab3s5x Accessed via http://wwwgbiforg/dataset/50c9509d-22c7-4a22-a47d-8c48425ef4a7 on 2017-03-16	1
Individual Sightings	Records provided by Individual Sightings accessed through ALA website	1
MDFRC Macroinvertebrate Survey	Murray-Darling Freshwater Research Centre	93
Museum and Art Gallery of the Northern Territory	Records provided by Museum and Art Gallery of the NT accessed through ALA website	15
Museums Victoria	Records provided by Museums Victoria accessed through ALA website	294
NatureMap	Records provided by NatureMap accessed through ALA website	213
OZCAM	Records provided by Online Zoological Collections of Australian Museums accessed through ALA website	2255

Name	Citation	No. of records
Queen Victoria Museum and Art Gallery	Records provided by the Queen Victoria Museum and Art Gallery accessed through ALA website	65
Queensland Museum	Records provided by Queensland Museum accessed through ALA website	42
Questagame	Records provided by Questagame accessed through ALA website	2
SA Museum	Records provided by the SA Museum accessed through ALA website	2
Sustainable Rivers Audit	Murray-Darling Basin Authority	406
Tasmanian Museum and Art Gallery	Records provided by Tasmanian Museum and Art Gallery accessed through ALA website	31
Tasmanian Natural Values Atlas	State of Tasmania Natural Values Atlas (wwwnaturalvaluesatlastasgovau)	4
Western Australian Museum	Records provided by WA Museum accessed through ALA website	68

Chapter 2 – Literature review

Current Threat of Triclabendazole Resistance in Fasciola hepatica

2.0 Preface

The scope of the thesis is to better understand the epidemiology and management of *F. hepatica* on dairy farms in Victorian.

In Chapter 1 I reviewed the literature and reported on our current understanding of the epidemiology of *F. hepatica* in Australia. This Chapter describes the ecology, lifecycle, and distribution of *F. hepatica* and *A. tomentosa* in Australia and how the *F. hepatica* infection is detected and the impact infection has on cattle production in Australia. This Chapter provided the epidemiological background for the thesis and identified the gaps in knowledge, some of which will be addressed in subsequent research in Chapters 3, 4, and 5.

In Chapter 2 I reviewed the literature and captured how *F. hepatica* historically has been managed and treated globally. This Chapter has two themes: chemical and non-chemical controls strategies and their effectiveness at controlling *F. hepatica*. The focus of chemical control centred around the use of triclabendazole (TCBZ) and the development of resistance to this chemical. The review of non-chemical control options identified strategies that could be used to control *F. hepatica* via the development and implementation of integrated parasite management (IPM) plans. The IPM plans take into account the climatic, flukicide, pasture, animal and water management factors that influence the prevalence of *F. hepatica* on-farm and identify what control strategies should be implemented when

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2.1 Published Article and Contribution

Published article

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends in Parasitology 32, 458-469. https://doi.org/10.1016/j.pt.2016.03.002

Contribution

This literature review was peer reviewed and published. J. Kelley prepared and compiled the data shown in Tables 1 and 2 and Figures 1 and 2: all authors contributed to the drafting of the text. J. Kelley and T. Spithill were responsible for the final editing of the manuscript and for the reply to the reviewers' comments. Trends in Parasitology applies the Creative Commons Attribution-Non Commercial-No Derivatives (CC BY-NC-ND) license to works we publish. The complete article is available here.

J. Kelley contributed to approximately 75% of the production of this literature review. J. Kelley made the tables and figures for the paper, assisted with writing and editing of the manuscript and responding to reviewers' comments, under the supervision of Professor Terry Spithill.

2.2 TCBZ-R Is a Current and Widespread Threat to Livestock Production Systems

2.3 Current Global Status of TCBZ-R

Trends in Parasitology

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Review

Current Threat of Triclabendazole Resistance in *Fasciola hepatica*

Jane M. Kelley,[1] Timothy P. Elliott,[2] Travis Beddoe,[1] Glenn Anderson,[3] Philip Skuce,[4] and Terry W. Spithill[1,*]

Triclabendazole (TCBZ) is the only chemical that kills early immature and adult *Fasciola hepatica* (liver fluke) but widespread resistance to the drug greatly compromises fluke control in livestock and humans. The mode of action of TCBZ and mechanism(s) underlying parasite resistance to the drug are not known. Due to the high prevalence of TCBZ resistance (TCBZ-R), effective management of drug resistance is now critical for sustainable livestock production. Here, we discuss the current status of TCBZ-R in *F. hepatica*, the global distribution of resistance observed in livestock, the possible mechanism(s) of drug action, the proposed mechanisms and genetic basis of resistance, and the prospects for future control of liver fluke infections using an integrated parasite management (IPM) approach.

Trends

TCBZ-R in *Fasciola hepatica* has now been demonstrated on at least 30 properties worldwide.

TCBZ-R in *F. hepatica* is now reported in cattle in Australia and South America.

Human infections with TCBZ-resistant *F. hepatica* have been reported in The Netherlands, Chile, Turkey, and Peru.

The coproantigen ELISA may allow an estimate of the intensity of fluke infection in livestock.

The mode of action of TCBZ may involve inhibition of adenylate cyclase activity.

Genome-wide single nucleotide polymorphism (SNP) analysis to map TCBZ-R in progeny of a genetic cross of parental TCBZ-resistant and TCBZ-susceptible clones has identified several scaffolds within the draft *F. hepatica* genome sequence associated with the TCBZ-R phenotype.

Appearance of resistance to closantel, clorsulon, and nitroxynil emphasises the importance of protecting the efficacy of existing flukicides.

A liver fluke vaccine for livestock is a major current area of research.

IPM on farms is a key strategy to improve livestock productivity.

TCBZ-R Is a Current and Widespread Threat to Livestock Production Systems

Fasciola species (*Fasciola hepatica* and *Fasciola gigantica*), also known as liver fluke parasites, are distributed worldwide in sheep and cattle and their **prevalence** (see Glossary) in some regions is so great that serious clinical disease, termed '**fasciolosis**', occurs [1,2]. Globally, fasciolosis due to both fluke species is conservatively thought to cause production losses of over US$3 billion per annum [3]. The benzimidazole derivative TCBZ, one of the major drugs used to control fasciolosis (Table 1), was first introduced during the early 1980s as a **flukicide** to treat and control acute and chronic fasciolosis in ruminants [4,5]. It had high efficacy (>98%) against adult flukes and, more importantly, unique efficacy against early-**immature** and immature flukes. Other single flukicides only target more mature flukes ranging in age from 8 to 14 weeks (Table 1) [4]. As a result, TCBZ rapidly became the drug of choice for treating fluke infections, especially in sheep, because it was safe and allowed producers the relative luxury of not having to test for the stage of fluke present in their livestock [6,7]. This over-reliance on TCBZ to treat sheep and, to a lesser extent, cattle, has resulted in selection for flukes resistant to TCBZ [6–8].

The status of TCBZ-R in *F. hepatica* has been reviewed elsewhere [6–13]. Here, we focus on the current status of TCBZ-R worldwide, the possible mechanism(s) of action of the drug, the current knowledge of the genetics of resistance, the prospects for future control of liver fluke infections, and the implementation of an **IPM** plan to manage fasciolosis.

Current Global Status of TCBZ-R

Cases of TCBZ-R in Livestock

Since the first appearance of TCBZ-R in *F. hepatica* [14], TCBZ-R has compromised fluke control in livestock in 11 countries or regions (Table 2; Figure 1, Key Figure). Resistance has likely

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Table 1. List of Currently Available TCBZ Products and other Drugs to Control *Fasciola hepatica* in Cattle and Sheep Worldwide[a]

Flukicides: Active Compound(s)	Method of Administration Available	Age of *F. hepatica* Killed[b]	Reports of Resistance On-Farm	Refs
TCBZ and TCBZ-based combinations	Oral, **pour-on**	From early immature	30 cases	Table 2
Albendazole	Oral, intraruminal	From adult	3 cases	[76,90,91]
Clorsulon	Injectable, oral	From adult; from late immature for oral	3 cases	[36,76,91]
Closantel	Pour-on, injectable, oral	From late immature	1 case	[75]
Nitroxynil	Injectable	From adult	1 case	[4]
Nitroxynil + clorsulon	Injectable	From early immature	No reports to date	
Oxyclozanide	Oral	From adult	No reports to date	

[a]Label claims for efficacy of products containing each compound can vary as a result of differences in formulation and regulatory systems in different countries.
[b]Age of parasites: early immature, 1–4 weeks, parenchyma migration stage; late immature, 6–8 weeks, prepatent stage in bile ducts; adult, 12–14 weeks, bile duct stage [18].

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[3]Virbac (Australia) Pty Ltd, Milperra, NSW, Australia
[4]Moredun Research Institute, Pentlands Science Park, Bush Loan, Midlothian, UK

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appeared due to a generally poor understanding of liver fluke biology by farmers and confounding factors, such as incorrect dosing, inappropriate product choice, and lack of testing for **efficacy** [11,12,15]. The high frequency of TCBZ use, effectively TCBZ monotherapy with no anthelmintic rotation, was a major contributing factor towards the development of TCBZ-R [14,16]. Since TCBZ is not a persistent chemical, resistance was likely due to **head selection** in contrast to **tail selection** observed with **roundworms** [17].

The main method used to identify TCBZ-R in the field has been the **faecal egg count reduction test** (FECRT), with the recommended post-treatment sample collection time point at 21 days [18,19]. Other studies using experimental infections have used 14 days for post-treatment sample collection, which may not allow sufficient time for all eggs from dead parasites to pass out of the gall bladder and be excreted [20,21]. The use of the FECRT and the new **coproantigen ELISA** (cELISA) [22], in the form of a **coproantigen reduction test** (CRT), is now becoming common research practice [19–21,23], but has yet to be routinely used in the field. Given that TCBZ kills most stages of a fluke infection in the host animal, a significant reduction (>95%) in egg count or coproantigen should occur if using an oral TCBZ formulation in a susceptible fluke population. However, when adult flukicides are tested, the egg counts and coproantigen levels may not be reduced to zero at the time of retesting (21 days post-treatment), even in a susceptible population, since young parasites not targeted by the adult flukicide will subsequently mature and release eggs or coproantigens. Several studies have shown that the signal in the cELISA is related to fluke numbers and could be used to indicate the relative level of fluke infection (low, moderate, or high), allowing the targeted treatment of animals, but further work is required to validate the cELISA under field conditions [2,22,24,25]. The **bulk milk tank ELISA** is also used to detect fluke infections; however, this method only detects antibodies to whole-fluke antigen and, since antibodies can persist following treatment, this ELISA cannot be used to evaluate drug efficacy against *F. hepatica* [2,10].

Before 2011, peer-reviewed reports of TCBZ-R were historically described in livestock on only six properties in Australia, Scotland, Wales, The Netherlands, Spain, and the Republic of Ireland [8,10] (Table 2). Since then, TCBZ-R has been reliably reported in sheep or cattle on a further 24 properties in Northern Ireland, Scotland, Wales, Australia, New Zealand, Peru, and Argentina (Table 2) [13,19,26–32]. Four human cases of TCBZ-R are discussed below. In total, cases of TCBZ-R have been reported on at least 30 properties (Table 2). Several non-peer-reviewed

Table 2. Published Peer-Reviewed Reports of TCBZ-R in Livestock On-farm or in Humans, and Methods of Assessment of Resistance

Year	Country or Region	Host		Diagnostic Method											Number of Farms	Refs
		Sheep	Cattle	Human	FECRT or FEC[a]	CRT[a]	PM[a] and TFC[a]	GLDH[a]	GGT[a]	PCV[a]	GOT[a]	Histo[a]	ELISA – Serum	*Fasciola hepatica* isolated		
1995	Australia	•			•		•							•	1	[14]
1998	Scotland	•			•		•		•						1	[92]
2000	The Netherlands	•	•		•									•	1	[16,93]
2000	Wales	•			•		•								1	[94]
2006	Spain	•			•										1	[91]
2009	Republic of Ireland	•			•			•	•						1	[33]
2011	Scotland	•			•		•		•		•			•	1	[26]
2011	Argentina		•		•										1	[26]
2012	Wales and Scotland	•			•		•					•			7	[30]
2012	Scotland	•			•	•	•	•	•				•		2	[29]
2012	The Netherlands[b]			•	•				•				•		N/A	[40]
2012	New Zealand	•			•		•								1	[32]
2013	Peru		•		•									•	1	[27]
2014	Chile[b,c]			•	•								•		N/A	[41]
2014	Australia[d]		•		•	•									5	[19]
2015	Australia		•		•	•									1	[28]
2015	Northern Ireland	•			•	•						•			6	[31]
2015	Turkey[b]			•					•			•	•		N/A	[42]
2016	Peru[b]				•								•		N/A	[43]

[a]FECRT, faecal egg count reduction test; CRT, coproantigen reduction test; PM, postmortem; TFC, total fluke counts; GLDH, glutamate dehydrogenase; GGT, gamma-glutamyl transferase; PCV, packed cell volume; GOT, glutamic oxaloacetic transaminase; Histo, Histology.
[b]N/A: not applicable, these are human cases of TCBZ-R.
[c]Only the abstract was available in English.
[d]Five farms were found to have resistance (<90%) by CRT; only four farms were confirmed with resistance by FECRT

Glossary

Affinity purification: the purification of a protein based on its ability to bind to a substrate or drug.
Anthelmintic: a chemical drug used to treat a worm infection.
Bulk milk tank ELISA: the level of specific worm antibody detected by an ELISA assay in the combined milk samples from several dairy cattle.
Coproantigen: a worm antigen found in faeces.
Coproantigen reduction test (CRT): the evaluation of the level of a worm antigen in faeces before and after chemical treatment of the host.
Efficacy: the effectiveness of a drug to kill worms in an infected animal.
ELISA: a technique used to measure the level of an antigen or antibody in an animal.
Faecal egg count (FEC): the number of worm eggs found in the faeces of an infected animal.
Fasciolosis: the disease caused by infection with *Fasciola hepatica* or *Fasciola gigantica*.
Faecal egg count reduction test (FECRT): the evaluation of the level of worm eggs in faeces before and after chemical treatment of an animal.
Flukicide: a chemical that kills *F. hepatica*.
Haplotype: set of DNA sequence variations in a chromosome that are inherited together.
Head selection: selection for anthelmintic-resistant roundworms at the time of treatment.
Immature fluke: a liver fluke aged up to 5-weeks old.
Integrated parasite management (IPM): the use of a range of methodologies to manage the incidence of parasites in a herd.
Mass spectrometry: the analysis of the mass:charge ratio of a molecule.
Metabolomics: the analysis of metabolic perturbations in a cell upon exposure to drugs.
Nuclear magnetic resonance: an analytical chemistry technique to determine the content and purity of a sample and its molecular structure.

2.4 Biochemistry of TCBZ Action

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cases of TCBZ treatment failure on-farm (possible TCBZ-R) are not listed in Table 2. These were cited in [6,10] as Anon. 1995 (the Sligo isolate); 1998; 2006; 2008; 2009. Mooney *et al.* [33] also cited two anecdotal reports for TCBZ-R in 1995 and 1998. Recent data from Great Britain revealed TCBZ-R on seven out 25 farms tested using a composite FECRT method [30]. Six of these farms were in South Wales and one in Scotland. In some cases, resistance is absolute (i.e., no reduction in FEC observed). On certain farms, egg counts increased post-TCBZ treatment, which is of major concern. In Northern Ireland, a recent FECRT and CRT survey of sheep flocks, supported by fluke histology, found demonstrable TCBZ-R on five out of 13 farms tested, with absolute resistance on two farms [31]. Interestingly, a subsequent questionnaire study revealed a change in farmer behaviour over recent years (2005–2011), presumably as a result of perceived TCBZ-R [34]. For example, there was a significant shift away from the use of TCBZ over time in favour of closantel, with farmers also tending to treat earlier in the year.

Despite being the source of one of the earliest TCBZ-resistant fluke isolates (the Sligo isolate) [11,35,36], there are few reliable published reports of TCBZ-R in the Republic of Ireland. Again, the general perception is that resistance is widespread. In 2009, Mooney *et al.* [33] reported a lack of efficacy of TCBZ in sheep on a hill farm in the west of Ireland. FECRT were in the region of 49–66% based on arithmetic means and considered to be highly indicative of TCBZ-resistant *F. hepatica* in sheep on this farm. Mooney *et al.* [33] cited two earlier reports of TCBZ-R in Ireland from 1995 and 1998, but these were anecdotal with no data or evidence of TCBZ-R provided.

In mainland Europe, most reports of TCBZ-R have come from the lower-lying northwestern countries, such as The Netherlands [16]. There are few, if any, reports of confirmed TCBZ-R from central or southern Europe. This most likely reflects the general prevalence of fluke and the perceived need to treat. There is a growing gradient in the prevalence of *F. hepatica* west-to-east and south-to-north in Europe, with prevailing climatic and/or underlying geological conditions probably pivotal. Fox *et al.* [37] predicted that fluke incidence will increase and spread west-to-east in the UK over the coming decades, based on modelling the **Ollerenshaw Indices** and UK Climate Projections (UKCP09). Similar trends are predicted to occur across Europe [38]. The implications of this spread of liver fluke is of serious concern in relation to TCBZ-R, since farmers in traditionally fluke-free regions will need to treat animals that may have been exposed to TCBZ-resistant flukes.

Human Cases of TCBZ-Resistant Fluke Infections

TCBZ is also the drug of choice for treating fasciolosis in humans and it is conceivable that TCBZ-resistant fluke populations, selected in livestock, could pose a zoonotic risk to human health, especially in areas such as Peru and Bolivia, where there is a high incidence of human infections [39]. The first incidence of TCBZ treatment failure in humans was reported in a livestock farmer in The Netherlands, with further recent reports of four cases from Chile, one case from Turkey, and seven cases from Peru [40–43]. Clearly, TCBZ-resistant zoonotic infections are a serious emerging issue (Figure 1).

Biochemistry of TCBZ Action

Despite years of research, the precise mode of action of TCBZ is still unclear (reviewed in [6–10]). TCBZ is a benzimidazole (BZ) derivative and all available evidence from gastrointestinal roundworms indicates that BZ **anthelmintics** bind to α- and β-tubulins within the cells of the parasite, causing disruption of vital processes, such as feeding and digestion. Several morphological studies of the effects of TCBZ and its active metabolites on *F. hepatica*, have examined the **tegument, vitellaria**, and testis of the fluke; all three tissues showed significant signs of ultrastructural disruption, consistent with inhibition of microtubule-based processes [7]. There is also a concurrent loss of tubulin immunostaining in the tegumental syncytium, further implicating an interaction with tubulin as the primary mode of action of TCBZ [44,45]. That

Ollerenshaw Indices: an estimate of the risk of liver fluke disease, based on the calculated evapotranspiration rate and its likely impact on intermediate host snails and shedding of metacercarial cysts.
P-glycoprotein: a protein involved in pumping a chemical from a cell.
Pour-on: a chemical delivered externally on the back of an animal.
Prevalence: the percentage of animals in a herd infected by a parasite.
Proteome: the full protein content of a cell or parasite.
Resistance: the ability of a worm to resist the effects of a chemical normally lethal to it.
Roundworm: a nematode parasite.
Single nucleotide polymorphisms (SNPs): DNA sequence variations at one position in a chromosome.
Tail selection: selection for anthelmintic-resistant infective roundworm larvae following treatment.
Tegument: the surface layer of tissue on the liver fluke.
Vitellaria: tissues in a fluke that produce egg-shell material.

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Key Figure

Global Distribution of Reports of Triclabendazole Resistance (TCBZ-R) in Livestock and Humans 1995–2015

Figure 1. Grey, 1 reported case of TCBZ-R; purple, 2–4 reported cases; yellow, ≥5 reported cases of TCBZ-R. Hashed lines indicate reported human cases of TCBZ-R.

said, this has not helped inform our understanding of TCBZ-R, because TCBZ-resistant flukes do not carry the F200Y/E198A or F167Y mutations in β-tubulin, implicated in BZ resistance in nematodes [46], suggesting that alterations to β-tubulin are not a key component of TCBZ-R [8].

Recently, TCBZ was reported to inhibit adenylate cyclase activity in yeast and/or inhibit the association of GTP-Ras with adenylate cyclase [47]. Interestingly, *F. hepatica* has one of the most active adenylate cyclase activities in biology and this activity is associated with the membrane fraction of the parasite [48]. One of the first signs of TCBZ-induced damage in *F. hepatica* is tegumental blebbing and disruption of the tegumental ultrastructure [10,49,50]. If TCBZ was shown to inhibit adenylate cyclase activity, the effects of TCBZ on the metabolism of the fluke would likely be pleiotropic due to the second messenger function of cAMP and its effects on protein kinases, carbohydrate metabolism, and motility [48]. These results suggest that an evaluation of the sensitivity of fluke adenylate cyclase to inhibition by TCBZ would be informative. In addition, an analysis of sequence polymorphisms in adenylate cyclase gene(s) and/or GTP-Ras gene(s) in resistant and susceptible fluke populations is warranted, to determine whether there is any selection on those sequences in resistant flukes.

New Approaches to Understanding the Mode of Action of TCBZ

The multiplicity of studies reporting different mechanisms of resistance to TCBZ [7,8,10] suggests that the mode of action of TCBZ and/or the effects on fluke metabolism are complicated, but the advent of new technologies could allow the target of TCBZ to be unravelled in the foreseeable future. One approach is **affinity purification** of the putative protein target, whereby TCBZ is immobilised to a solid support and a protein extract is passed over the column, followed by elution of any bound target proteins. This has resulted in the identification of protein targets against several types of drug [51–53]. However, these methods seem best suited for situations where a high-affinity ligand binds a relatively abundant target protein.

2.5 Mechanism(s) of TCBZ-R

2.6 Genetics of TCBZ-Resistant Fluke Populations

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A new approach to understanding the mode of action of small molecules is the application of **metabolomics,** a whole-organism assay approach that identifies metabolic perturbations in a cell upon exposure to drugs. This technique identifies the metabolomic compounds via **mass spectrometry** or **nuclear magnetic resonance** and has been applied to several drug studies in various parasites [54]. One such study with the protozoan parasite *Trypanosoma cruzi* and the drug benznidazole (BZN) revealed that metabolised BZN generated covalent adducts of redox active thiols, such as glutathione and cysteine, that were toxic to *T. cruzi* [55]. This is in contrast to affinity purification studies that identified the Sec23A orthologue, the bacterial YjeF homologue, and aldo-ketoreductase as proteins that could bind immobilised BZN [53]. Thus, a combination of approaches may be required to fully characterise on-target and off-target effects of TCBZ and to clearly define the mechanism(s) of TCBZ action.

Mechanism(s) of TCBZ-R

The actual biochemical mechanism underlying TCBZ-R in *F. hepatica* is not clear (reviewed in [6–10]). Evidence for metabolic differences between TCBZ-susceptible and -resistant isolates has been reported (e.g., sulfoxidation of TCBZ to TCBZ.SO and on to TCBZ.SO_2), suggesting that altered uptake, efflux, and/or metabolism of TCBZ has a more important role in TCBZ-R than tubulin-based processes. **P-glycoprotein** (Pgp) activity has also been shown to have an effect on the uptake and efficacy of TCBZ *in vitro* using both resistant and susceptible isolates [56–60], with resistance to TCBZ reversed in the presence of the Pgp inhibitor R(+)-verapamil. A role for mutations in one *Pgp* gene in TCBZ-R was proposed but the few mutations that have been described [61] appear to be more related to variation between strains and isolates of *F. hepatica* than a true TCBZ-R phenotype, since these mutations were not associated with resistance in Australian or Scottish TCBZ-resistant isolates [62,63]. A single amino acid substitution in glutathione *S*-transferase (GST) has been implicated in a TCBZ-resistant fluke isolate, but this has not yet been confirmed in any other TCBZ-resistant isolates [64]. An analysis of the total soluble **proteome** of TCBZ-resistant and -susceptible flukes was also performed, with and without exposure to TCBZ-SO, to search for key differences in the protein response to TCBZ exposure in adult flukes [65]. The majority of differentially expressed proteins identified, such as molecular chaperones, fatty acid-binding proteins, thioredoxin peroxidase, and GSTs, were thought to be related to a general stress response. It appears that TCBZ treatment causes the upregulation of proteins that protect cells from damage, which is not surprising given that several drug studies in other systems have shown similar responses [52,66,67].

Genetics of TCBZ-Resistant Fluke Populations

Molecular studies of well-defined TCBZ-resistant fluke populations, using both nuclear and mitochondrial DNA (mtDNA), have demonstrated that such populations are genetically diverse [8,68,69]. Infrapopulation diversity (diversity within one animal) was illustrated in a calf and ewe infected with the laboratory TCBZ-resistant isolates Oberon and Sligo, respectively, with ten mtDNA **haplotypes** identified from 18 Oberon flukes analysed [68]. A high level of diversity was also seen in two TCBZ-resistant fluke populations (seven to nine haplotypes in 24–30 flukes) in Australian cattle [69], suggesting that drug selection pressure does not lead to genetic bottlenecking of TCBZ-resistant fluke populations [69,70]. The genetic analysis of 48 TCBZ-resistant flukes recovered from cattle and sheep in Australia showed that mtDNA haplotypes are shared between the hosts, indicating that there is no host selection being applied to fluke populations [69], as seen in Northern Ireland [71].

Following a considerable research effort, a draft genome assembly has recently been published for *F. hepatica*, revealing that the liver fluke has a large, polymorphic genome and one of the largest pathogen genomes sequenced to date, at 1.3 Gb [72]. As a result, *F. hepatica* is likely to be genetically predisposed to developing resistance to anthelmintics. Moreover, any resistant individual *F. hepatica* that is selected in the field will undergo clonal amplification when parasites

2.7 Anthelmintic Control of TCBZ-Resistant Flukes: Role for Dual-Active Flukicide Formulations

2.8 What Can Be Done to Manage the Impact of TCBZ-R on Livestock Production?

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develop within the snail intermediate host, potentially leading to rapid dissemination of resistant parasites [10]. Hodgkinson *et al.* [8] reported a strategy to use genome-wide **single nucleotide polymorphisms** (SNPs) to map TCBZ-R in recombinant *F. hepatica* progeny derived from genetic crossing of parental TCBZ-resistant and TCBZ-susceptible clones. Initial results from this genome-wide approach were reported at the World Association for the Advancement of Veterinary Parasitology Conference at Liverpool in August 2015[i]. Pooled genotyping and subsequent analysis of SNP allele frequencies from several replicates of TCBZ-exposed F2 parasites relative to untreated controls identified a number of scaffolds within the draft *F. hepatica* genome sequence associated with the TCBZ-R phenotype. Early indications suggest that there is currently no evidence that the obvious candidate TCBZ-R genes lie in the regions of the genome under selection. The precise loci and, therefore, genes involved are still to be defined, but the approach being taken is a powerful one and likely to provide novel insights into the molecular basis of TCBZ-R [8].

Anthelmintic Control of TCBZ-Resistant Flukes: Role for Dual-Active Flukicide Formulations

The only chemical options for the control of TCBZ-resistant fluke are, depending on the host species, treatment with clorsulon, nitroxynil, closantel, albendazole, or oxyclozanide [4,10] (Table 1). The fact that these chemicals are available to control TCBZ-resistant flukes is of benefit, but none of these chemicals is administered as a single active dose that is able to kill the early immature stage of the parasite (i.e., <5 weeks of age). This raises the issue of what to do, practically, in the face of an acute fluke outbreak, especially in sheep, when the flukes are likely, or confirmed, to be TCBZ resistant. This scenario was realised recently in Northern Ireland during the winter of 2012–2013; the advice given by veterinary practitioners and advisors was to use TCBZ as an emergency treatment, followed up by (but not mixed with) a different flukicide (e.g., closantel or nitroxynil) to kill any fluke that survived the initial TCBZ treatment[ii] [73]. Closantel is a viable alternative for TCBZ in sheep, but it is only effective against >8-week-old flukes [74]. However, the first case of flukes resistant to closantel was recently reported in cattle in Sweden [75] and resistance to albendazole, clorsulon, and nitroxynil has been reported (Table 1). These results highlight the importance of protecting existing adult flukicides by effective on-farm fluke management.

The use of dual-active flukicides has been recommended to control a *F. hepatica* isolate that was resistant to albendazole and clorsulon when these drugs were administered individually; this isolate was susceptible to these drugs when given as a dual-active formulation [76]. When such formulations have a synergistic effect (i.e., have greater efficacy than the sum of the actives), this may increase the lifespan of the respective actives. Synergy has been seen with several dual-active flukicides (e.g., TCBZ+ clorsulon or TCBZ+ luxabendazole) against TCBZ-resistant fluke in sheep (reviewed in [4,10]).

What Can Be Done to Manage the Impact of TCBZ-R on Livestock Production?

Vaccines as an Alternative Control Strategy for Fluke Control

An alternative approach to control TCBZ-R would be the development of a livestock vaccine for *F. hepatica*, which would reduce fluke burdens irrespective of the drug-resistance status of the flukes and would not compromise fluke control during lactation. The need for a vaccine is becoming urgent, particularly to protect sheep from acute fluke infections in Northern Ireland, Republic of Ireland, northwest Europe, and Australia, as exemplified by the 2012–2013 fluke season in Northern Ireland[ii] [73]. This need will be exacerbated by the widespread prevalence of TCBZ-R (Figure 1). However, no commercial liver fluke vaccine exists, although several experimental vaccines for livestock are under development [77]. No vaccine has shown reproducibly high enough efficacy (>60%) in cattle to warrant commercial production, although the leucine

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aminopeptidase (LAP) vaccine has shown high efficacy (up to 89%) in sheep [77]. It is important to note that a vaccine with only partial efficacy (50–60%) may still provide economic benefits to producers experiencing infection with TCBZ-resistant fluke, depending on the intensity of fluke infection in a herd, since production losses are associated with fluke burden: the threshold for economic losses in cattle is approximately 30–40 flukes [10,78,79], but may be as low as ten flukes [24]. Thus, until a new anthelmintic is developed that kills all developmental stages, including the early immature fluke, a vaccine is the only alternative treatment that could provide ongoing control of fluke infections in livestock in regions where TCBZ-R is endemic.

IPM for Farms

Farms are not closed systems and there are many factors that influence the prevalence of *F. hepatica*, such as soil type, irrigation, temperature, rainfall, pasture management, and livestock management practices [2,73,80,81] (Figure 2). The management practices on farms generally rely solely upon anthelmintics and appear to have contributed to the development of resistance [13,82]. Management practices must change to preserve the longevity of existing flukicides, because the likelihood of any new flukicides coming to market in the near future is low [73].

Throughout the year, there are periods in which the risk of fluke infection is higher and these periods fluctuate depending upon location and prevailing climatic conditions, but do provide a set of guidelines to determine when treatment may be required [37,38,83,84]. If farmers combine strategic treatments with FECs and the cELISA during high-risk periods, this approach could be used to determine when to drench, which drench to use, or whether treatment is required at all, based on the known thresholds for economic loss [2,24,79]. Well-executed strategic treatments will minimise the need for further treatments throughout the year and, therefore, help to preserve the efficacy of existing flukicides [73]. Regular drug efficacy testing, using FECRT and/or CRT, to preserve the efficacy of existing flukicides or TCBZ is essential to

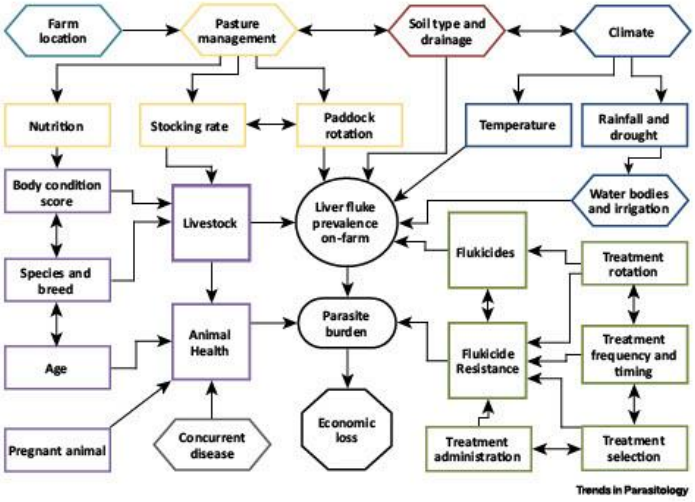

Figure 2. Integrated Parasite Management. A schematic of the factors that influence *Fasciola hepatica* prevalence on a farm. The key areas are colour coded: climate, water bodies, and irrigation in dark blue; pasture management in yellow; anthelmintic practices in green; concurrent disease in grey; and livestock in purple.

2.9 Concluding Remarks and Future Perspectives

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allow producers to avoid using products with reduced efficacy and prevent economic losses resulting from unidentified resistance [73]. Movement of livestock can also contribute to the spread of resistant *F. hepatica*. All newly purchased livestock or livestock housed over winter (e.g., in Europe) should be quarantine treated upon arrival and cleaned out with a dual-active formulation [10,13,73,82]. Flukicides should always be administered according to the product specifications and best-practice methods, which include: weighing individual animals or the heaviest in the herd to determine dose, calibrating drench equipment before use and during treatments, selecting the most potent formulations of product, and, where possible, regularly rotating effective products [73].

In addition, we must also look at how pastures, drinking water, and irrigation can be better managed to decrease the likelihood of *F. hepatica* infection (Figure 2). Pasture management can allocate low-risk pastures (such as newly sown paddocks, hay, or silage paddocks) to young animals during the high-risk periods, to limit the chances of parasite transmission [73]. Often these practices are difficult to implement within an existing system due to the timing of harvest and high-risk periods. Previous studies suggest that metacercariae in herbage can be infective for 270–340 days, whereas, in silage, metacercariae will be killed if ensiled for a period of 35–57 days, and well-cured hay with low moisture content poses less risk [85,86]. There is limited recent literature investigating the viability of metacercariae on pasture or in hay or silage and this requires further investigation. Through major overhauls of irrigation infrastructure, it may be possible to better target the intermediate host of the liver fluke, the mud snail *Galba* spp. in Europe and the UK, or *Austropeplea* spp. (formerly *Lymnaea* spp.) and *Pseudosuccinea* spp., and, thus, break the life cycle [83]. However, such intervention is costly and, therefore, unappealing to farmers. Despite this, there have been successes: cementing of ditches near rice fields was used to assist the elimination of schistosomiasis in Japan in 1950–1970 [87]. Alternative cheaper options, such as fencing off high-risk areas and regular maintenance of irrigation channels and/or drains, will help to reduce the likelihood of liver fluke infection in livestock [73,83], but are set against a trend towards the introduction of agrienvironment wetland schemes, at least in Europe, aimed at increasing biodiversity and reducing greenhouse gas emissions (e.g., [10,88]). The successful implementation of IPM strategies is inherently difficult because the program will undergo continual change over the first few years while the strategy is fine-tuned to suit the requirements of farms and the unique local issues affecting each property. This requires the time, skill, and knowledge of expert extension staff to continually assist the farmer throughout the transitional period [73].

Concluding Remarks and Future Perspectives

The widespread incidence of TCBZ-R in livestock is a major threat to global livestock production and producers need alternative treatments, such as new flukicides or vaccines, to control infections. Some key outstanding questions need to be addressed if we are to fully understand the basis of TCBZ-R and provide producers with better management options to control fasciolosis and reduce economic losses (see Outstanding Questions). Affinity purification of target proteins using TCBZ as ligand, or metabolomic analysis of the parasite response to TCBZ, may allow the identification of the protein target (s) of TCBZ. The current search for SNPs associated with TCBZ-R in the fluke genome, using carefully selected parasite lines, will likely provide new insights into the possible mechanism of TCBZ-R as well as tools to monitor the appearance of TCBZ-R in herds [8]. The cELISA could be used to estimate the approximate intensity of fluke infections, allowing targeted treatment of livestock with moderate–high fluke burdens, especially in cattle [2,22,24,25]. Farmers must be more proactive and use an IPM approach to optimise their practices, monitor flukicide efficacy, and test regularly for liver fluke prevalence to preserve the longevity of existing flukicides [10,73,82]. Given the increasing awareness of the impact of fasciolosis on production and the prevalence of TCBZ-R [2,73], we anticipate that future control of fasciolosis will occur in three stages: (i) in the short term, better

Outstanding Questions

What is the true prevalence of TCBZ-R in the Republic of Ireland, Northern Ireland, and Great Britain, given the large number of anecdotal reports of TCBZ-R?

How widespread is TCBZ-R in liver fluke in regions such as Africa, Asia, and South America?

How widespread are human infections with TCBZ-resistant liver fluke, given the global appearance of TCBZ-R in livestock?

Can affinity purification of a TCBZ protein target from *Fasciola hepatica* be used to identify the protein that is the site of action of TCBZ in the fluke?

Can the careful use of metabolomics be used to identify the metabolic pathway that is perturbed by the action of TCBZ in *F. hepatica*?

Does TCBZ bind to more than one target and perturb more than one metabolic pathway?

Is adenylate cyclase a target of TCBZ in *F. hepatica* and is there evidence for selection on adenylate cyclase genes in the genome of TCBZ-R fluke populations?

Is TCBZ-R in *F. hepatica* due to selection on a single gene or several genes?

Can we develop fluke DNA markers for TCBZ-R to allow better monitoring of resistance on-farm using DNA from fluke eggs or faeces as a biomarker?

Can the cELISA be used to estimate the approximate intensity of fluke infection in herds to assist with the more targeted use of flukicides by producers and reduce selection pressure for resistance?

How widespread is resistance to closantel, clorsulon, and nitroxynil in liver fluke populations?

Is the development of a new flukicide that mimics the efficacy of TCBZ a futile exercise that will only provide a short-term control option due to the inevitable selection for drug resistance in fluke populations?

Will a vaccine of even partial (50%) efficacy be accepted by producers as a useful new tool to provide economic

2.10 Acknowledgement

2.11 References

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delivery of advice to producers from veterinarians and government or private extension advisors that will lead to more effective IPMs tailored to address the issue of fasciolosis and TCBZ-R on each farm and local region; (ii) in the medium term, the development of a new drench that has a similar efficacy spectrum to TCBZ; and (iii) in the longer term, the application of a livestock vaccine would provide a more sustainable control strategy [77,89]. The world market for a liver fluke vaccine is estimated at approximately US$182m per annum[ii], suggesting that a major commercial opportunity exists for this niche vaccine that would likely overcome the futile cycle of flukicide development and implementation followed by the inevitable selection for drug resistance.

benefits in the interim for farmers confronted with TCBZ-R?

Is a vaccine the only long-term alternative for dairy farmers to control liver fluke during lactation, given the trend to reduce drug residues in milk?

Will producers take a more proactive approach and work with veterinarians and extension officers to develop and adopt IPM plans, fine-tuned to their property and local environment, to better manage fluke infections and TCBZ-R on their farms?

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Resources

[i] www.waavp2015.com/bio_jane_hodgkinson.php

[ii] http://tinyurl.com/o73chqt

[iii] www.ucd.ie/nova/ucdlicensingopportunities/novel%20vaccine%20for%20protection%20of%20liver%20fluke.pdf

References

1. Dargie, J.D. (1987) The impact on production and mechanisms of pathogenesis of trematode infections in cattle and sheep. *Int. J. Parasitol.* 17, 453–463
2. Charlier, J. *et al.* (2014) Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. *Parasitology* 141, 326–335
3. Spithill, T.W. *et al.* (1999) *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In *Fasciolosis* (Dalton, J., ed.), pp. 465–525, CAB International
4. Fairweather, I. and Boray, J.C. (1999) Fasciolicides: efficacy, actions, resistance and its management. *Vet. J.* 158, 81–112
5. Boray, J.C. *et al.* (1983) Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. *Vet. Rec.* 113, 315–317
6. Fairweather, I. (2005) Triclabendazole: new skills to unravel an old (ish) enigma. *J. Helminthol.* 79, 227–234
7. Brennan, G.P. *et al.* (2007) Understanding triclabendazole resistance. *Exp. Mol. Pathol.* 82, 104–109
8. Hodgkinson, J. *et al.* (2013) Identification of putative markers of triclabendazole resistance by a genome-wide analysis of genetically recombinant *Fasciola hepatica*. *Parasitology* 140, 1523–1533
9. Fairweather, I. (2009) Triclabendazole progress report 2005-2009: an advancement of learning? *Helminthology* 83, 139–150
10. Fairweather, I. (2011) Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? *Vet. Parasitol.* 180, 133–143
11. Fairweather, I. (2011) Liver fluke isolates: a question of provenance. *Vet. Parasitol.* 176, 1–8
12. Fairweather, I. (2011) Raising the bar on reporting 'triclabendazole resistance'. *Vet. Rec.* 168, 514–515
13. Sargison, N.D. and Scott, P.R. (2011) Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. *Vet. Rec.* 168, 159–165
14. Overend, D.J. and Bowen, F.L. (1995) Resistance of *Fasciola hepatica* to triclabendazole. *Aust. Vet. J.* 72, 275–276
15. Anon (2015) Raising awareness of sustainable management of liver fluke in sheep. *Vet. Rec.* 177, 246
16. Moll, L. *et al.* (2000) Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. *Vet. Parasitol.* 91, 153–158
17. Le Jambre, L.F. *et al.* (1999) Selection for anthelmintic resistance by macrocyclic lactones in *Haemonchus contortus*. *Int. J. Parasitol.* 29, 1101–1111
18. Wood, I.B. *et al.* (1995) World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). *Vet. Parasitol.* 58, 181–213
19. Brockwell, Y.M. *et al.* (2014) Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. *Int. J. Parasitol. Drugs Drug Res.* 4, 48–54
20. Flanagan, A.M. *et al.* (2011) Comparison of two assays, a faecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. *Vet. Parasitol.* 176, 170–176
21. Flanagan, A.M. *et al.* (2011) Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. *Vet. Parasitol.* 176, 34–42
22. Mezo, M. *et al.* (2004) An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). *J. Parasitol.* 90, 845–852
23. Gordon, D.K. *et al.* (2012) On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. *Vet. Parasitol.* 187, 436–444
24. Charlier, J. *et al.* (2008) Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. *Vet. Parasitol.* 153, 44–51
25. Brockwell, Y.M. *et al.* (2013) Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. *Vet. Parasitol.* 198, 417–426
26. Olaechea, F. *et al.* (2011) Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). *Vet. Parasitol.* 178, 364–366
27. Ortiz, P. *et al.* (2013) Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an *in vivo* efficacy test in sheep. *Vet. Parasitol.* 195, 118–121
28. Elliott, T.P. *et al.* (2015) High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Balmedale districts of Gippsland, Victoria, Australia. *Vet. Parasitol.* 209, 117–124

29. Gordon, D. *et al.* (2012) Confirmation of triclabendazole resistance in liver fluke in the UK. *Vet. Rec.* 171, 159–160
30. Daniel, R. *et al.* (2012) Composite faecal egg count reduction test to detect resistance to triclabendazole in *Fasciola hepatica*. *Vet. Rec.* 171, 153–157
31. Hanna, R.E.B. *et al.* (2015) *Fasciola hepatica*: a comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. *Vet. Parasitol.* 207, 34–43
32. Hassell, C. and Chapman, V. (2012) Case report: suspect lack of triclabendazole efficacy in liver fluke in sheep in the Taranaki. *Proceedings of the Society of Sheep and Beef Cattle Veterinarians of the New Zealand Veterinary Association* 2012, 4.14.1–4.14.2
33. Mooney, L. *et al.* (2009) The comparative efficacy of four anthelmintics against a natural acquired *Fasciola hepatica* infection in hill sheep flock in the west of Ireland. *Vet. Parasitol.* 164, 201–205
34. McMahon, C. *et al.* (2015) Liver fluke control on sheep farms in Northern Ireland: a survey of changing management practices in relation to disease prevalence and perceived triclabendazole resistance. *Vet. Parasitol.* 216, 72–83
35. Coles, G.C. *et al.* (2000) Activity of closantel against adult triclabendazole-resistant *Fasciola hepatica*. *Vet. Rec.* 146, 504–507
36. Coles, G.C. and Stafford, K.A. (2001) Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole resistant *Fasciola hepatica*. *Vet. Rec.* 148, 723–724
37. Fox, N.J. *et al.* (2011) Predicting impacts of climate change on *Fasciola hepatica* risk. *PLoS ONE* 6, e16126
38. Caminade, C. *et al.* (2015) Modelling recent and future climatic suitability for fascioliosis in Europe. *Geospat. Health* 9, 301–308
39. Mas-Coma, S. *et al.* (2005) Fascioliasis and other plant-borne trematode zoonoses. *Int. J Parasitol.* 35, 1255–1278
40. Winkelhagen, A.J.S. *et al.* (2012) Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. *Emerg. Infect. Dis.* 18, 1028–1029
41. Gil, L.C. *et al.* (2014) Resistant human fascioliasis: report of four patients. *Rev. Med. Chil.* 142, 1330–1333
42. Gülhan, B. *et al.* (2015) Partial hepatectomy for the resistant *Fasciola hepatica* infection in a child. *APSP J. Case Rep.* 6, 27
43. Cabada, M.M. *et al.* (2016) Treatment failure after multiple courses of triclabendazole among patients with fascioliasis in Cusco, Peru: a case series. *PLoS Negl. Trop. Dis.* 10, e0004361
44. Robinson, M.W. *et al.* (2002) Triclabendazole-resistant *Fasciola hepatica*: β-tubulin and response to *in vitro* treatment with triclabendazole. *Parasitology* 124, 325–338
45. McConville, M. *et al.* (2006) Adult triclabendazole-resistant *Fasciola hepatica*: surface and subsurface tegumental responses to *in vitro* treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. *Parasitology* 133, 195–208
46. von Samson-Himmelstjerna, G. *et al.* (2009) Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. *Parasitology* 136, 349–358
47. Lee, Y.J. *et al.* (2013) The small molecule triclabendazole decreases the intracellular level of cyclic AMP and increases resistance to stress in *Saccharomyces cerevisiae*. *PLoS ONE* 8, e64337
48. Mansour, T.E. (1979) Chemotherapy of parasitic worms: new biochemical strategies. *Science* 205, 462–469
49. Stitt, A.W. and Fairweather, I. (1993) *Fasciola hepatica*: tegumental surface changes in adult and juvenile flukes following treatment *in vitro* with the sulphoxide metabolite of triclabendazole (Fasinex). *Parasitol. Res.* 79, 529–536
50. Stitt, A.W. and Fairweather, I. (1994) The effect of the sulphoxide metabolite of triclabendazole ("Fasinex") on the tegument of mature and immature stages of the liver fluke, *Fasciola hepatica*. *Parasitology* 108, 555–567
51. Brehmer, D. *et al.* (2004) Proteome-wide identification of cellular targets affected by bisindolylmaleimide-type protein kinase C inhibitors. *Mol. Cell Proteomics* 3, 490–500
52. Argyrou, A. *et al.* (2006) Proteome-wide profiling of isoniazid targets in *Mycobacterium tuberculosis*. *Biochemistry* 45, 13947–13953
53. Trochine, A. *et al.* (2014) *Trypanosoma cruzi* chemical proteomics using immobilized benznidazole. *Exp. Parasitol.* 140, 33–38
54. Creek, D.J. and Barrett, M.P. (2014) Determination of antiprotozoal drug mechanisms by metabolomics approaches. *Parasitology* 141, 83–92
55. Trochine, A. *et al.* (2014) Benznidazole biotransformation and multiple targets in *Trypanosoma cruzi* revealed by metabolomics. *PLoS Negl. Trop. Dis.* 8, e2844
56. Devine, C. *et al.* (2011) Inhibition of triclabendazole metabolism *in vitro* by ketoconazole increases disruption to the tegument of a triclabendazole-resistant isolate of *Fasciola hepatica*. *Parasitol. Res.* 109, 981–995
57. Devine, C. *et al.* (2011) Piperonyl butoxide enhances triclabendazole action against triclabendazole-resistant *Fasciola hepatica*. *Parasitology* 138, 224–236
58. Devine, C. *et al.* (2012) Potentiation of triclabendazole action *in vivo* against a triclabendazole-resistant isolate of *Fasciola hepatica* following its co-administration with the metabolic inhibitor, ketoconazole. *Vet. Parasitol.* 184, 37–47
59. Meaney, M. *et al.* (2013) Increased susceptibility of a triclabendazole (TCBZ)-resistant isolate of *Fasciola hepatica* to TCBZ following co-incubation *in vitro* with the P-glycoprotein inhibitor, R (+)-verapamil. *Parasitology* 140, 1297–1303
60. Savage, J. *et al.* (2013) Increased action of triclabendazole (TCBZ) *in vitro* against a TCBZ-resistant isolate of *Fasciola hepatica* following its co-incubation with the P-glycoprotein inhibitor, R (+)-verapamil. *Exp. Parasitol.* 135, 642–653
61. Wilkinson, R. *et al.* (2012) An amino acid substitution in *Fasciola hepatica* P-glycoprotein from triclabendazole-resistant and triclabendazole-susceptible populations. *Mol. Biochem. Parasitol.* 186, 69–72
62. Elliott, T.P. and Spithill, T.W. (2014) The T687G SNP in a P-glycoprotein gene of *Fasciola hepatica* is not associated with resistance to triclabendazole in two resistant Australian populations. *Mol. Biochem. Parasitol.* 198, 45–47
63. Kotze, A.C. *et al.* (2014) Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. *Int. J. Parasitol. Drugs Drug Res.* 4, 164–184
64. Fernández, V. *et al.* (2015) A single amino acid substitution in isozyme GST mu in triclabendazole resistant *Fasciola hepatica* (Sligo strain) can substantially influence the manifestation of anthelmintic resistance. *Exp. Parasitol.* 159, 274–279
65. Chemale, G. *et al.* (2010) Comparative proteomic analysis of triclabendazole response in the liver fluke *Fasciola hepatica*. *J. Proteome Res.* 9, 4940–4951
66. Singh, A.K. *et al.* (2014) A quantitative proteomic screen to identify potential drug resistance mechanism in α-difluoromethylornithine (DFMO) resistant *Leishmania donovani*. *J. Proteomics* 102, 44–59
67. Brotherton, M.C. *et al.* (2014) Quantitative proteomic analysis of amphotericin B resistance in *Leishmania infantum*. *Int. J. Parasitol. Drugs Drug Res.* 4, 126–132
68. Walker, S.M. *et al.* (2007) Evidence for multiple mitochondrial lineages of *Fasciola hepatica* (liver fluke) within infrapopulations from cattle and sheep. *Parasitol. Res.* 101, 117–125
69. Elliott, T. *et al.* (2014) Evidence for high genetic diversity of NAD1 and COX1 mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. *Vet. Parasitol.* 200, 90–96
70. Walker, S.M. *et al.* (2011) Population dynamics of the liver fluke, Fasciola hepatica: the effect of time and spatial separation on the genetic diversity of fluke populations in the Netherlands. *Parasitology* 138, 215–223
71. Dosay-Akbulut, M. *et al.* (2005) Understanding genetic diversity of the liver fluke *Fasciola hepatica*. *Z. Naturforsch. C.* 60, 774–778
72. Cwiklinski, K. *et al.* (2015) The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. *Genome Biol.* 16, 71–84
73. Crilly, J.P. *et al.* (2015) Triclabendazole-resistant liver fluke: issues and strategies. *Livestock* 20, 6–14

74. Boray, J.C. *et al.* (2007) Liver fluke disease in sheep and cattle. Department of Primary Industries NSW, Australia. *Primefacts* 446, 1–10
75. Novobilský, A. and Höglund, J. (2015) First report of closantel treatment failure against *Fasciola hepatica* in cattle. *Int. J. Parasitol. Drugs Drug Res.* 5, 172–177
76. Martínez-Valladares, M. *et al.* (2014) Efficacy of an anthelmintic combination in sheep infected with *Fasciola hepatica* resistant to albendazole and clorsulon. *Exp. Parasitol.* 136, 59–62
77. Toet, H. *et al.* (2014) Liver fluke vaccines in ruminants: strategies, progress and future opportunities. *Int. J. Parasitol.* 44, 915–927
78. Hope Cawdery, M.J. *et al.* (1977) Production effects of liver fluke in cattle. The effects of infection on liveweight gain, feed intake and food conversion efficiency in beef cattle. *Brit. Vet. J.* 133, 145–159
79. Vercruysse, J. and Claerebout, E. (2001) Treatment vs non-treatment of helminth infections in cattle: defining the threshold. *Vet. Parasitol.* 98, 195–214
80. Bennema, S.C. *et al.* (2011) Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. *Int. J. Parasitol.* 41, 225–233
81. Selemetas, N. *et al.* (2015) The effects of farm management practices on liver fluke prevalence and the current internal parasite control measures employed on Irish dairy farms. *Vet. Parasitol.* 207, 228–240
82. Sargison, N. (2012) Diagnosis of triclabendazole resistance in *Fasciola hepatica*. *Vet. Rec.* 171, 151–152
83. Boray, J.C. (1969) The epidemiology of fascioliosis in two representative endemic regions of Australia. *Aust. Vet. J.* 45, 549–553
84. Kenyon, F. *et al.* (2009) Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. *Vet. Parasitol.* 163, 293–297
85. Kendall, S.B. (1965) Relationships between the species of *Fasciola* and their molluscan hosts. *Adv. Parasitol.* 3, 59–98
86. Wikerhauser, T. and Erglez, J. (1961) Viability of *Fasciola hepatica* metacercariae in silage *Vet. Arhiv.* 31, 315–318
87. Tanaka, H. and Tsuji, M. (1997) From discovery to eradication of schistosomiasis in Japan: 1847–1998. *Int. J. Parasitol.* 27, 1465–1480
88. Pritchard, G.C. *et al.* (2005) Emergence of fascioliosis in cattle in East Anglia. *Vet. Rec.* 157, 578–582
89. Molina-Hernández, V. *et al.* (2015) *Fasciola hepatica* vaccine: we may not be there yet but we're on the right road. *Vet. Parasitol.* 208, 101–111
90. Sanabria, R. *et al.* (2013) Identification of a field isolate of *Fasciola hepatica* resistant to albendazole and susceptible to triclabendazole. *Vet. Parasitol.* 193, 105–110
91. Alvarez-Sanchez, M. *et al.* (2006) Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. *Vet. Rec.* 159, 424–425
92. Mitchell, G.B.B. *et al.* (1998) Triclabendazole-resistant liver fluke in Scottish sheep. *Vet. Rec.* 143, 399
93. Gassenbeek, C. *et al.* (2001) An experimental study on triclabendazole resistance of *Fasciola hepatica* in sheep. *Vet. Parasitol.* 95, 37–43
94. Thomas, O. *et al.* (2000) Triclabendazole resistant *Fasciola hepatica* in south-west Wales. *Vet. Rec.* 146, 200

2.12 Postscript

Since this Chapter's publication in 2016, more cases of TCBZ resistance have been reported in animals (reviewed in Fairweather et al.,2020) and humans (reviewed in Marcos et al.,2021). The mechanisms of drug resistance are still unclear, but new research suggests there may be a polygenic basis to resistance (weather et al., 2020). Several advances have been made in on-farm *F. hepatica* control strategies as discussed in Coyne et al. (2020), John et al. (2019), Takeuchi-Storm et al. (2018) and reviewed in Fairweather et al. (2020). However, there are still no clear guidelines on how to test for TCBZ-resistant *F. hepatica,* and several other barriers discussed in Castro-Hermida et al. (2021) have yet to be addressed to improve the effectiveness of pharmacological treatment options for on-farm control. A complete list of flukicide products registered for Australian cattle has been provided in supplementary table 5.2.

References

Castro-Hermida, J.A., González-Warleta, M., Martínez-Sernández, V., Ubeira, F.M., Mezo, M., 2021. Current Challenges for Fasciolicide Treatment in Ruminant Livestock. Trends in Parasitology 37, 430-444.

Coyne, L.A., Bellet, C., Latham, S.M., Williams, D., 2020. Providing information about triclabendazole resistance status influences farmers to change liver fluke control practices. Veterinary Record 187, 357-357.

Fairweather, I., Brennan, G.P., Hanna, R.E.B., Robinson, M.W., Skuce, P.J., 2020. Drug resistance in liver flukes. International Journal for Parasitology: Drugs and Drug Resistance 12, 39-59.

John, B.C., Davies, D.R., Williams, D.J., Hodgkinson, J.E., 2019. A review of our current understanding of parasite survival in silage and stored forages, with a focus on Fasciola hepatica metacercariae. Grass and Forage Science 74, 211-217.

Marcos, L., Maco, V., Terashima, A., 2021. Triclabendazole for the treatment of human fascioliasis and the threat of treatment failures. Expert Review of Anti-infective Therapy 19, 817-823.

Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A.-S., Sengupta, M.E., Beesley, N.J., Hodgkinson, J., Williams, D., Thamsborg, S.M., 2018. Patterns of Fasciola hepatica infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasites & vectors 11, 674.

Chapter 3 – Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties

3.0 Preface

The scope of the thesis is to better understand the epidemiology and management of *F. hepatica* on Victorian dairy farms. In Chapters 1 I reviewed the epidemiology of *F. hepatica* in Australia and in Chapter 2, the management of *F. hepatica* globally. I found that in Australia, there was a limited understanding of the current prevalence of *F. hepatica* across all States and Territories. In Victoria, the last state-wide study was conducted in the late 1970s by Watt (1979). The study identified several endemic regions where *F. hepatica* was a significant problem but limited to no follow-up studies were conducted to assess the true extent of the problem. In the Chapter 2 review, I further identified that one of the endemic regions identified as the Macalister irrigation district in Watt (1979) study also had a reported case of TCBZ resistance (Brockwell et al., 2014).

The aims of Chapter 3 were to establish the prevalence of *F. hepatica* in Victoria, determine where the parasite was endemic and to assess whether there more cases of TCBZ *F. hepatica* in other dairy regions of Victoria.

In Chapter 3, I describe the state-wide prevalence study that I undertook in Victoria's irrigated dairy regions in 2014 and 2016. In the study, I utilised the coproantigen ELISA (cELISA) and LFEC to test the dairy cattle in these regions to determine where *F. hepatica* was endemic in Victoria. The tests were also used to perform coproantigen reduction tests (CRT) and faecal egg count reduction tests (FECRT) to screen farms with a high prevalence of *F. hepatica* for flukes that were resistant to triclabendazole.

To access a higher quality copy of the Chapter, use the DOI link below.

3.1 Published article and contribution

Published article

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Veterinary Parasitology 277, 109019. https://doi.org/10.1016/j.vetpar.2019.109019

Contribution

This paper was peer reviewed and published as an original research article. J. Kelley designed the trial and recruited veterinarians to collect the samples with assistance from T. Elliott and G. Rawlin. J. Kelley managed and analysed all samples using the LFEC and cELISA with assistance from V. Rathinasamy. J. Kelley analysed the results with assistance from M. Stevenson and T. Spithill. J. Kelley wrote the paper with editing from all other authors. Veterinary Parasitology applies the Creative Commons Attribution-Non Commercial-No Derivatives (CC BY-NC-ND) license to works they publish. The complete article is available here.

J. Kelley contributed approximately 85% to the production of this research article. She made a significant input in concept development, experimental design, writing of manuscript and responses to reviewers' comments, under the supervision of Professor Terry W Spithill.

3.2 Abstract

3.3 Introduction

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Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties

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ABSTRACT

Fasciola hepatica (liver fluke) is a widespread parasite infection of livestock in Victoria, South-eastern Australia, where high rainfall and a mild climate is suitable for the main intermediate host *Austropeplea tomentosa*. The aims of this study were to quantify the prevalence and intensity of *F. hepatica* in dairy cattle in the irrigated dairy regions of Victoria and determine if triclabendazole resistance was present in infected herds. Cattle in 83 herds from the following six irrigation regions were tested for *F. hepatica*: Macalister Irrigation District (MID), Upper Murray (UM), Murray Valley (MV), Central Goulburn (CG), Torrumbarry (TIA) and Loddon Valley (LV). Twenty cattle from each herd were tested using the *F. hepatica* faecal egg count (FEC) as well as the coproantigen ELISA (cELISA). The mean individual animal true prevalence of *F. hepatica* across all regions was 39 % (95 % credible interval [CrI] 27%–51%) by FEC and 39 % (95 % CrI 27%–50%) by cELISA with the highest true prevalence (75–80 %) found in the MID. Our results show that 46 % of the herds that took part in this study were likely to experience fluke-associated production losses, based on observations that herd productivity is impaired when the true within-herd prevalence is > 25 %. Using the FEC and cELISA reduction tests, triclabendazole resistance was assessed on 3 herds in total (2 from the 83 in the study; and 1 separate herd that did not take part in the prevalence study) and resistance was confirmed in all 3 herds. This study has confirmed that *F. hepatica* is endemic in several dairy regions in Victoria: triclabendazole resistance may be contributing to the high prevalence in some herds. From our analysis, we estimate that the state-wide economic loss associated with fasciolosis is in the order of AUD 129 million (range AUD 38–193 million) per year or about AUD 50,000 (range AUD 15,000–75,000) per herd per year.

1. Introduction

Fasciola hepatica, commonly known as liver fluke, is a trematode parasite that causes significant worldwide production losses in domestic ruminants (Charlier et al., 2013). Ruminants with liver fluke experience reductions in milk volume, milk quality, growth rates, food conversion efficiency, wool quality and reproductive performance (Schweizer et al., 2005; Charlier et al., 2013). In 1999 it was estimated that livestock producers in Australia spent approximately AUD 10 million (equivalent to AUD 16.6 million in 2018) on flukicides per year to reduce production losses in the approximately 6 million cattle and 40 million sheep that grazed the fluke endemic regions of Australia (Boray and Love, 2007). Lost production as a consequence of liver fluke infection was estimated to cost the Australian sheep industry in the order of AUD 25 million per year in 2015 (Lane et al., 2015) and infected dairy farms in Gippsland were expected to lose approximately AUD 20,700 worth of milk per lactation (Elliott et al., 2015), or about 2.8 % of a total average annual production per farm of about AUD 715,000. The three largest dairy areas in Australia are in Victoria: the Northern area, the Eastern (Gippsland) area and the South West area. These areas

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3.4 Material and Methods

account for 66 % of Australia's total milk production, valued at AUD2.3 billion in 2014 (Agriculture Victoria, 2014).

Within these areas irrigation is commonly used to offset water deficiencies and maintain pasture quantity and quality from late spring to autumn (Nash and Barlow, 2008). The greater Goulburn-Murray irrigation region and the Upper Murray (UM) irrigation region are located in the North and the Macalister Irrigation District (MID) region is located in Gippsland. The greater Goulburn-Murray irrigation region is made up of six smaller irrigation regions: the Murray Valley (MV), Central Goulburn (CG), Torrumbarry (TIA), Loddon Valley (LV), Rochester and Shepparton irrigation regions. In 1979 an abattoir survey of 39,499 cattle established that 68 % of livers inspected from the greater Goulburn-Murray region, 55 % from the MID region and 68 % from the UM region were infected with *F. hepatica*, which was considerably higher than the Victorian average *F. hepatica* infection prevalence of 41 % estimated by Watt (1979). The distribution and high intensity of infection was best explained by the use of irrigation in the regions from which the slaughtered cattle were sourced (Watt, 1979; Durr et al., 2005). Based on these findings, McCausland et al. (1980) investigated a further 98 cattle from 36 dairy farms in the MID and found that 64 % were infected with mature *F. hepatica* with 98 % of livers showing evidence of bile duct damage due to *F. hepatica*.

Border-check (flood) irrigation is the most widely used method for irrigating pastures in South-eastern and Northern Victoria. Using this technique, water is released from an irrigation channel outlet to flow across a paddock. This irrigation method creates ideal micro-habitats for the main intermediate host of *F. hepatica* in Australia, *Austropeplea tomentosa* (*A. tomentosa*) and ample water for *F. hepatica* eggs to hatch. The ecology of *A. tomentosa* has been reviewed by Boray (1969). Low lying areas accumulate surface water allowing *F. hepatica* eggs to be dispersed from bovine faecal material.

Control of *F. hepatica* in dairy cattle has relied on the use of flukicides most notably triclabendazole which, since its release in Australia in 1983, has been the preferred treatment at dry off because label restrictions prevent use during lactation and it is the only registered drug with the ability to kill both juvenile and adult fluke stages in dairy cattle (Boray et al., 1983). The first case of triclabendazole resistance in *F. hepatica* was identified in sheep grazing in the greater Goulburn-Murray irrigation region of Victoria and many cases have now been reported in sheep and cattle globally (Overend and Bowen, 1995; Kelley et al., 2016). The first cases of triclabendazole resistance in *F. hepatica* infecting cattle in Australia was reported by Brockwell et al. (2014) who identified resistance in four beef herds in New South Wales, as well as one dairy herd in the MID region. An additional case of triclabendazole resistance was identified in another dairy herd in the MID region by Elliott et al. (2015). In both studies the coproantigen reduction test (CRT) and liver fluke faecal egg count reduction test (FECRT) were used to confirm resistance. The two cases of triclabendazole resistance in the MID region led to 15 dairy herds across the Eastern (Gippsland) area being screened for *F. hepatica* using the coproantigen ELISA (Elliott et al., 2015). *F. hepatica* was found in six of the 15 herds and the herd prevalence of the infected herds ranged from 47 % to 100 % (Elliott et al., 2015).

The cELISA has not been widely used for screening large numbers of cattle for *F. hepatica* as most studies in dairy herds have used bulk tank milk ELISA samples which are easy to collect and from which treatment thresholds have been clearly defined (Charlier et al., 2013). However, the bulk tank milk ELISA only measures the presence of antibodies to fluke antigens so this test only confirms prior exposure to *F. hepatica* as opposed to the presence of active infection. The bulk tank milk ELISA threshold for identifying herds with a herd prevalence greater than 25 % was determined by Salimi-Bejestani et al. (2005). An increased prevalence above this threshold was subsequently found to be associated with reduced milk production, reduced milk fat percentages and increases in the inter-calving interval (Charlier et al., 2007); the 25 % threshold has since been adopted in many studies (McCann et al., 2010; Bennema et al., 2011; Mezo et al., 2011; Kuerpick et al., 2013; Howell et al., 2015; Novobilský et al., 2015). The 25 % threshold for production loss was observed in earlier research discussed by Vercruysse and Claerebout (2001) and has been applied to research utilising the FEC (Malone and Craig, 1990) and the cELISA (Elliott et al., 2015). Thresholds to assess individual fluke burdens using the cELISA have not been investigated in detail even though studies have identified a strong correlation between coproantigen release and total fluke burden (Mezo et al., 2004; Charlier et al., 2008; Brockwell et al., 2013). The thresholds based on FECs to detect losses due to fluke infections have been well defined. Egg counts that exceed 5 eggs per 2 g (ep2g) result in limited economic loss and counts that exceed 40 ep2g are associated with the presence of clinical disease (Malone and Craig, 1990; Vercruysse and Claerebout, 2001).

With this background, the aims of this study were to: (1) quantify the prevalence and intensity of F. hepatica in dairy cows in the irrigated dairy regions of Victoria; and (2) determine if triclabendazole resistance was present in infected herds.

2. Material and methods

2.1. Study design

This was a cross-sectional study to determine the prevalence of *F. hepatica* in Victorian dairy cows. The source population comprised dairy cows in dairy herds that produced milk for human consumption in Victoria, South-eastern Australia in 2014 and 2016. The eligible population comprised those members of the source population located in each of the six selected irrigation regions from the Northern and Eastern (Gippsland) areas of Victoria, had more than 20 cattle and where cattle had not been treated for *F. hepatica* in the three months prior to sample collection. The study population comprised those members of the eligible herd population whose managers agreed to take part in the study when approached by the local veterinarian between March and April. Dairy herds were recruited from 6 irrigation regions by local veterinarians as follows: 25 herds in the Central Goulburn (CG), 10 in the Loddon Valley (LV), 20 in the MID, 9 in the Murray Valley (MV), 9 in the Torrumbarry Irrigation Area (TIA) and 10 farms in the Upper Murray (UM) (Table 1; Fig. 1).

Between May and August each of the study herds were visited by the participating veterinarian. At each visit faecal samples were collected rectally from 20 randomly selected cattle in the milking herd. At the same time the identifier of each cow, the herd identifier and the date on which the herd was last treated for liver fluke was recorded. Faecal samples were initially stored at 4 °C; 2 faecal aliquots were then weighed (2 g ± 0.05) and the bulk sample was frozen at −20 °C. One 2 g aliquot was stored at 4 °C and analysed using the FlukeFinder®; the other was stored at −20 °C until the sample could be analysed using the cELISA.

2.2. F. hepatica faecal egg counts

The FlukeFinder® faecal sedimentation method was used to determine the *F. hepatica* egg count (Elliott et al., 2015). Each 2 (± 0.05) g faecal sample was sieved through a coarse sieve (125 nm) and a fine sieve (30 nm) (Nzalawahe et al., 2015). The faecal matter collected in the fine sieve was washed into a 50 mL plastic beaker and then poured into a 15 mL falcon tube. The remaining faecal material on the coarse sieve was discarded. The water column in the 15 mL falcon tube was filled to 11 mL and allowed to sediment for 2 min, then the supernatant was poured off and water poured in to refill it to 11 mL. This step was repeated three times and the final pellet was then poured into a small petri dish. The pellet was stained with 1 drop of 1 % methylene blue and eggs were counted using a dissecting microscope at 25 × . The FlukeFinder® was washed between samples and results are reported as the number of eggs per 2 g of faeces (ep2g).

Table 1
Characteristics of herds studied in irrigation regions of Victoria, South-eastern Australia, 2014–2016. Average herd size, calving system and drug used for the last flukicide treatment, stratified by region.

Region	# herds	Average herd size	Calving system				Last flukicide treatment				
			Split	Season	Unk	Total	TCBZ	CLOR	None	Unk	Total
Central Goulburn (CG)	25	404	21	1	3	25	4	17	2	2	25
Loddon Valley (LV)	10	250	9	1	0	10	6	3	0	1	10
Macalister (MID)	20	401	7	3	10	20	6	5	1	8	20
Murray Valley (MV)	9	520	1	1	7	9	0	3	0	6	9
Torrumbarry (TIA)	9	373	8	0	1	9	1	6	0	2	9
Upper Murray (UM)	10	340	7	3	0	10	1	7	0	2	10
Total	83	381	53	9	21	83	18	41	3	21	83

Unk: Calving system unspecified; or unknown last flukicide treatment.
TCBZ: triclabendazole.
CLOR: clorsulon.

2.3. Coproantigen ELISA

The commercial cELISA BIO K 201/2 was used. The kit batch used for samples analysed in 2014 was FASA14M02 and the kit batch used for samples analysed in 2016 was FASA16I07. During this 2-year period the kit was optimised, increasing the analytical sensitivity from 600 pg/mL in 2014 to 150 pg/mL in 2016 (Martínez-Sernández et al., 2016). We used the modified cELISA protocol discussed in Brockwell et al. (2013) by storing faecal aliquots at −20 °C and extracting samples overnight at room temperature on a rocking platform. All faecal samples were analysed in duplicate. Each cELISA plate included a duplicate of the kit positive and negative control (dilution buffer). The background optical density (OD) was deducted from the sample OD and the duplicate was averaged for each cow tested. The OD was then converted to a percentage of the positive control antigen: for the 2014 batch the positive control OD was 1.979 and, for the 2016 batch, the OD was 1.815. The negative cut-off was determined to be 1.3 % in Brockwell et al. (2014). We opted to use a cut-off of 1.6 % as described in Elliott et al. (2015) to allow for field variability.

2.4. Triclabendazole resistance

Cattle in two of the study herds (farms 1 and 2) included in the prevalence study, as well as one herd that did not take part in the prevalence study (farm 3), were tested for triclabendazole resistance using FECRT and a CRT. This study was performed in Winter (June,

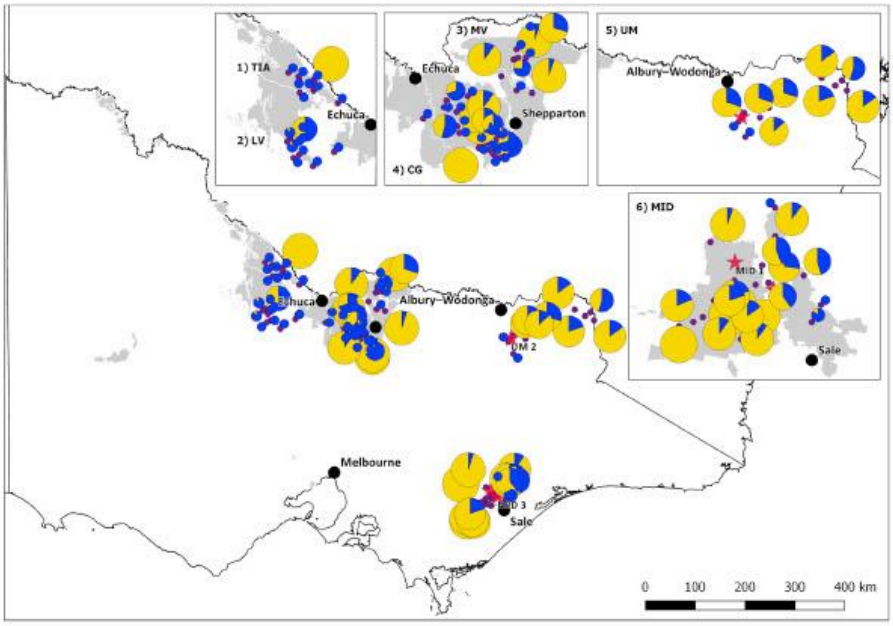

Fig. 1. Map of Victoria showing the location of the 83 dairy herds tested in the six Victorian irrigation regions: (1) TIA; (2) LV; (3) MV; (4) CG; (5) UM; and (6) MID. Each herd location is represented as a pie chart with the size of the pie proportional to the number of cows tested in each herd. The colours within each pie chart indicate the number of *F. hepatica* positive (yellow) and *F. hepatica* negative (blue) to the cELISA (FEC data not shown). The grey shading shows the managed irrigation regions in Victoria. Farms tested for triclabendazole resistance are represented by the red stars (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.5 Results

July, August). The two herds were chosen based on the willingness of the managers to participate, the herd numbers and that the heifers were positive for liver fluke. All animals included in the triclabendazole resistance study were heifers between 8 and 14 months of age with bodyweights between 143 and 421 kg. Sixty heifers on farms 1 and 2 were pre-screened for liver fluke: the thirty individuals with the highest FEC were selected and randomly allocated to either the control or treatment group. The single herd located in the MID on farm 3 was included because there had been a death from severe fasciolosis in that herd. The heifers in this herd were not pre-screened and a control group was not included due to animal welfare concerns. On day 0, 13 or 14 heifers were treated with 12 mg/kg triclabendazole (Flukare C™) with the treatment dose calculated based on the individual weight of heifers determined using a set of Tru-Test™ livestock scales. The dose of triclabendazole was rounded up to the nearest 1 mL and administered orally using a 60 mL syringe. At the time of treatment, a faecal sample was collected and the animal's identification number, bodyweight, the dose of triclabendazole administered, and time and date of administration were recorded. The control group (14 heifers) was left untreated and faecal samples were collected, as described above. In the herd on farm 3, faecal samples were collected from 13 randomly selected heifers and then all heifers in the herd were treated due to concerns for animal welfare. Twenty-one days post-treatment faecal samples were collected rectally from the same heifers sampled on day 0.

2.5. Triclabendazole efficacy

The efficacy of triclabendazole was determined by using a CRT and FECRT, using either method 1 or 2 as described by Brockwell et al. (2014). Method 1 (used on farms 1 and 2) was the RESO technique as recommended by the World Association of the Advancement of Veterinary Parasitology. The RESO technique compares post-treatment arithmetic means of treated and control groups (Coles et al., 1992). Method 2 (used on farm 3) compares the means of individual animal pre- and post-treatment counts to derive individual FECR reductions. Efficacy for both tests was calculated using the following formula:

$$Efficacy = \left(\frac{FEC_0 - FEC_{21}}{FEC_0}\right) \times 100 \quad (1)$$

In Eq. (1) FEC_{21} and FEC_0 represents the mean faecal egg count at 21 days post treatment or at the start of treatment, respectively. A calculated triclabendazole efficacy of < 90 % is considered to reflect drug resistance in the group analysed (Brockwell et al., 2014; APVMA, 2015). All cELISA kits used for the CRT were from batch FASA15C05 and the OD of the positive control was 1.653.

2.6. Statistical analyses

We report both the apparent and true prevalence of fasciolosis using FEC and cELISA diagnostic test methods. Apparent prevalence (AP) equals the number of test-positive individuals divided by the total number of individuals tested. The true prevalence (TP) estimates take into account the imperfect diagnostic sensitivity (Se) and specificity (Sp) of each test method using the approach described by Rogan and Gladen (1978) and modified for the extreme (i.e. low or high) prevalence situation using Bayesian methods as described by Messam et al. (2008). The Bayesian approach was important in this study because it allowed us to account for uncertainty in the change in the diagnostic test sensitivity of the cELISA that occurred when the kit was optimised to increase the analytical sensitivity from 0.6 ng/mL in 2014 to 150 pg/mL in 2016 (Martínez-Sernández et al., 2016).

A description of the Bayesian approach for estimating true prevalence is as follows. If x equals the number of cows testing positive using a diagnostic test of sensitivity Se and specificity Sp and n equals the number of cows tested, the distribution of the number of test-positive cows is $x|(TP, Se, Sp) \sim binomial(n, AP)$ where $AP = TP \times Se + (1 - TP) \times (1 - Sp)$.

To estimate the true prevalence of fasciolosis beta prior distributions for Se and Sp were used. For FEC we assumed the mode of the diagnostic sensitivity was 0.85 and that we were 95 % confident that the diagnostic sensitivity was greater than 0.75 (Mazeri et al., 2016). For the specificity of FEC we assumed the mode of specificity was 0.98 and that we were 95 % confident that diagnostic specificity was greater than 0.90 (Mazeri et al., 2016). For the 2014 cELISA we assumed that the mode of the diagnostic sensitivity was 0.94 and that we were 90 % confident that the diagnostic sensitivity was greater than 0.90 (Charlier et al., 2008). For the 2016 cELISA we assumed a marginal improvement in diagnostic sensitivity. Here, the mode of diagnostic sensitivity was set to 0.96 and we were 90 % confident that the diagnostic sensitivity was greater than 0.92 (Martínez-Sernández et al., 2016).

The logit of the true prevalence of *F. hepatica* was modelled as a function of irrigation region (a categorical variable comprised of six levels) and a herd-level random effect term to account for clustering of *F. hepatica*-positive cows within individual herds, similar to the approach taken by Dhand et al. (2010). This allowed us to provide an estimate of the prevalence of *F. hepatica* in each of the six irrigation regions, accounting for the confounding effect of herd.

Markov chain Monte Carlo (MCMC) methods were used to derive posterior estimates of the TP of *F. hepatica* in the study population using JAGS (Plummer, 2003). Using JAGS, the MCMC sampler was run for 100,000 iterations and the first 5000 'burn in' samples discarded. The posterior distribution of TP was obtained by running sufficient iterations to ensure that the Monte Carlo standard error of the posterior means were at least one order of magnitude smaller than their posterior standard deviation (Wakefield et al., 2000). The point estimate and 95 % credible intervals (CrI) for TP are reported as the median and 0.025 and 0.975 quantiles of the posterior distribution of P.

2.7. Ethics

The triclabendazole resistance study was approved by La Trobe University Ethics Committee AEC14-29. The prevalence study was carried out as an adjunct to a disease investigation program by veterinarians of the Department of Jobs, Precincts and Regions, Centre for AgriBioscience, La Trobe University, Bundoora Victoria 3083, Australia.

3. Results

The 83 herds that took part in this study had an average herd size of 381 cattle and most herds were managed using a split calving system (Table 1). In total, 1669 cattle were tested and the individual animal true prevalence of *F. hepatica* across all herds studied was 39 % (95 % CrI 27%–51%) by FEC and 39 % (95 % CrI 27%–50%) by cELISA (Tables 2 and 3). Based on FEC data, there was a considerable range in both the *F. hepatica* infection intensity among individual cows (Fig. 2) as well as the true within-herd prevalence of *F. hepatica* across each of the 83 study herds (Table 2; Fig. 3). Overall, our results show that 46 % (38 of 83) of the herds that took part in this study exhibited a within-herd true prevalence of fluke (based on FEC data) > 25 % (Table 2) which is above the accepted threshold for impaired herd productivity and economic loss (Vercruysse and Claerebout, 2001; Charlier et al., 2007).

3.1. Macalister Irrigation District

Dairy cows in the MID region had the highest individual animal true prevalence of *F. hepatica* with 80 % (95 % CrI 64 %–88 %) of the 414 cattle testing positive by FEC (Table 2) and 75 % (95 % CrI 58 %–85 %) testing positive by cELISA (Table 3). At the herd level, 17 of the 20 MID herds (85 %) had a within-herd true FEC prevalence > 25 %; 15 of the

3.6 Discussion

Table 2
Prevalence of *Fasciola hepatica* in dairy cattle in irrigation regions of Victoria, South-eastern Australia, 2014–2016. Number of cows tested, egg counts of tested cows by faecal egg count ep2g categories, individual cow faecal egg count apparent prevalence of fasciolosis and individual cow faecal egg count true prevalence of fasciolosis, stratified by region.

Region	Tested (*n*)	ep2g range (*n*)				Prevalence[a]		% herds with > 25 % true prevalence
		0	1-5	6-40	> 40	Apparent (95 % CI)	True (95 % CrI)	
Central Goulburn	500	378	77	43	2	24 (21–28)	21 (13–36)	24 %
Loddon Valley	200	187	8	4	1	6 (4–11)	5 (0–14)	10 %
Macalister	414	113	136	139	26	73 (68–77)	80 (64–88)	85 %
Murray Valley	180	122	26	28	4	32 (25–40)	34 (19–49)	44 %
Torrumbarry	180	160	6	11	3	11 (7–17)	11 (9–17)	11 %
Upper Murray	195	83	68	37	7	57 (50–64)	64 (35–85)	90 %
Total	1669	1043	321	262	43	38 (35–40)	39 (27–51)	46 %

CI: confidence interval; CrI: Bayesian credible interval.
[a] Number of *F. hepatica*-positive cows per 100 cows at risk.

Table 3
Prevalence of *Fasciola hepatica* in dairy cattle in irrigation regions Victoria, South-eastern Australia 2014–2016. Total number of cows tested, number of tested cows with cELISA less than 1.6 % and greater than or equal to 1.6 %, individual cow cELISA apparent prevalence of fasciolosis and individual cow cELISA true prevalence of fasciolosis, stratified by region.

Region	Tested (*n*)	cELISA		Prevalence[a]	
		< 1.6 %	≥1.6 %	Apparent (95 % CI)	True (95 % CrI)
Central Goulburn	500	372	128	26 (22–30)	22 (12–36)
Loddon Valley	200	186	14	7 (4–11)	3 (0–12)
Macalister	414	117	297	72 (67–76)	75 (58–85)
Murray Valley	180	104	76	42 (35–50)	41 (30–53)
Torrumbarry	180	159	21	12 (7–17)	11 (9–17)
Upper Murray	195	80	115	59 (52–66)	62 (40–77)
Total	1669	1018	651	39 (37–41)	39 (27–50)

CI: confidence interval; CrI: Bayesian credible interval.
[a] Number of *F. hepatica*-positive cows per 100 cows at risk.

20 MID herds had a within-herd true FEC prevalence > 90 % (Fig. 3). Twenty-six of the 414 cows tested (6 %; 95 % CI 4 %–8 %) were categorised as having a clinical level of infection based on their crude FECs being > 40 ep2g (Table 2; Fig. 2).

3.2. Upper Murray

The second highest individual animal true prevalence of *F. hepatica* was in the UM region with 64 % (95 % CrI 35 %–85 %) of the 195 cattle testing positive by FEC (Table 2) and 62 % (95 % CrI 40 %–77 %) testing positive by cELISA (Table 3). At the herd level, 9 of the 10 UM herds tested (90 %) had a within-herd true FEC prevalence > 25 %; 4 of the 10 UM herds had a within-herd true FEC prevalence > 90 % (Fig. 3). Of the 195 cattle in the UM region that were tested, 7 (4 %; 95 % CI 1 %–7 %) were categorised as having a clinical level of *F. hepatica* infection based on their crude FECs being > 40 ep2g (Table 2; Fig. 2).

3.3. Murray Valley

The largest discrepancy between diagnostic tests in the regional prevalence of *F. hepatica* was observed in the MV region. The individual animal true prevalence was 34 % (95 % CrI 19 %–49 %) by FEC and 41 % (95 % CrI 30 %–53 %) by cELISA (Table 2; Table 3). At the herd level, 4 of the 9 MV herds tested (44 %) had a within-herd true FEC prevalence > 25 %; 2 of the 9 herds had a within-herd true FEC prevalence of > 90 % (Fig. 3). Even though the prevalence of *F. hepatica* in the MV region was moderate, 4 of the 180 cattle tested (2 %; 95 % CI 1 %–6 %) had FECs greater than the clinical cut-off of 40 ep2g (Table 2; Fig. 2).

3.4. Central Goulburn

The individual animal true prevalence of *F. hepatica* in the CG region was 21 % (95 % CrI 13 %–36 %) by FEC and 22 % (95 % CrI 12 %–36 %) by cELISA (Table 2; Table 3). At the herd level, the FEC identified 6 herds (24 %) and the cELISA identified 7 herds (28 %) that exceeded the 25 % cut-off for production loss, respectively; 3 of the 25 herds had a within-herd true FEC prevalence > 90 % (Fig. 3). However, the overall intensity of infection in the CG region was relatively low with only two cows (0.4 %; 95 % CI 0 %–1.4 %) exceeding the clinical cut-off of 40 ep2g (Table 2; Fig. 2).

3.5. Torrumbarry and Loddon Valley

In the TIA and LV regions the individual animal true prevalence of *F. hepatica* by FEC was 11 % (95 % CrI 9 %–17 %) and 5 % (95 % CrI 0 %–14 %), respectively (Table 2), and 11 % (95 % CrI 9 %–17 %) and 3 % (95 % CrI 0 %–12 %) by cELISA, respectively (Table 3). At the herd level, in the TIA region all the positive cattle were isolated to a single herd where the within-herd true FEC prevalence was > 90 % using both diagnostic test methods (Fig. 3). In the LV region, 1 of 10 herds exhibited a within-herd true FEC prevalence of > 25 % with no herds showing a within-herd true FEC prevalence > 90 % (Fig. 3). Of the cows that were tested, only 1.7 % (95 % CI 0.3 %–4.8 %) in the TIA region and 0.5 % (95 % CI 0 %–2.8 %) in the LV region had an egg count > 40 ep2g (Table 2; Fig. 2).

3.6. Triclabendazole resistance

Triclabendazole was the second most popular flukicide treatment after clorsulon in this group of herds (Table 1). Two herds in the MID region and one herd in the UM region were tested for triclabendazole resistance. The efficacy of triclabendazole was less than 90 % in all three herds using both diagnostic test methods: a 2%–69% reduction using FECRT and a 0%–86% reduction using CRT, confirmed the presence of triclabendazole resistance in these herds (Table 4; Fig. 1).

4. Discussion

4.1. Regional findings

There were distinct differences in the within-herd true prevalence of *F. hepatica* between irrigation regions. The MID region had the highest individual animal true prevalence, with 80 % and 75 % of cows tested identified as fluke positive by FEC and cELISA, respectively. The true prevalence estimates for the MID region reported here are in the same order of magnitude but slightly less than the 81 % apparent prevalence of *F. hepatica* reported in a smaller study by Elliott et al. (2015). Two earlier abattoir studies in the MID region found the prevalence of *F.*

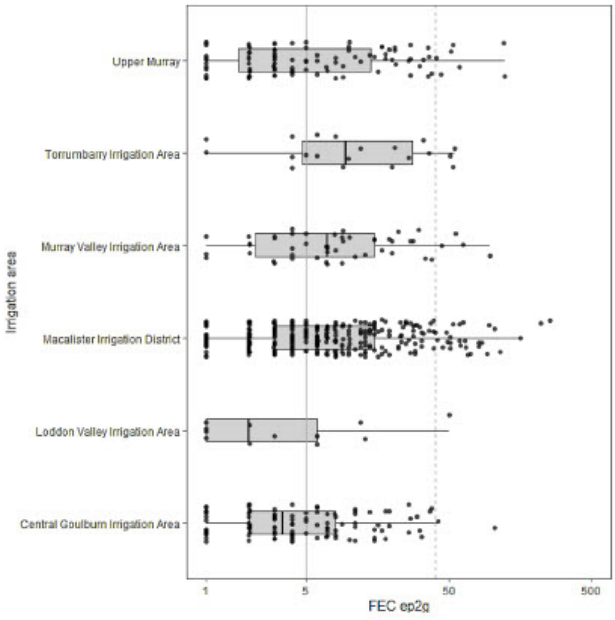

Fig. 2. Horizontal box and whisker plot showing the distribution of individual cow *F. hepatica* faecal egg counts (log 10 scale), by irrigation region. The left and right upper bound of each box represent the 25th and 75th quantiles of the distribution of FECs and the horizontal lines extending from the boxes represent the lower and upper bounds of the 95 % confidence interval for the FECs. Superimposed are points representing the FECs for each cow. The solid vertical line indicates the FEC ep2g value (5 ep2g) at which milk production is impaired; the dashed vertical dashed line indicates the FEC ep2g value (40 ep2g) at which clinical signs are apparent.

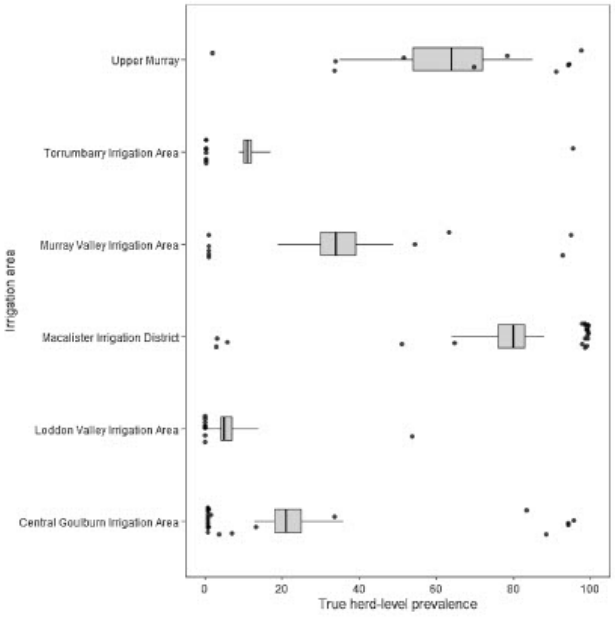

Fig. 3. Horizontal box and whisker plot showing the distribution of within-herd true prevalence of *F. hepatica* (expressed as the number of fluke-positive cows per 100 cows at risk) based on FEC, by irrigation region. In the above plot the left and right upper bound of the boxes represent the 25th and 75th quantiles of the distribution of within-herd true prevalence of *F. hepatica*, the vertical lines within each box represents the median within-herd true prevalence and the horizontal lines extending from the boxes represent the lower and upper bounds of the 95 % confidence interval for within-herd true prevalence. Superimposed are points representing the within-herd true prevalence of *F. hepatica* for each herd.

Table 4
Three farms were tested for *F. hepatica* triclabendazole resistance using the both the CRT and FECRT. The FEC was determined in 2 g of faeces and the cELISA results are shown as the percentage of positive control. Triclabendazole resistance is defined as a less than < 90 % reduction in triclabendazole efficacy.

Region	Farm number	*Number of cattle*		FEC		FECRT	cELISA		CRT
		TCBZ	Control	Day 0	Day 21		Day 0	Day 21	
Macalister[a]	1	13	14	30	16	+47 %	19 %	32 %	−68%
Upper Murray[a]	2	14	14	16	5	+69 %	22 %	3 %	+86 %
Macalister[b]	3	13	NA	97	95	+2 %	32 %	31 %	+3 %

TCBZ: triclabendazole.
[a] Efficacy determined using method 1 of Brockwell et al. (2013).
[b] Efficacy determined using method 2 of Brockwell et al. (2013) as no control group was available: no control group was included in this herd because of suspected clinical deaths due to *F. hepatica* prior to the study commencement.

hepatica to be 64 % (based on recovery of mature flukes; McCausland et al., 1980) and 55 % (based on liver condemnation; Watt, 1979). Prevalence analysis based on the number of liver condemnations alone is not a conclusive diagnostic for liver fluke infection, with a sensitivity of only 63 % (Rapsch et al., 2006). In contrast, recovery of mature adult flukes is definitive suggesting that the 64 % prevalence observed by McCausland et al. (1980) is a true estimate. We conclude that since the early 1980s there has been an increase in the prevalence *F. hepatica* in dairy cattle in the MID region. Most herds in the MID region (85 %) and UM region (90 %) had within-herd true prevalence estimates exceeding 25 % (Table 2; Fig. 3) with 75 % (MID) and 40 % (UM) of herds showing a within-herd true prevalence greater than 90 %. These values far exceed the threshold for economic loss of 25 % prevalence suggesting that production losses are likely to be occurring in these regions (Vercruysse and Claerebout, 2001; Schweizer et al., 2005; Charlier et al., 2013).

In the UM region, Watt (1979) reported that, in 1977, the individual animal (true) prevalence of *F. hepatica* was 68 %, slightly higher than the 64 % and 62 % individual animal true prevalence by FEC and cELISA (respectively) reported in this study. The results are similar despite the fact that the Watt (1979) study relied on abattoir workers scoring fluke infections based on gross liver pathology whereas we directly determined actual infections in each animal.

One key difference between the MID and the UM regions is that the UM region relies on rivers, groundwater and collected surface water for irrigation, whereas a border-check (flood) irrigation system provides water from the Macalister River (via the Glenmaggie Weir) and from the Thomson River (via Cowwarr Weir) to approximately 381 dairy farms in the MID region (SRW, 2019). This could explain the relatively lower prevalence in the UM region, as the MID flood irrigation system could contribute to the spread of fluke between farms and maintain the snail habitat for longer periods of time. In addition, a few farms located in the UM and MID regions are very close to or border national parks where kangaroos, wallabies, wombats could also be a significant reservoir for infection (Spratt and Presidente, 1981).

In Victoria the largest irrigation region is the greater Goulburn-Murray which is comprised of six irrigation regions providing water to approximately 1200 dairy farms. Four of these regions were studied here (MV, CG, TIA, LV). There were notable differences in the *F. hepatica* true prevalence estimates across the four irrigation regions: the MV was the only region in the greater Goulburn-Murray region to have an individual mean animal true prevalence greater than 25 %. In the TIA and LV regions the individual animal true prevalence by FEC was 11 % and 5 %, respectively (Table 2) and the intensity of infection by FEC was low, compared to the other study areas (Fig. 2; Tables 2 and 3). In the TIA region all of the fluke-positive cattle were isolated to a single herd. One possible explanation for this observation is that there may have been a breakdown in quarantine practices in this herd. Herd managers should be encouraged to quarantine new incoming stock and to test new stock for fluke infection to maintain a negative status, particularly in low prevalence regions such as the LV, TIA and CG.

In 1979 the prevalence of *F. hepatica* was found to be 68 % in the greater Goulburn-Murray region (Watt, 1979); however, in this study, the individual mean animal true prevalence of *F. hepatica* in this region was 18 % by FEC and 19 % by cELISA (calculated from the data in Tables 2 and 3). In the last 15 years, there have been two significant changes in the greater Goulburn-Murray region that could have contributed to the marked decrease in *F.hepatica* prevalence: salinity and irrigation infrastructure upgrades. The intermediate host *A. tomentosa* is highly sensitive to saline conditions and the infrastructure upgrades have included lining irrigation channels with rock or plastic and, in some cases, water is now piped to farms (GMW, 2015). This could have had a substantial impact on the snail population and reduced conditions favourable for *F. hepatica* transmission.

4.2. Triclabendazole resistance

Flukicides are frequently used by herd managers to control *F. hepatica* in irrigated farming regions. The most frequently used flukicide in this study was clorsulon (49 % of users) followed by triclabendazole (22 % of users) (Table 1). In Australia clorsulon is registered for use in lactating dairy cattle. When screening herds for inclusion in our study, herd managers were only asked to report the last flukicide treatment and the high use of clorsulon could reflect the fact that herd managers were choosing to treat their stock mid lactation to reduce sub-clinical production losses caused by *F. hepatica*. Orally administered triclabendazole has long been used as a dry-off treatment for dairy cattle, because of the milk withholding period. The first case of triclabendazole resistance was reported in 1995 in sheep in the LV region by Overend and Bowen (1995), and this drug has been widely used to treat fasciolosis in sheep in Australia, so further selection for drug resistance in sheep is possible. Our study has identified a further three triclabendazole resistant cattle herds in addition to two other herds in the UM or MID regions reported by Brockwell et al. (2014) and Elliott et al. (2015). The additional herd from farm 3 that was included in the triclabendazole resistance study was of particular concern as the calves had been treated 10 weeks prior to our study with triclabendazole and a *post mortem* examination of a calf that died showed evidence of severe fasciolosis.

4.3. Diagnostics tools: FEC and cELISA

Accurate diagnostic tests are essential for identifying infected cattle, informing *F. hepatica* control strategies and testing for resistance (Charlier et al., 2013). A large number of herds (68–94 %; Table 2) in the MV, CG, TIA and LV regions were found to be negative by FEC or cELISA but herd managers on these farms had been treating regularly for *F. hepatica*. Testing cows prior to treatment could have saved these herd managers considerable time, money and labour e.g. the current cost in Australia for treatment of an average herd of 381 animals with triclabendazole is in the order of AUD 1900 plus labour costs whereas the costs of an FEC or cELISA test on 10 animals to confirm herd infection is approximately AUD 250 plus sampling costs. Herds located in the MV, CG, TIA and LV regions could benefit from a yearly bulk tank

3.7 Conclusion

3.8 Acknowledgments

milk ELISA as it would provide timely and sensitive estimates of the likelihood of fluke exposure given the relatively low true prevalence of *F. hepatica* in these regions. If a herd was subsequently found to be positive on the basis of the bulk tank milk ELISA, the herd manager could then use FEC or cELISA for continued monitoring. In contrast, in the higher prevalence regions (such as the MID or UM), the routine use of the bulk tank milk ELISA could lead to false positives as most herds used a split-calving system (Table 1). Under split-calving management systems, cows within a herd are not treated at the same point in time which means that milk from both treated and untreated animals contribute to bulk milk samples on a given sampling day; since anti-*Fasciola* antibodies can persist in milk for at least 3–6 months post anthelmintic treatment, there is a risk of false positive bulk tank milk ELISA test results on milk from treated herds (Charlier et al., 2012; Takeuchi-Storm et al., 2018). This is complicated by the fact that clorsulon and oxyclozanide can be administered to lactating dairy cows in Australia. To obtain the best outcome to guide decision making, milk samples for bulk milk tank ELISA testing need to be collected based on when the last flukicide treatment occurred.

On some farms, we observed discrepancies in prevalence estimates based on FEC and cELISA analysis. For example, in the UM region, *F. hepatica* prevalence differed according to diagnostic test method on one farm where the true prevalence was 40 % using FEC and 0 % using the cELISA. One possible explanation is that this faecal sample was not stored and transported correctly during transit from the farm to the laboratory. Flanagan et al. (2011) showed that coproantigen stability was reduced by storage at 26 °C. In general, there is a moderate to good association between FEC and cELISA OD values in cattle ($r = 0.668$; Brockwell et al., 2013).

4.4. Estimated economic losses

The high prevalence and relatively high intensity of fluke infections in certain regions suggests that dairy producers in Victoria are experiencing significant production losses since milk production is negatively impacted by a herd prevalence of > 25 % (Vercruysse and Claerebout, 2001; Charlier et al., 2007). Schweizer et al. (2005) reviewed losses due to fasciolosis in dairy cattle in Switzerland and identified negative impacts of infection arising from suppressed milk production, reduced milk fat content, reduced fertility, reduced weight gain and condemnation of livers, in addition to the cost of drenching. Milk production losses due to fasciolosis range from 3 % to 15 % and vary with the intensity of infection and nutritional status of the animal (Boray, 1969; Hope Cawdery, 1984; Schweizer et al., 2005; Charlier et al., 2013).

Based on our data, 46 % of the herds tested were likely to have impaired milk production arising from fasciolosis, due a herd prevalence > 25 %. In 2017–2018 total milk production in the 6 regions studied here was 3898 million litres so 1793 million litres (46 %) were estimated to be affected by fasciolosis (Dairy Australia, 2019). Since fasciolosis is known to cause milk losses in the range 3–15 % (Schweizer et al., 2005; Charlier et al., 2013) we estimated mean losses at 10 % as per Schweizer et al. (2005) (range 3–15 %) to be 179 million litres (range 53–268 million litres). With a milk price currently at AUD 0.47/L (Dairy Australia, 2019), total milk losses are about AUD 84 million (range AUD 25–126 million) per year. Since milk losses only represent about 65 % of the total economic loss due to fasciolosis (Schweizer et al., 2005) the total annual loss is estimated to be AUD 129 million (range AUD 38–193 million). With 2561 herds in the regions studied, annual losses per herd are about AUD 50,000 (range AUD 15,000–75,000). These estimates demonstrate the likely positive benefits that would arise from effective liver fluke control on dairy farms in the irrigation regions of Victoria.

4.5. Future directions and recommended fluke control strategies

4.5.1. Triclabendazole resistance

Further work needs to be done to establish the extent of fluke triclabendazole resistance in the Victorian dairy industry, and a robust simple protocol must be designed for triclabendazole resistance testing, as uptake of routine testing by herd managers will continue to be hindered while testing guidelines for cattle remain poorly defined.

4.5.2. Regional screening

Previous regional screening of irrigation regions in Victoria has identified regions where *F. hepatica* is endemic (Watt, 1977, 1979; McCausland et al., 1980) and where drug resistance is a contributing factor (Brockwell et al., 2014; Elliott et al., 2015). The method of testing in our study was labour intensive and costly. A state-wide prevalence study screening herds using the cELISA or bulk milk tank ELISA could be a more effective way for identifying additional regions with a high incidence of fasciolosis. Our data could not be used to ascertain the factors contributing to the prevalence of liver fluke in each of the study herds. If a state-wide prevalence study were to be carried out, detailed information should be collected at the same time about herd management practices (e.g. quarantine and anthelmintic treatment of newly purchased stock) in addition to herd location information. This would allow herd-level risk factors for fasciolosis to be identified which could then be used to inform control strategies. Given new irrigation infrastructure works are planned for the MID in 2019, there is an opportunity in the future to evaluate whether these upgrades reduce fluke prevalence in the region. Regional studies often lack fine-scale spatial detail required by herd managers and their advisors to manage *F. hepatica* on-farm. A network of closely monitored farms across the irrigation regions in Victoria could provide valuable information about when herd managers need to test their stock.

4.6. Conclusions

This study has confirmed that *F. hepatica* is endemic in several dairy regions in Victoria, in particular in the MID and the UM regions, and that triclabendazole resistant *F. hepatica* is present in some herds in these regions. Our findings indicate that the state-wide annual economic loss associated with fasciolosis is in the order of AUD 129 million or about AUD 50,000/herd/year.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.9 References

References

Agriculture Victoria, 2014. Dairy Industry Profile, Department of Economic Development, Jobs, Transport and Resources. accessed 13/8/19. http://agriculture.vic.gov.au/agriculture/dairy.

APVMA, 2015. Efficacy Standards for Ruminant Anthelmintics in Australia (Australian Pesticides and Veterinary Medicines Authority Australian Pesticides and Veterinary Medicines Authority). accessed 13/8/19. https://apvma.gov.au/node/372.

Bennema, S., Ducheyne, E., Vercruysse, J., Claerebout, E., Hendrickx, G., Charlier, J., 2011. Relative importance of management, meteorological and environmental factors in the spatial distribution of *Fasciola hepatica* in dairy cattle in a temperate climate zone. Int. J. Parasitol. 41, 225–233.

Boray, J., 1969. Experimental fasciolíasis in Australia. Adv. Parasitol. 7, 95–210.

Boray, J., Crowfoot, P., Strong, M., Allison, J., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet. Rec. 113, 315–317.

Boray, J.C., Love, S., 2007. Liver fluke disease in sheep and cattle. NSW Agriculture. accessed 13/8/19. https://www.dpi.nsw.gov.au/_data/assets/pdf_file/0004/114691/liver-fluke-disease-in-sheep-and-cattle.pdf.

Brockwell, Y., M, Spithill, T.W., Anderson, G.R., Grillo, V., Sangster, N.C., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Vet. Parasitol. 196, 417–426.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int. J. Parasitol. Drugs Drug. Res. 4, 48–54.

Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Vet. Parasitol. 153, 44–51.

Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. Prev. Vet. Med. 78, 57–66.

Charlier, J., Hostens, M., Jacobs, J., Van Ranst, B., Duchateau, L., Vercruysse, J., 2012. Integrating fasciolosis control in the dry cow management: the effect of closantel treatment on milk production. PLoS One 7, e42216.

Charlier, J., Vercruysse, J., Morgan, E., van Dijk, J., Williams, D.J., 2013. Recent advances in the diagnosis, impact on production and prediction of *Fasciola hepatica* in cattle. Parasitology 141, 326–335.

Coles, G., Bauer, C., Borgsteede, F., Geerts, S., Klei, T., Taylor, M., Waller, P., 1992. World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.

Dairy Australia 2019. https://www.dairyaustralia.com.au/industry/production-and-sales/latest-production-and-sales-statistics, **accessed 5/8/19.**

Dhand, N.K., Johnson, W.O., Toribio, J.-A.L., 2010. A Bayesian approach to estimate OJD prevalence from pooled fecal samples of variable pool size. J. Agric. Biol. Environ. Stat. 15, 452–473.

Durr, P., Tait, N., Lawson, A., 2005. Bayesian hierarchical modelling to enhance the epidemiological value of abattoir surveys for bovine fasciolosis. Prev. Vet. Med. 71, 157–172.

Elliott, T.P., Kelley, J.M., Rawlin, G., Spithill, T.W., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica*in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Vet. Parasitol. 209, 117–124.

Flanagan, A.M., Edgar, H.W., Forster, F., Gordon, A., Hanna, R.E.B., McCoy, M., Brennan, G.P., Fairweather, I., 2011. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. Vet. Parasitol. 176, 34–42.

GMW, 2015. Goulburn-Murray Irrigation District Connections Project (Tatura, Goulburn-Murray Water). accessed 13/8/19. https://www.water.vic.gov.au/planning/environmental-contributions/third-tranche-of-the-environmental-contribution/irrigation-modernisation-and-water-recovery/goulburn-murray-water-connections-program-project-management.

Hope Cawdery, M.J., 1984. Review of the economic importance of fasciolosis in sheep and cattle. Irish Veterinary News, September 14–22.

Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepaticaexposure* in high-yielding dairy herds. Prev. Vet. Med. 121, 41–48.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 32, 458–469.

Kuerpick, B., Conraths, F.J., Staubach, C., Froehlich, A., Schnieder, T., Strube, C., 2013. Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. Parasitology 140, 1051–1060.

J. Lane , T. Jubb , R. Shephard , J. Webb-Ware , G. Fordyce . MLA Final Report: Priority List of Endemic Diseases for the Red Meat Industries Available from www.mla.com.au, accessed 13/8/19.

Malone, J., Craig, T., 1990. Cattle liver flukes: risk assessment and control. Compend. Contin. Educ. Vet. 12, 747–754.

Martínez-Sernández, V., Orbegozo-Medina, R.A., González-Warleta, M., Mezo, M., Ubeira, F.M., 2016. Rapid enhanced MM3-COPRO ELISA for detection of *Fasciolacoproantigens*. PLoS Negl. Trop. Dis. 10, e0004872.

Mazeri, S., Sargison, N., Kelly, R.F., Bronsvoort, B.M., Handel, I., 2016. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. PLoS One 11 e0161621.

McCann, C., Baylis, M., Williams, D., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. Vet. Rec. 166, 612–617.

McCausland, I., Vandegraaff, R., Nugent, L., 1980. Fasciolosis in dairy cows on irrigated pasture. Aust. Vet. J. 56, 324–326.

Messam, L.L.M., Branscum, A.J., Collins, M.T., Gardner, I.A., 2008. Frequentist and Bayesian approaches to prevalence estimation using examples from Johne's disease. Anim. Health Res. Rev. 9, 1–23.

Mezo, M., González-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). J. Parasitol. 90, 845–852.

Mezo, M., González-Warleta, M., Castro-Hermida, J.A., Muiño, L., Ubeira, F.M., 2011. Association between anti-*F. hepatica* antibody levels in milk and production losses in dairy cows. Vet. Parasitol. 180, 237–242.

Nash, D., Barlow, K., 2008. Impacts of irrigated dairying on the environment. In: McDowell, R. (Ed.), Environmental Impacts of Pasture-Based Farming. CAB International, Wallingford, UK, pp. 232–248.

Novobilský, A., Sollenberg, S., Höglund, J., 2015. Distribution of *Fasciola hepatica* in Swedish dairy cattle and associations with pasture management factors. Geospat. Health 9, 293–300.

Nzalawahe, J., Kassuku, A.A., Stothard, J.R., Coles, G.C., Eisler, M.C., 2015. Associations between trematode infections in cattle and freshwater snails in highland and lowland areas of Iringa Rural District. Tanzania. Parasitology 142, 1430–1439.

Overend, D., Bowen, F., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Aust. Vet. J. 72, 275–276.

Plummer, M., 2003. JAGS: a program for analysis of Bayesian graphical models using Gibbs sampling. Proceedings of the 3rd International Workshop on Distributed Statistical Computing (DSC 2003). March 20–22, Vienna, Austria.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. Int. J. Parasitol. 36, 1153–1158.

Rogan, W.J., Gladen, B., 1978. Estimating prevalence from the results of a screening test. Am. J. Epidemiol. 107, 71–76.

Salimi-Bejestani, M., Daniel, R., Felstead, S., Cripps, P., Mahmoody, H., Williams, D., 2005. Prevalence of *Fasciola hepaticain* dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Vet. Rec. 156, 729–731.

Schweizer, G., Braun, U., Deplazes, P., Torgerson, P., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet. Rec. 157, 188–193.

Spratt, D.M., Presidente, P.J., 1981. Prevalence of *Fasciola hepatica* infection in native mammals in southeastern Australia. Aust. J. Exp. Biol. Med. Sci. 59, 713–721.

SRW, 2019. Macalister Irrigation District. In Facts and Figures (Southern Rural Water). accessed 13/8/19. http://www.srw.com.au/water-systems/macalister-irrigation-district.

Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A., Sengupta, M.E., Beesley, N.J., Hodgkinson, J., Williams, D., Milan, Stig, Thamsborg, S.M., 2018. Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasit. Vectors 11, 674–692.

Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. Vet. Parasitol. 98, 195–214.

Wakefield, J., Best, N., Waller, L., 2000. Bayesian approaches to disease mapping. In: Elliott, P., Wakefield, J., Best, N., Briggs, D. (Eds.), Spatial Epidemiology: Methods and Applications. Oxford University Press, Oxford, UK, pp. 104–127.

Watt, G., 1977. An Abattoir Survey of the Prevalence of *Fasciola hepatica* Affected Livers in Cattle in Victoria. PhD, University of Melbourne, Melbourne.

G. Watt . An approach to determining the prevalence of liver-fluke in a large region Proceedings of the 2nd International Symposium on Veterinary Epidemiology and Economics May 1979, Canberra, Australia 152-155.

3.10 Postscript

A complete list of flukicide products registered for use in beef and dairy cattle in Australia can be found in Chapter 5, section 12, supplementary Table 5.2. The Table details the following: product, company, composition, use, age of fluke killed, host, meat with holding period, use in lactating cattle and finally, milk withholding period.

Chapter 4 – Analysis of daily variation in the release of faecal eggs and coproantigen of *Fasciola hepatica* in naturally infected dairy cattle and the impact on diagnostic test sensitivity

4.0 Preface

In Chapter 3, I found that there was a high prevalence of *F. hepatica* in the Macalister Irrigation District and the Upper Murray using the coproantigen ELISA (cELISA) and LFEC diagnostic tests. I subsequently used the same diagnostic tools and found that cattle on three farms located in these regions were infected with triclabendazole-resistant *F. hepatica.* In Chapter 1, I reported that the application of the cELISA and LFEC for assessing the prevalence and drug resistance had been established in the literature; however, I discovered several papers that suggested that sampling time during the day may affect the sensitivity of LFEC and cELISA (Dorsman, 1956; Brockwell et al., 2013). Brockwell et al. (2013) reported a 2-6 fold variation in the cELISA OD values and a 2-4 variation in LFEC over a five-day sampling period. To improve the management and control of *F. hepatica* in Victoria, farmers need robust diagnostic tools to make informed decisions about whether they need to treat their cattle, when to treat and what product to use on their cattle.

The aim of Chapter 4 was to assess the fluctuation in the diagnostic outputs from the cELISA and LFEC in naturally infected dairy cattle to ascertain whether the tests were robust and could be used to inform the on-farm management of *F. hepatica* in Victoria.

In the following Chapter, I describe the application of the cELISA and LFEC to cattle naturally infected with *F. hepatica* to assess if sampling time and day to day variation in coproantigen and egg release affects the sensitivity of the tests and the correlation with total fluke count. Ten cattle on a dairy farm in the Macalister Irrigation District (MID), which was found to have a high prevalence of *F. hepatica* in Chapter 3, were sampled and tested twice daily for five days and then euthanized to allow correlations between total fluke counts and diagnostic test outputs.

To access a higher quality copy of the Chapter, use the DOI link below.

4.1 Published Article and Contribution

Publication

This manuscript has been drafted for submission to Veterinary Parasitology, submitted on the 26/02/2021 (Vetpar-D-21-15033) the manuscript was accepted on the 23 June 2021 and a copy has been included in Chapter 7, section 7.1.

Kelley, J.M., Stevenson, M.A., Rathinasamy, V., Rawlin, G., Beddoe, T., and Spithill, T.W. (2021). Analysis of daily variation in the release of faecal eggs and coproantigen of *Fasciola hepatica* in naturally infected dairy cattle and the impact on diagnostic test sensitivity. *Veterinary Parasitology* 298**,** 109504. https://doi.org/10.1016/j.vetpar.2021.109504

Contribution

This paper was drafted and submitted as an original research article. J. Kelley designed the trial and recruited veterinarians to collect the samples with assistance from G. Rawlin. J. Kelley managed and analysed all samples using the LFEC and cELISA with assistance from V. Rathinasamy. V. Rathinasamy determined the total fluke count. J. Swan and V. Rathinasamy assisted J. Kelley in the dissection of livers. J. Kelley analysed the results with the assistance from M. Stevenson and T. Spithill. J. Kelley wrote the paper with editing from all other authors.

J. Kelley contributed to approximately 80% of the production of this research article. She made a significant input in concept development, experimental design, analysis, and the writing of manuscript submission to Veterinary Parasitology under the supervision of Professor Terry W Spithill.

Analysis of daily variation in the release of faecal eggs and coproantigen of *Fasciola hepatica* in naturally infected dairy cattle and the impact on diagnostic test sensitivity

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Keywords: *Fasciola hepatica*, cELISA, faecal egg counts, diagnostic test sensitivity, daily faecal antigen release

4.2 Abstract

23 **Abstract**

24 The liver fluke, *Fasciola hepatica (F. hepatica,)* is a widespread parasite infection in dairy cattle in Victoria,
25 South-eastern Australia. Robust diagnosis of fluke infection is needed in dairy cattle to identify sub-clinical
26 infections which often go unnoticed, causing significant production losses. We tested the coproantigen ELISA
27 (cELISA) and the FlukeFinder faecal egg count kit® on naturally infected cows in a fluke endemic region of
28 Victoria. The aim of the study was to investigate the variation in the release of coproantigens and eggs into
29 faeces over a 5-day period, at the morning (AM) and afternoon (PM) milkings, and to assess the impact of the
30 timing of faecal sample collection on diagnostic test sensitivity. Ten cows were enrolled into the study based
31 on positive *F. hepatica* faecal egg counts (LFEC), and faecal samples from the ten cows were collected twice
32 daily, at the 7-9 AM and 4-6 PM milking, for five consecutive days. At the conclusion of the sampling period,
33 the cows were euthanised, and *F. hepatica* burden was determined at necropsy. A moderate negative
34 correlation between cow age and cELISA optical density (OD) was observed using data from all samples (R -
35 0.63; 95% CI -0.68 to -0.57). Over the 5-day sampling period, we observed within-animal variation between
36 days for both the cELISA OD (2.6- 8.9 fold) and LFEC (5-16 fold), with more variation in values observed in the
37 PM samples for both tests. The correlation with total fluke burden was higher in the AM sampling using both
38 the cELISA and LFEC (R= 0.64 and 0.78, respectively). The sensitivity was 100% for the cELISA using various cut-
39 offs from the literature (0.014 OD, 0.030 OD, and 1.3% or 1.6% of the positive control). The sensitivity of the
40 FlukeFinder kit® (based on 588 faecal samples and not accounting for lack of independence in the data) was
41 88% (95% CI 85% to 90%). Seventy-one false negatives were recorded from the 588 LFEC tests, all of which
42 were observed in the cows with fluke burdens <14 flukes; 42 of the 71 false negative LFECs occurred in one
43 individual cow, which had the lowest burden of nine flukes. In dairy cows, the cut-off for production losses
44 due to fasciolosis is estimated at > 10 fluke. Both the cELISA and the LFEC identified all cows with burdens

4.3 Introduction

45 equal to or greater than this cut-off. Five of the ten cows also exhibited relatively high paramphistome egg
46 counts.

47 **1. Introduction**

48 *Fasciola hepatica* (*F. hepatica*), more commonly known as liver fluke, has serious production limiting impacts
49 in dairy cattle affecting milk production, milk quality, weight gain and fertility (Schweizer et al. 2005). Lost milk
50 production due to fasciolosis in the UK alone is estimated (using 2020 consumer price indices) to be about
51 USD 529 per cow per year (Howell et al., 2015) or about USD 1005 million per year based on a 2019 national
52 herd size of 1.9 million cows (Uberoi, 2020). In Australia, in 2020, reduced milk production due to *F. hepatica*
53 infection was estimated to cost the Victorian dairy industry USD 101 million per year (Kelley et al., 2020). A
54 robust quantitative diagnostic test that can identify *F. hepatica*-infected individuals and herds would allow for
55 prompt intervention and treatment of cattle with flukicides. Europe has moved towards screening dairy herds
56 with an ELISA detecting antibodies using bulk tank milk (BTM) samples (BTM ELISA) which are readily available
57 and easy to collect (Pritchard et al., 2005; Salimi-Bejestani et al., 2005; Charlier et al., 2007; Bennema et al.,
58 2009; McCann et al., 2010; Kuerpick et al., 2013; Selemetas et al., 2014; Bloemhoff et al., 2015; Howell et al.,
59 2015; Novobilský et al., 2015). The BTM sELISA was first described by Salimi-Bejestani et al. (2005): this assay
60 has a high sensitivity (96%) and moderate specificity (80%) and can accurately identify herds that are incurring
61 production losses when herd prevalence is more than 25% (Charlier et al., 2007). However, the BTM ELISA has
62 its drawbacks as anti-*Fasciola* antibodies in milk can persist for up to six months even after successful
63 treatment with a flukicide (Salimi-Bejestani et al., 2005). In Australia, most dairy herds use a split calving
64 system, which means that flukicide treatments occur at different times of the year for individual herds. As a
65 result, antibodies found in milk could arise from treated and untreated cattle, complicating the interpretation
66 of a positive BTM ELISA test.

3

As a consequence, a different approach has been used to screen dairy cattle in Australia. Brockwell et al. (2014), Elliott et al. (2015) and Kelley et al. (2020) each used the commercial coproantigen ELISA test to screen multiple herds for *F. hepatica* as coproantigen release ceases seven days after effective treatment with a flukicide (Brockwell et al., 2013). The coproantigen ELISA (cELISA) detects infection in cattle from >6 weeks post-infection (PI) (Mezo et al., 2004; Brockwell et al., 2013) and has a high sensitivity 77%-100% and specificity >99%(Mezo et al., 2004; Mazeri et al., 2016). In addition, correlations between *F. hepatica* burden and OD were observed in cattle by Charlier et al. (2008) ($R = 0.60$) and Brockwell et al. (2013) ($R^2 = 0.8718$), although recent work by Martínez-Sernández et al. (2016) found a somewhat weaker correlation ($R^2 = 0.2998$). However, Brockwell et al. (2013) observed a 2 to 6 fold variation in coproantigen release from cattle over a five day period. To address the variable release of coproantigens the cELISA kit was modified by Martínez-Sernández et al. (2016), increasing the sensitivity from 0.60 ng/mL to 0.15 ng/mL; however, the variability in the cELISA in daily samples increased by 6 to 12 fold (Mezo et al., 2004). There is a consensus in the literature that the cELISA kit-cut-off recommended by the commercial manufacturer is too high to accurately distinguish between positive and negative cattle. As a result, studies have used various ELISA OD cut-offs for detecting *F. hepatica* infections in cattle: 0.114 OD (Mezo et al., 2004), 0.030 OD (Charlier et al., 2008), 0.014 OD (Brockwell et al., 2013), kit-cut-off × 0.67 (Palmer et al., 2014), 0.084 OD (Martínez-Sernández et al., 2016) as well as 1.3% (Brockwell et al., 2014) or 1.6% (Elliott et al., 2015) of the OD value of the positive control. The lack of consistency between reports makes it difficult to determine the sensitivity of the cELISA, but several studies in cattle have reported that the assay can detect as few as 1, 2, and 15 flukes in the liver (Mezo et al., 2004; Brockwell et al., 2013; Martínez-Sernández et al., 2016).

Similar problems occur when using *F. hepatica* faecal egg counts (LFEC). In cattle with low *F. hepatica* burdens (<10 flukes) false negatives frequently occur (Martínez-Sernández et al., 2016). There are many variations on the LFEC technique, but sedimentation is the most accurate in cattle (Happich and Boray, 1969; Kajugu et al.,

2015). Two studies reported correlations between LFEC and *F. hepatica* burden. In cattle, the correlation was $R^2 = 0.836$ and in sheep $R^2 = 0.571$ (Brockwell et al., 2013; George et al., 2017). LFECs are highly specific >97.5% (97.5-100), and egg shedding does not persist after treatment with a flukicide (Ibarra et al., 1998; Anderson et al., 1999; Rapsch et al., 2006; Brockwell et al., 2013; Mazeri et al., 2016). However, weekly, daily, and hourly variation *in F. hepatica* egg shedding has been observed in several studies (Dorsman, 1956, 1960; Hagens and Over, 1966; Brockwell et al., 2013). Based on hourly faecal sampling in cattle, Dorsman (1956) proposed that faecal collection should occur at 1:30 pm when the highest egg release was more likely to represent the burden of *F. hepatica* within the liver. Hagens and Over (1966) reached the same conclusion observing the peak release of eggs between 12:00 pm – 8:00 pm, similarly suggesting that this was the most suitable time for sampling cattle. However, the sensitivity of the LFEC is affected by the volume of faeces sampled, the faecal output by the animal, the burden of *F. hepatica* within the animal, the experience of the technician and the duration of the *F. hepatica* infection as the test only detects *F. hepatica* from > 8 PI weeks in cattle (Boray, 1969; Conceição et al., 2002; Rapsch et al., 2006; Charlier et al., 2008; Brockwell et al., 2013; Martínez-Sernández et al., 2016).

Objectives

Previous studies have investigated the level of variability in coproantigen shedding in animals between weeks and on consecutive days, but not variations within a day. Monitoring of *F. hepatica* egg shedding variation has been extensive. However, it has never been determined if peak egg shedding from 12:00 pm - 8:00 pm actually correlates with *F. hepatica* burden in the liver and if this is, therefore, a better time to collect faecal samples from cattle. In this study, recognising the variable release of both coproantigens and eggs, we investigated the sensitivity of two sample points in the morning (AM) and afternoon (PM) milking, the variation in coproantigen and LFEC shedding over a consecutive five day period and the correlation of coproantigen levels and LFEC with *F. hepatica* burden in ten naturally infected dairy cows.

5

4.4 Material and Methods

2. Methods

2.1 Study design

One pasture-fed, split calving dairy herd in Victoria, Australia identified by Kelley et al. (2020), was purposively selected for this study based on the herd owner's willingness to participate. Thirty cows were screened using the FlukeFinder® kit to determine if they were infected with *F. hepatica*. Ten cows were selected based on positive LFEC and purchased from the owner. The age of the cows ranged from 2.9 to 11.1 years, and the predominant breed was Holstein. Following the purchase, the ten selected cows remained on the farm and were kept separately from the main milking herd but grazed pasture and received grain and concentrate at milking as for the main milking herd. The study group was milked twice daily after the main herd: a morning milking (AM) between 7:00 am, and 9:00 am and an afternoon milking (PM) between 4:00 pm and 6:00 pm. Sample collection over five consecutive days began on the 30th March 2017 and concluded on 03rd April 2017. At the AM and PM milkings on each of the five sampling days, 50 g faecal samples were collected rectally from each of the ten study group cows; a new rectal examination sleave was used for each cow. On day six (04th April 2017), nine of the ten cows were euthanised. One cow #(3491) was unable to be yarded on day six and was instead euthanised on day 14 (12th April 2017). Cows were euthanised in two groups: group 1 between 10:00 am, and 11:00 am and group 2 between 1:00 pm and 2:00 pm. Livers and gallbladders were removed within 30 minutes of death, and livers were stored at 4 °C until they could be sectioned.

This study was approved by the La Trobe University Ethics Committee, AEC16-62 and ran in conjunction with the State Government of Victoria, Department of Jobs, Precincts and Regions pathology and quarantine training program for veterinarians and animal health officers.

6

133 *2.2 Faecal sample collection over five days*

134 Faecal samples from each cow at the AM and PM milkings were split into two replicates. Replicate A and B
135 each weighed approximately 25 g. Faecal matter was mixed thoroughly from each replicate, and then two 2 g
136 samples were weighed for each cow; aliquot 1 for the cELISA and aliquot 2 for the LFEC. cELISA replicates were
137 stored at 4°C until aliquots were weighed, the aliquots were then frozen at -20 °C, which occurred within 72
138 hours of collection. LFEC aliquots were weighed and stored at 4 °C until counts were completed. On two
139 occasions, faecal samples were unable to be collected: cow #3294 on day 4 PM and cow # 844 on day 5 PM.
140 Faecal samples collected on day 6 (the day of euthanasia) were not included in the analyses presented in this
141 paper because faecal samples were collected outside of the time window for AM and PM milkings and were
142 not replicates.

143 2.3 Total fluke count

144 The total liver fluke counts (TFC) were completed following the guidelines outlined by Reichel (2002) and
145 Brockwell et al. (2013). Before removing the liver from the abdominal cavity, the small intestines were tied off
146 using cable ties approximately 30 cm on either side of the gallbladder, ensuring the gallbladder was left intact.
147 The livers were removed and stored in individual eskies on ice. The livers were then moved to a 4°C fridge and
148 remained there until sectioned, which occurred within 72 hours. The livers were then cut into 5 to 10 mm
149 strips and squeezed to remove *F. hepatica* from the bile ducts. Once the liver was sectioned, the liver slices
150 were soaked in PBS (1 × PBS including 16 mM Na_2HPO_4, 5 mM $NaH_2PO_4.H_2O$, 120 mM NaCl; pH 7.4) and left at
151 room temperature overnight. The following morning individual livers slices were soaked in a tub of warm
152 water. The PBS and water were sieved, and all containers were inspected for *F. hepatica*. The TFC was
153 determined by counting whole intact *F. hepatica*, plus the highest count for either head or tails from partially
154 recovered *F. hepatica*. Animal 4316 liver was pale and deformed with bile duct thickening (fibrosis) (data not

155 shown). No noticeable *F. hepatica* damage was present in the other nine livers. The researcher conducting the
156 diagnostic testing remained blind to the results of the TFC until the completion of all LFEC and cELISA.

157 *2.4 cELISA*

158 Faecal aliquots were stored at -20 °C until analysis using the cELISA Faecal BIO K 201 kit from Bio X Diagnostics,
159 Belgium. The method used is described in Kelley et al. (2020), which includes details of modifications based
160 on Brockwell et al. (2013). The cELISA plates were loaded with samples from one cow per strip (column): two
161 duplicates of replicate A and B were added. The cELISA plate was also loaded with the following controls:
162 negative control (dilution buffer), positive control (positive manufacturer control provided with the kit batch,
163 no dilution), high positive extract from a known infected animal (cELISA OD >1.0) and a low positive extract
164 from a known infected animal (cELISA OD <0.8) sourced from several artificially infected cattle from a separate
165 study conducted by our laboratory (data not shown). All values are presented as either OD (450 nm) or a
166 percentage of the positive control provided with the kit batch. All cELISA plates were from the same batch
167 FASA16I07, and the positive control OD was 1.815.

168 *2.5 F. hepatica and paramphistome faecal egg counts*

169 The FlukeFinder® kit was used to determine the LFEC and paramphistome faecal egg count (PFEC) as high
170 paramphistome egg counts were observed. Eggs were distinguished based on egg colour when stained with
171 methylene: *F. hepatica* eggs appear golden or orange, whereas paramphistome remain colourless (clear). The
172 sedimentation method was used as described by Kelley et al. (2020). LFEC and PFEC are reported as the
173 number of eggs per 2 g of faeces (ep2g). Each LFEC sample was counted three times to assess technician
174 accuracy, given the high numbers of paramphistome eggs present in the samples. PFEC were not recorded for
175 the following cows: cow #536 (day 4 AM replicate A), cow #836 (day 5 PM replicate A), cow #844 (day 1 PM

176 replicate A and day 5 PM), cow #1100 (day 1 AM replicate A and day 5 AM replicate A), cow #3294 (day 4 PM,
177 replicate A and B) and cow #3491 (day 5 AM replicate B).

178 *2.6 Statistical analyses*

179 Correlations between age, TFC and AM and PM cELISA OD and AM and PM LFECs were quantified using
180 Spearman's rank correlation (R). Scatterplots were constructed to show cELISA OD estimates for each AM and
181 PM as a function of sampling day and time. To quantify the difference between cELISA OD estimates for AM
182 and PM sampling, accounting for lack of independence in the data arising from repeated cELISA OD estimates
183 from the same cow over the 5-day study period, we used a mixed-effects linear regression model with
184 sampling time (a categorical variable comprised of two levels: AM and PM) as a fixed effect, sampling day as
185 a random slope and cow identity as a random intercept term. A similar approach was taken for the LFEC
186 estimates. Scatterplots were constructed to show LFEC counts for each AM and PM as a function of sampling
187 day. To quantify the difference between LFEC estimates for AM and PM sampling, a mixed-effects linear
188 regression model was used with Box-Cox transformed LFECs (Box and Cox 1964) as the outcome variable,
189 sampling time (AM or PM) as a fixed effect, sampling day as a random slope and cow identity as a random
190 intercept term. Analyses were carried conducted using the contributed nlme package (Pinheiro et al., 2020) in
191 R (R Core Team 2020). The presence of TFCs provided a rare opportunity to quantify the diagnostic sensitivity
192 of LFEC for AM and PM samplings. To allow our results to be compared with other similar studies where faecal
193 samples were collected from individual cows on multiple occasions, the diagnostic sensitivity of the LFEC was
194 calculated without accounting for the lack of independence in the data. Confidence intervals for diagnostic
195 sensitivity were calculated using the exact method (Collett 1999). The sensitivity was calculated using the
196 method in Estuningsih et al (2009).

4.5 Results

197 **3. Results**

198 *3.1 Summary correlation statistics*

199 All ten cows in this study were *F. hepatica* positive, with the number of flukes in the liver ranging from 9 to 72
200 (Table 1). No correlation was observed between age and TFC (R -0.32; 95% CI -0.86 to -0.41) or between age
201 and LFEC (Pearson's rho -0.23; 95% CI -0.30 to -0.15). However, a moderate negative correlation between age
202 and the cELISA OD was observed using data from both AM and PM samples (R -0.63; 95% CI -0.68 to -0.57)
203 (Figure 1). At the AM and PM milkings, there was a higher positive correlation between the cELISA and TFC at
204 the AM milking (R 0.64; 95% CI 0.54 to 0.73) relative to the PM milking (R 0.58; 95% CI 0.44 to 0.67) (Figure 2).
205 A higher positive correlation was also observed between TFC and LFEC at the AM milking (R 0.78; 95% CI 0.73
206 to 0.82) relative to the PM milking (R0.66; 95% CI 0.59 to 0.72) (Figure 3).

207 *3.2 cELISA controls and sensitivity*

208 Three true positive controls (the kit positive control, a high positive faecal extract control and a low positive
209 faecal extract control) and one negative control were included on all cELISA plates (Table 2). The high and low
210 control extracts were sourced from local artificially infected cattle with a known TFC. The sensitivity of the
211 cELISA was calculated using all the published cut-offs based on OD, batch % positive, kit % positive, and high
212 extract % positive (Table 3). The highest sensitivity (100%) was obtained using a number of cut-offs and
213 positive controls (Table 3). Of the 392 samples tested using the cELISA, all samples were positive using a cut-
214 off of 0.014 (Brockwell et al., 2013), 0.030 OD (Charlier et al., 2008), 1.3% (Brockwell et al., 2014) and 1.6%
215 (Elliott et al., 2015): lower sensitivity was observed when the kit-cut-off of >8% was used or using the >5.4%
216 cut-off of Palmer et al. (2014)(Table 3).

3.3 cELISA OD variability at AM and PM sampling over five days

The kinetics of the cELISA OD values over the 5-day sampling period for each cow is shown in Figure 4. Afternoon cELISA OD values were, on average, 0.21 (95% CI 0.04 to 0.12) OD units less than AM cELISA OD values. There was a 2.6 to 8.9-fold variation in the OD values for the cELISA over the five days, with the greatest variation observed in cows #412, #650, #844 and #3294 (Table 4). In general, there was more variation in the PM samples (Figure 5). The sensitivity of the cELISA was higher using the AM samples (Table 3).

3.4 LFEC counts and sensitivity

In the case of LFEC, 588 samples were counted, and all ten cows were found to be positive for *F. hepatica,* with the LFEC ranging from 0 to 32 ep2g (Figures 3 and 6). On 189 occasions, the LFEC exceeded the 5 ep2g cut-off for production loss in cattle described in Malone and Craig (1990) and Vercruysse and Claerebout (2001). The sensitivity of the FlukeFinder® Kit (based on 588 faecal samples and not accounting for lack of independence in the data) was 88% (95% CI 85% to 90%) with a total of 71 false negative results recorded: 13 for cow #412; 11 for cow #536; 42 for cow #650 which had the lowest *F. hepatica* burden (n=9); and five for cow #3294. Thirty of the false negative samples occurred at the AM sampling, and 41 false negatives occurred at the PM sampling.

3.5 LFEC output variability at AM and PM sampling over five days

There was a 5 to 16-fold variation in LFECs over the 5-day sampling period with the greatest variation in cows #836, #1100, #2300, #3491 and #4316 (Table 4; Figure 7). Afternoon LFEC estimates were, on average, 0.81 (95% CI 0.62 to 1.01) eggs less than AM LFEC estimates. In general, there was more variation in the PM samples (Figure 7).

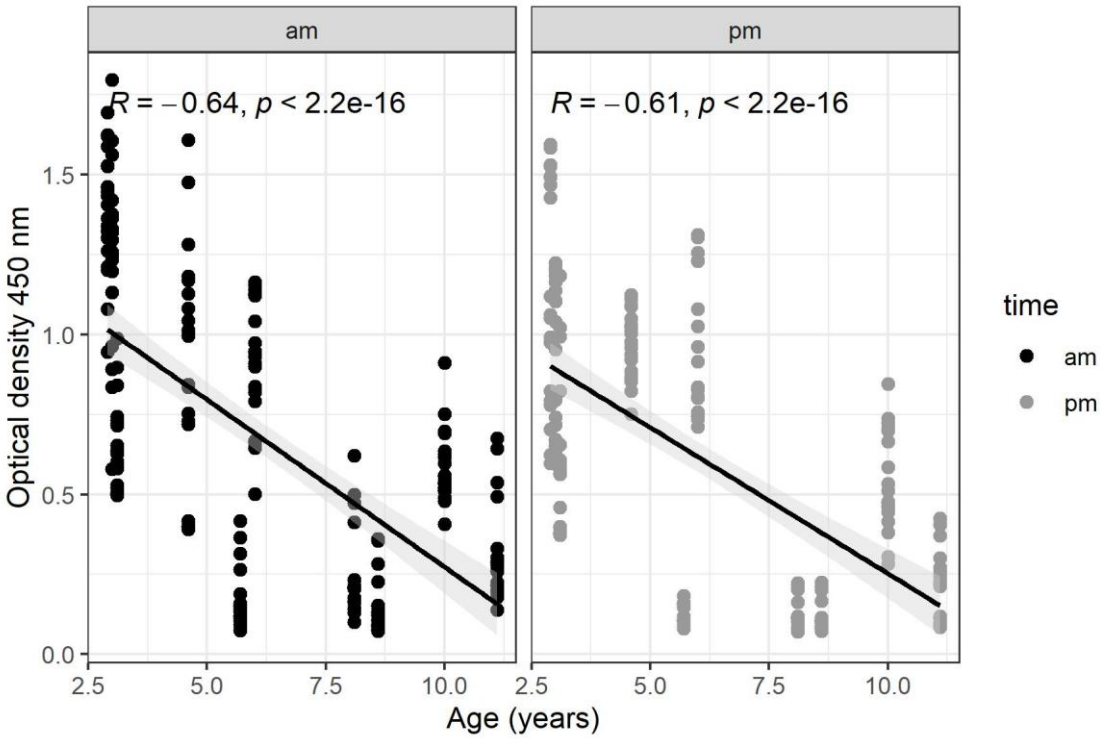

Figure 4.1. Scatterplot showing the cELISA OD as a function of cow age for AM and PM sampling events. Superimposed is a line of best fit to the data.

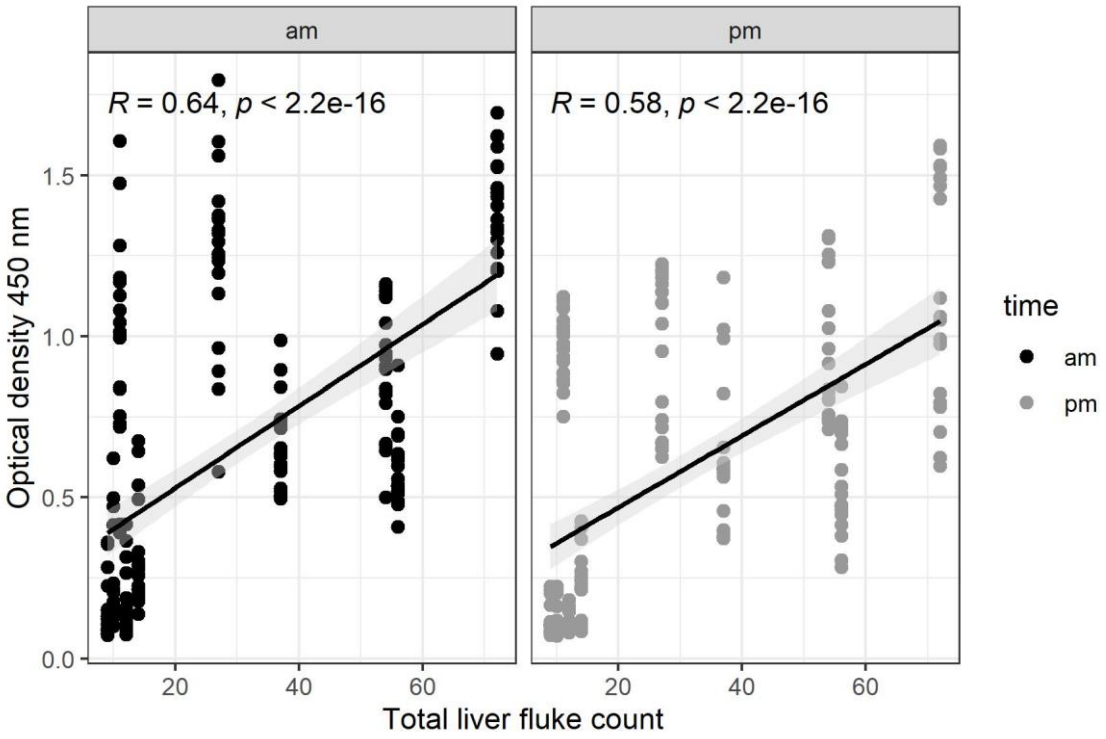

Figure 4.2. Scatterplot showing the cELISA OD as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

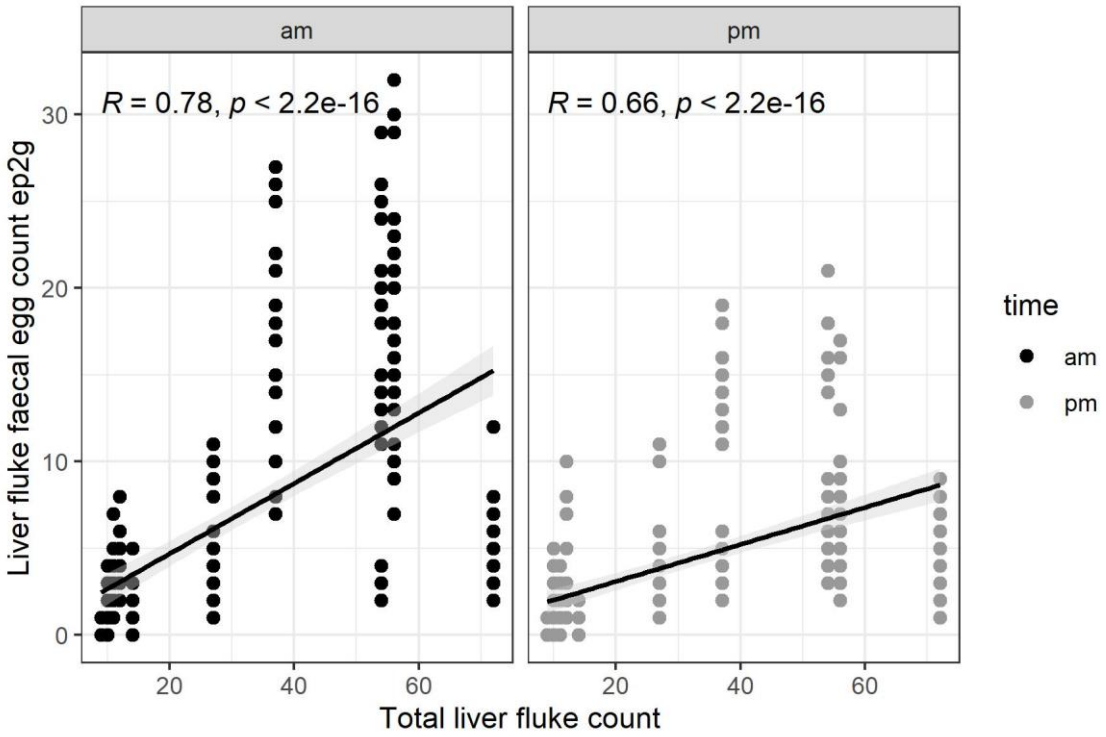

Figure 4.3. Scatterplot showing LFEC (ep2g) as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

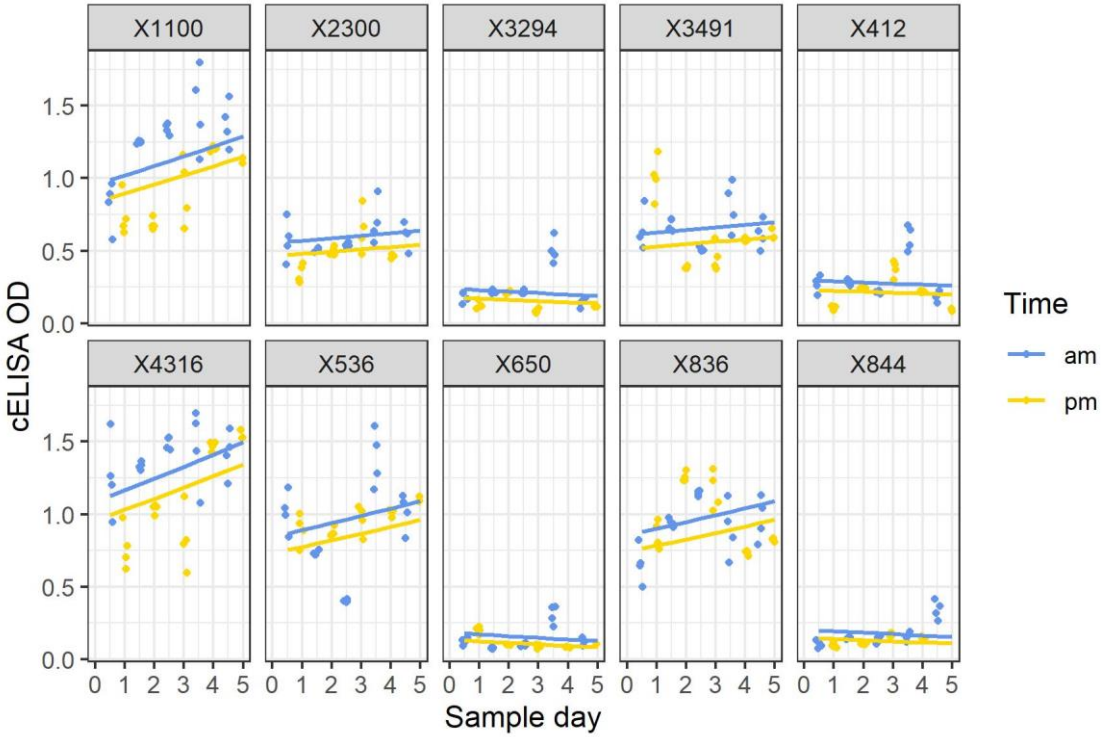

Figure 4.4. Scatterplots showing cELISA OD as a function of sampling event over 5 consecutive days for each of the ten cows included in this study. Superimposed on each plot are the cELISA OD values predicted using the mixed-effects linear regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on day 4.

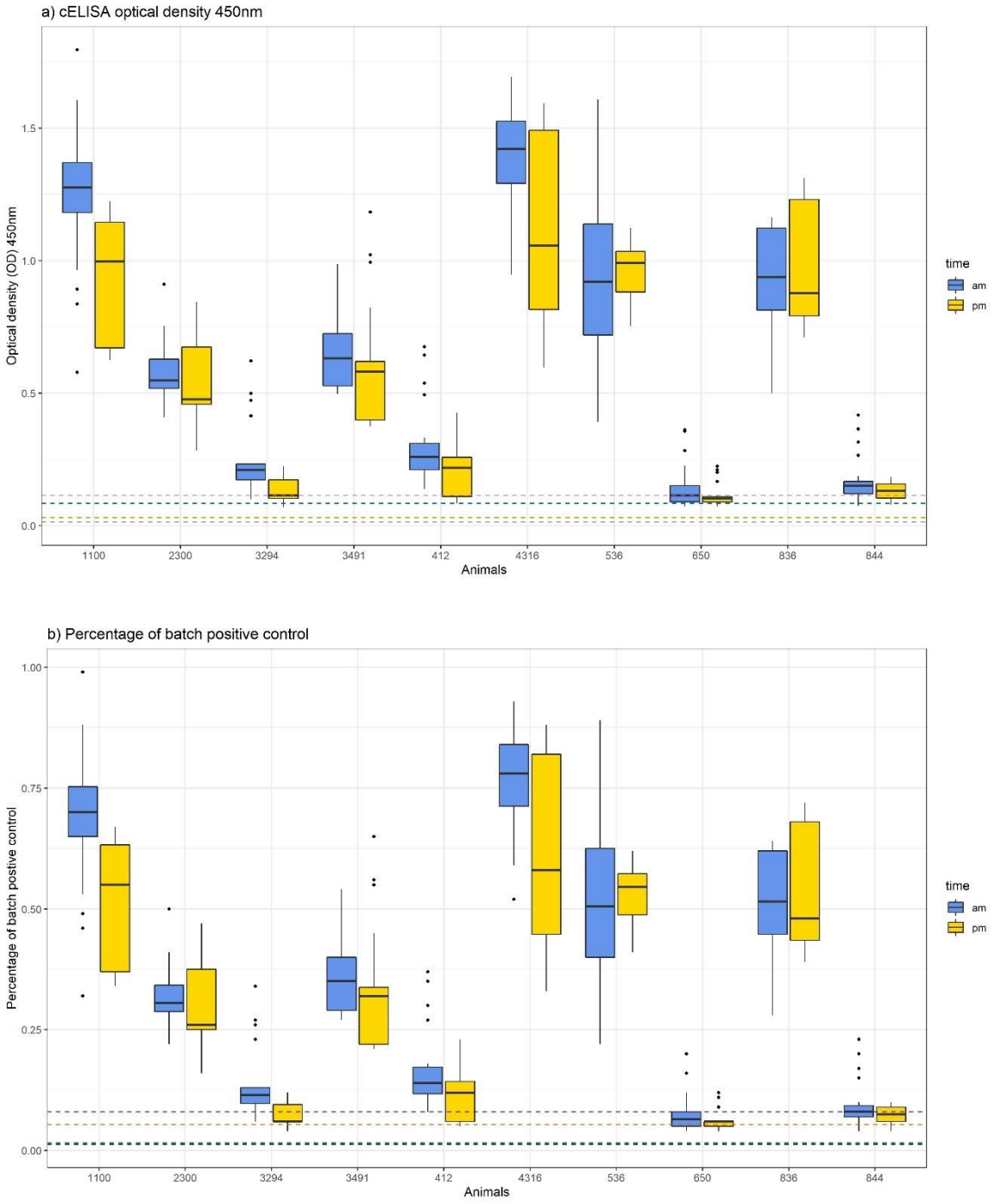

Figure 4.5. Box and whisker plots showing the distribution of: (a) cELISA OD estimates at AM and PM sampling events for each of the ten cows included in this study; and (b) the percentage of the batch positive control for the same samplings. In plot (a) the four horizontal dashed lines represent the cut-off ODs described in: (i) Brockwell et al. (2013), 0.014, grey; (ii) Charlier et al. (2008), 0.030, gold; (iii) Martínez-Sernández et al. (2016), 0.084, blue; and (iv) Mezo et al. (2004), 0.114, pink. In plot (b) the four horizontal dashed lines represent the cut-off % positive values described in: (i) Brockwell et al. (2014), 1.3%, black; (ii) Elliott et al. (2015,) 1.6%, green; (iii) Palmer et al. (2014), 5.36%, red; (iv) the recommended kit cut-off of 8%, purple.

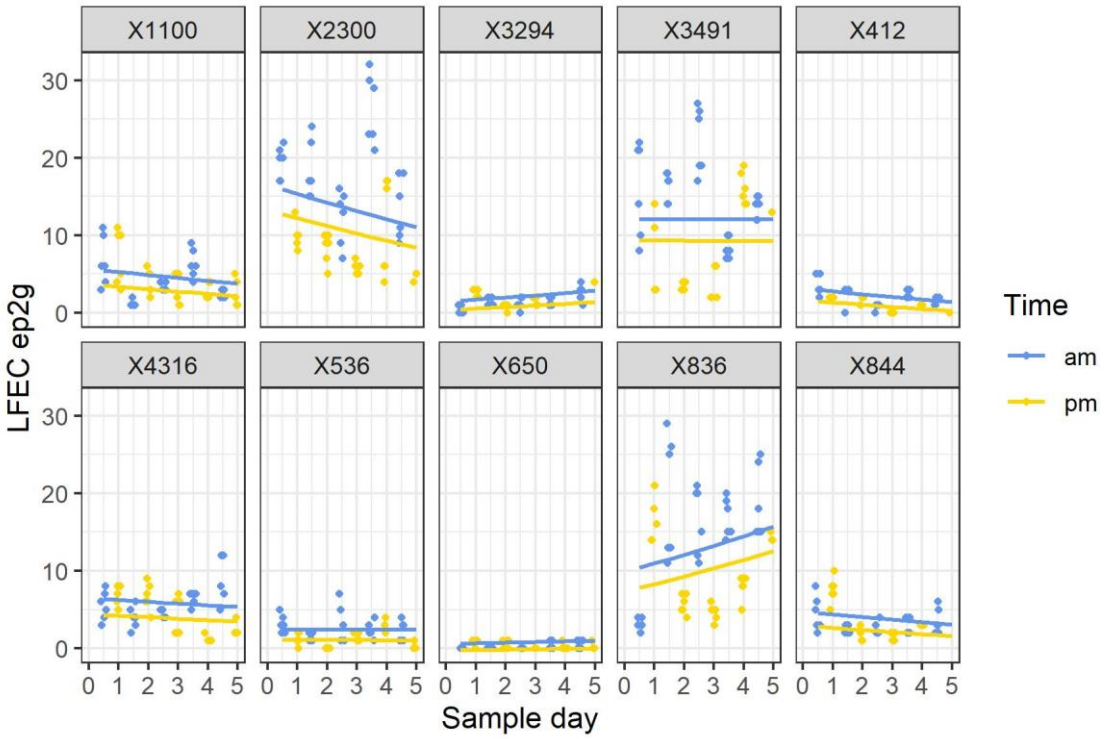

Figure 4.6. Scatterplots showing LFEC (ep2g) as a function of sampling event for each of the ten cows included in this study. Superimposed on each plot are the LFEC estimates predicted using the mixed-effects linear regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on day 4.

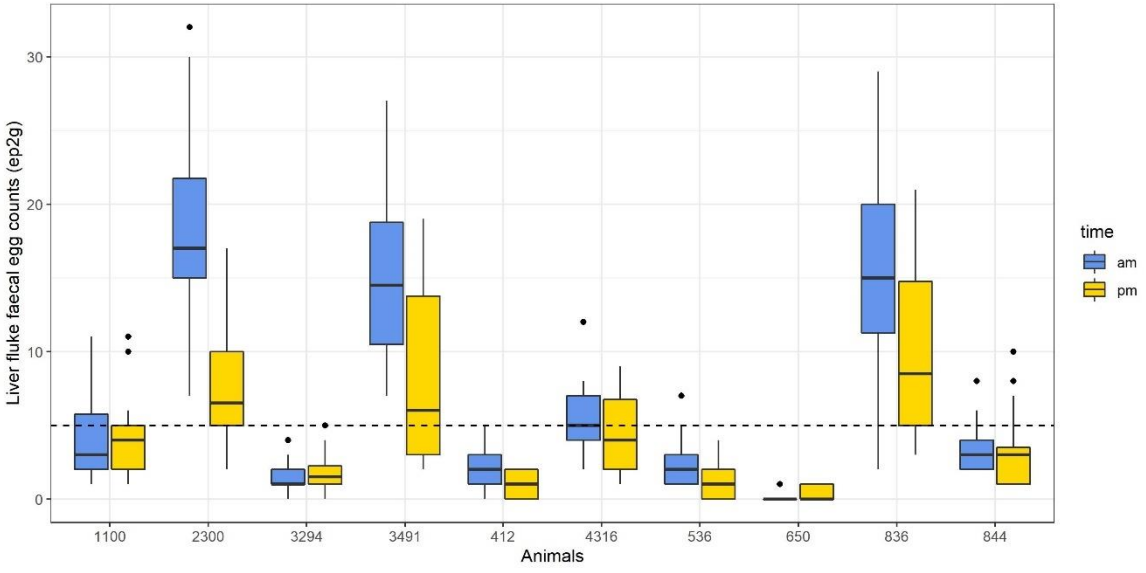

Figure 4.7. Box and whisker plots showing the distribution of LFEC (ep2g) at AM and PM sampling events for each of the ten cows included in this study. The horizontal dashed line shows the 5 ep2g cut-off for production loss in cattle described by Malone and Craig (1990) and Vercruysse and Claerebout (2001).

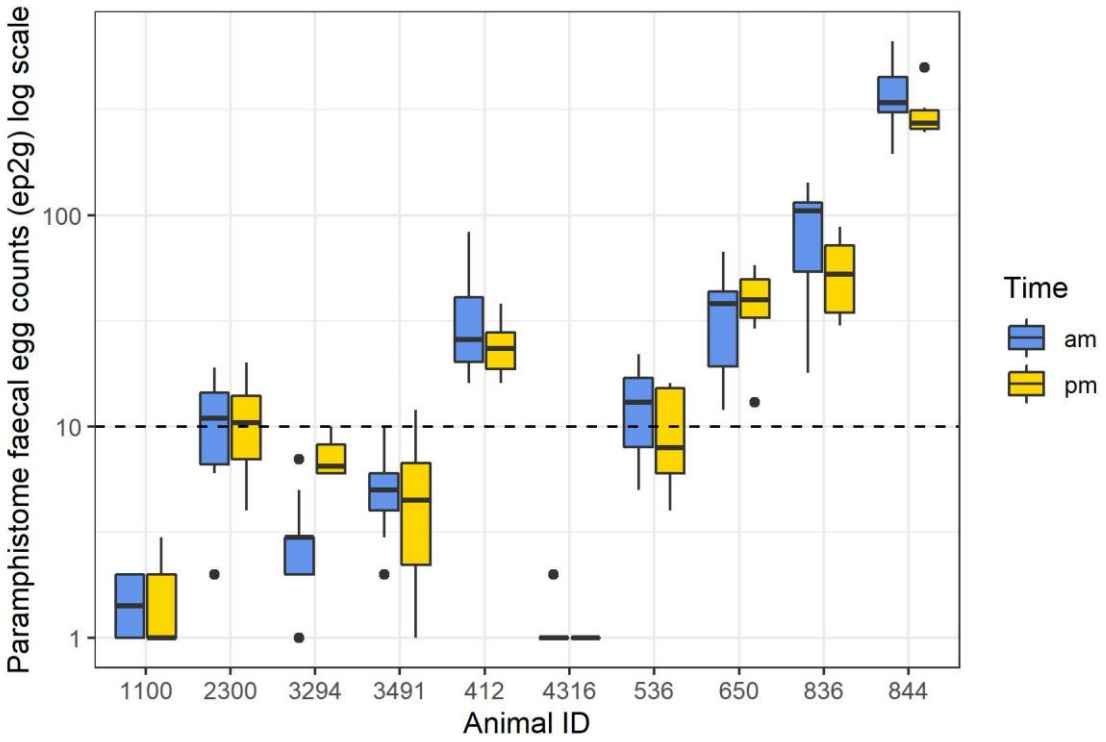

Figure 4.8. Box and whisker plots showing the distribution of PFEC (ep2g) on the log scale for each of the ten cows included in this study. The horizontal dashed line shows the 10 ep2g cut-off indicating that there are approximately 143 to 145 adult paramphistomes in the rumen (Willmott and Pester, 1952).

4.6 Discussion

3.6 PFEC

In total, 190 PFECs were performed, and all ten cows were found to be positive for paramphistomes (Figure 8). The PFEC ranged from 0 to 664 ep2g with the highest counts in cow #836. Cow #4316 was positive on only one occasion of the 20 samples counted. Cows #1100, #3294, and #3491 each recorded false negatives. Adult paramphistomes were collected from cows #844 and #836. DNA from four paramphistomes were sequenced by the Moredun Research Institute, and each was found to be *Calicophoron calicophorum*. At every collection point, cows #412, #650, #836, and #844 exceeded the 10 ep2g cut-off described in Willmott and Pester (1952), which represents a paramphistome burden of >140 (Figure 8).

4. Discussion

In this study, we investigated the sensitivity of two fluke diagnostic tests (cELISA and LFEC) in naturally infected dairy cows using faecal samples collected at the morning (AM) and afternoon (PM) milking in order to determine the daily variation in coproantigen and egg shedding over five consecutive days, to assess the correlation between coproantigen and LFEC levels with *F. hepatica* burden and determine the impact of this variation on test sensitivity.

4.1 Animal age vs TFC, cELISA OD and LFEC

The TFC is commonly used to determine the true liver fluke burden in animals and validate diagnostic tests with a sensitivity of 99% and specificity of 98% (Mazeri et al., 2016). We used the TFC in this study since it is the most reliable method for assessing the sensitivity and on-farm application of the cELISA and the FlukeFinder® kit under Australian farm management practices. Ten naturally infected cows were euthanised, and the fluke counts ranged from 9 to 72 flukes which allowed for an assessment of the tests in cows showing low to moderate infection levels. No correlation was observed between animal age and TFC (R = -0.32) and

12

258 animal age and LFEC (R = -0.23) (data not shown). This is consistent with Clery et al (1996) work, who found
259 no evidence of resistance to infection in cattle with increasing age, whilst Gonzalez-Lanza et al. (1989) found
260 that the rate of *F. hepatica* egg release and parasite prevalence generally increased with animal age. The
261 reason for the differences is likely due to the study design, Gonzalez-Lanza et al. (1989) tested 1301 animals
262 compared to the ten cows in this study. A moderate negative correlation (R = -0.63) was observed between
263 animal age and cELISA OD (Figure 1). This is consistent with findings reported by Takeuchi-Storm et al. (2018),
264 who found on all but one farm that the cELISA OD, serum ELISA OD and LFEC peaked in cows at 2 to 4 years of
265 age and declined as cows aged.

266 *4.2 cELISA OD daily variation and sensitivity*

267 Fluctuations in the cELISA OD estimates were observed in *F. hepatica* infections in sheep by Mezo et al. (2004)
268 and Valero et al. (2009) and in cattle by Brockwell et al. (2013). As a result, the commercial cELISA kit was
269 modified by increasing the analytical sensitivity from 0.6 ng/mL to 0.15 ng/mL (Mezo et al., 2004; Martínez-
270 Sernández et al., 2016). However, changing the detection limit of the cELISA kit increased the OD variability in
271 cattle from a factor of 2 to 6 (Brockwell et al., 2013) to 6 to 12 (Martínez-Sernández et al., 2016). We observed
272 a 2.6 to 8.9 fold variation in cELISA values in the ten cows in this study, with the greatest variation seen in the
273 PM samples (Table 4; Figure 2). Mazeri et al. (2016), using the modified cut-off described in Palmer et al.
274 (2014), found seasonal differences in the sensitivity of the cELISA: summer 80%, winter 85% and autumn 87%.
275 In Australia, Spring and Autumn are considered the highest risk periods for acquiring new *F. hepatica*
276 infections, whereas cool temperatures in winter and hot temperatures in summer reduce the release of
277 infective metacercariae. Given that the cows used in this study were naturally infected and sampling took
278 place in autumn, some of the variability we observed could be explained by the presence of immature *F.*

279 *hepatica* in the cows. In sheep, a marked increase in coproantigen release occurred as *F. hepatica* reach
280 patency (Valero et al., 2009).

281 We established the sensitivity of the cELISA assay to be 100% using several published cut-offs of 1.3%
282 (Brockwell et al., 2014), 1.6% (Elliott et al., 2015), 0.014 OD (Brockwell et al., 2013) and 0.030 OD (Charlier et
283 al., 2008). Our findings are consistent with four other studies that found the manufacturer's cut-off for the
284 cELISA (8% of the positive control) is too high, resulting in a high incidence of false negatives in low burden
285 cattle (< 10 flukes) (Charlier et al., 2008; Novobilský et al., 2012; Brockwell et al., 2013; Palmer et al., 2014).
286 False positives have also been observed in sheep after treatment with a flukicide (George et al., 2017). The
287 impact of applying all published cut-offs on the sensitivity of cELISA using our dataset can be seen in Table 3,
288 where sensitivity varies from 75-100%. The specificity of the cELISA was not determined in this study as it has
289 been found to be >99% (Mezo et al., 2004; Kajugu et al., 2015). Moreover, it has been established by Brockwell
290 et al. (2013), Kajugu et al. (2015) and Mazeri et al. (2016) that the cELISA does not cross-react with
291 paramphistomes which were present in the ten cows tested (Figure 8).

292 *4.3 cELISA OD daily variation and correlation with TFC*

293 In this study, we assessed the variation in cELISA OD within a day and over five consecutive days. The variation
294 was found to be higher at the PM sampling time than AM sampling, but the cause of the OD variation is not
295 clear. However, it is likely to be a result of a combination of biological processes and cELISA plate variability,
296 given the variability in OD values observed with the positive controls between the plates obtained from a
297 single commercial batch (Table 2). Brockwell et al. (2013) suggested that some of the biological causes were
298 the intermittent release of coproantigens by *F. hepatica,* pathological changes to the bile ducts gall bladder
299 that may impact egg release and liver well as variation in faecal consistency. Kajugu et al. (2015) also suggested
300 that expulsion of coproantigens from the fluke gut is likely to be episodic, impacting the level of coproantigen

301 in faeces over time. Factors affecting within plate variability are a pipetting, washing, and mixing of the faecal
302 aliquot, whereas the between plate variability was managed by the addition of positive controls and known
303 positive extracts to all plates and statistically by calculating the % of positive control OD based on the batch
304 value (Table 3). The observed correlation between the cELISA OD and TFC was R = 0.64 at the AM sampling
305 and R = 0.58 at the PM sampling. These correlations are similar to those reported by Charlier et al. (2008) in
306 cattle (R = 0.6) but lower than that reported by Brockwell et al. (2013)(R^2 = 0.8368). More recently, Martínez-
307 Sernández et al. (2016) observed the correlation to be R = 0.2998, which is less than our findings, but that
308 correlation was only calculated in cattle that had less than ten adult *F. hepatica.*

309 *4.4 cELISA detection limit*

310 Before modifying the cELISA (BIO X kit), the original kit could detect burdens in cattle with more than two
311 flukes and 2/7 cattle that had one fluke (Mezo et al., 2004). In this study, the new version of the cELISA
312 detected the lowest TFC of nine flukes (cow #650) at every sampling point. All samples collected for cows #
313 412 (14 fluke), # 536 (11 fluke), # 844 (12 fluke) and # 3294 (10 fluke) were also positive at every sampling
314 point. Given that the lowest reported cut-off for production loss in dairy cattle is ten flukes, the data indicates
315 that the cELISA kit can consistently identify individual cattle as fluke positive before production losses occur
316 (Charlier et al., 2008). The aim of the Martínez-Sernández et al. (2016) study was to reduce false negatives by
317 increasing the assay's sensitivity. Given that the old version of the kit was already capable of detecting 1-2
318 fluke, which is less than the production cut-off of ten *F. hepatica*, the reports of false negatives in the literature
319 were likely a result of the cut-off used, not the detection limit of the cELISA kit.

320 In the pursuit of trying to improve the sensitivity of the assay, we have lost sight of the value of the cELISA,
321 which is to detect low (≤10) and moderate to high (≥11) fluke burdens in animals as well as detecting *F.*
322 *hepatica* that survives flukicide treatment (Kelley et al., 2016). The most critical outcome for practically

managing *F. hepatica* in cattle is identifying whether the herd is incurring production losses, determining whether the *F. hepatica* present are drug resistant, and managing the herd to reduce the level of pasture contamination. The cELISA appears to be a robust tool to help achieve these outcomes.

4.5 LFEC sensitivity

The first published use of the FlukeFinder® sedimentation method was in Malone and Craig (1990); however, the kit's sensitivity has never been reported. We have determined that the sensitivity of the FlukeFinder® in cattle to be 88% (95% CI 85% to 90%) with 71 of 588 LFEC samples false negatives, all of which were observed in the cows with the lowest fluke counts. Our sample collection took place in autumn, which has been found to decrease the sensitivity of the LFEC due to the fact that cattle were infected with pre-patent *F. hepatica* that had not reached sexual maturity (Mazeri et al., 2016). The presence of immature *F. hepatica* could have contributed to elevating the coproantigen level without increasing the LFEC (Mezo et al., 2004). Due to time constraints, the size of *F. hepatica* recovered was not measured, but smaller fluke (<12 mm) consistent with the size of immature fluke were observed in some cows.

4.6 LFEC daily variation

The over-dispersion of *F. hepatica* in cattle and the considerable variation in egg shedding with fluke burden intensity increases the variability in *F. hepatica* egg numbers in faecal matter. The host immune response could also affect egg shedding, but limited research has investigated this effect. Vaccination of cattle and sheep with fluke antigens has been shown to reduce the egg counts, egg viability and egg maturation in some studies but the basis for the vaccine-induced effect is not clear (Wijffels et al., 1994; Dalton et al., 1996). Happich and Boray (1969) established that the faecal sedimentation technique is the most suitable for quantitative diagnosis in cattle, particularly in low burden infections (<10 flukes). Previous work by Brockwell et al. (2013) observed a 2 to 4 fold variation in egg output over a five day sampling period in cattle which is not consistent

345 with our findings of a 5 to 16 fold variation. A relatively low egg output was observed for cow #4316 (Figure 3;
346 Figure 6) even though it had the highest burden of *F. hepatica* (*n* = 72) (Table 1). Happich and Boray (1969)
347 found a negative association between the number of *F. hepatica* in the liver and egg shedding capacity
348 suggesting that a crowding effect could occur in cattle. The variation in egg output in cow #4316 may have
349 been due to crowding effects and mechanical barriers in the liver caused by scarring and fibrosis that were
350 observed when dissecting that liver (data not shown) which may have hampered egg release. The fibrosis was
351 only present in animal #4316, likely due to heavy infections with immature *F. hepatica.* The burrowing of
352 immature *F. hepatica* had caused haemorrhagic tracts and fibrosis to form as well as causing the liver to be
353 enlarged, pale and hard in animal #4316 (Boray, 1969).

354 *4.7 LFEC daily variation and TFC*

355 Three previous studies have observed daily fluke egg shedding trends in cattle. All studies concluded that the
356 highest egg output occurs between 12:00 pm to 12:00 am (Dorsman, 1956, 1960; Hagens and Over, 1966).
357 Based on these observations, Dorsman (1956) and Hagens and Over (1966) proposed that faecal sample
358 collection should take place in the afternoon, assuming that the highest egg output reflects the fluke burden
359 within the animal. Our findings show that sampling at AM has a greater correlation of LFEC with fluke burden
360 (Figure 3), where the observed correlation between LFEC and TFC was R = 0.78 (AM) and R = 0.66 (PM). This
361 finding is not consistent with correlations of R^2 = 0.84 reported by Brockwell et al. (2013) in cattle. The
362 differences in the correlation observed may be due to differences in the study designs. Brockwell et al. (2013)
363 used naive artificially infected cattle of the same age, whereas our cows were naturally infected and of various
364 ages. Our study's findings are more consistent with work in sheep by George et al. (2017), who observed a
365 correlation of R^2 = 0.571 between LFEC and TFC.

17

4.7 Conclusion

366 *4.8 Paramphistomes*

367 In this study, every cow was positive for paramphistomes. In Figure 8, four cows (#412, #650, #836 and #844)
368 exceeded the 10-13ep2g ep2g, indicating that there are approximately 143 to 145 adult paramphistomes in
369 the rumen (Willmott and Pester, 1952). The burden within the four highly infected cows was likely more than
370 >140 adult paramphistomes as the average PFEC was 28, 36.2, 68.3 and 350 ep2g. Work by Sargison et al.
371 (2016) in the United Kingdom established that the burdens of more than 1000 adult paramphistomes had no
372 impact on production in beef cattle. Little work has been undertaken in dairy production systems globally.
373 Paramphistomes were collected from cows # 836 and 844: the Moredun Research Institute sequenced
374 genomic DNA (ITS-2 region) from four paramphistomes, and each sample was found to be *C. calicophorum*
375 (Gordon et al., 2013). A high incidence of paramphistomes has been observed in two studies in Australia. Co-
376 infections with paramphistomes were common in Kelley et al. (2020) (data not shown), and Molloy et al.
377 (2005) determined that the prevalence of paramphistomes in cattle in South-Eastern Queensland was 46.2%.
378 In recent years Europe has seen a dramatic increase in paramphistome prevalence in cattle and now considers
379 it an emerging disease (Morley, 2018; Sargison et al., 2019). Paramphistomes have previously been considered
380 a relatively benign parasite in Australia, but given the high counts observed in this study and previous studies
381 indicating a high incidence, further work is needed in Australia to assess their impact on production.

382 4.9 Conclusion

383 In conclusion, the cELISA and the LFEC (FlukeFinder kit®) were found to be robust diagnostic tools in naturally
384 infected dairy cows. Variation in the daily cELISA and LFEC were observed within cows over the 5-day faecal
385 sampling period. The cELISA returned no false negatives. The cELISA and the LFEC showed a higher correlation
386 to TFC when samples were collected at the AM milking. False negatives were observed using the FlukeFinder
387 kit®, with 42/71 false negatives observed in cow #650, which had the lowest *F. hepatica* burden (*n* =9). Both

18

4.8 Acknowledgments

4.9 References

388 tests accurately detected cows with burdens >10 fluke, which is the production cut-off in cattle. In addition to
389 these findings, each of the study group cows was infected with *C. calicophorum,* with PFEC counts ranging
390 from 1 to 664 ep2g. Counts this high have not been reported previously in Australia and warrant further
391 investigation to determine the effect of high-intensity infections of *C. calicophorum* on productivity in dairy
392 cattle.

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399 **6. References**

400 Anderson, N., Luong, T., Vo, N., Bui, K., Smooker, P.M., Spithill, T.W., 1999. The sensitivity and specificity of two methods
401 for detecting *Fasciola* infections in cattle. Vet. Parasitol. 83, 15-24.
402 Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. lme4: Linear mixed-effects models using Eigen and S4. J.
403 Statistical Software 67, 1 - 48.
404 Bennema, S., Vercruysse, J., Claerebout, E., Schnieder, T., Strube, C., Ducheyne, E., Hendrickx, G., Charlier, J., 2009. The
405 use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and
406 *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). Vet. Parasitol. 165, 51-57.
407 Bloemhoff, Y., Forbes, A., Danaher, M., Good, B., Morgan, E., Mulcahy, G., Sekiya, M., Sayers, R., 2015. Determining the
408 prevalence and seasonality of *Fasciola hepatica* in pasture-based dairy herds in Ireland using a bulk tank milk
409 ELISA. Irish Vet. J. 68, 16-25.

410 Boray, J.C., 1969. Experimental fascioliasis in Australia. Advances Parasitol. 7, 95-210.
411 Box, G., Cox, D., 1964. An analysis of transformations (with discussion). J. Royal Statistical Society Series B 26, 211 -
412 252.
413 Brockwell, Y., Spithill, T., Anderson, G., Grillo, V., Sangster, N., 2013. Comparative kinetics of serological and
414 coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following
415 treatment with triclabendazole. Vet. Parasitol. 196, 417-426.
416 Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola*
417 *hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int. J. Parasitol.: Drugs
418 Drug Res. 4, 48-54.
419 Castro, E., Freyre, A., Hernandez, Z., 2000. Serological responses of cattle after treatment and during natural re-infection
420 with *Fasciola hepatica*, as measured with a dot-ELISA system. Vet. Parasitol. 90, 201-208.
421 Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative
422 evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Vet. Parasitol. 153,
423 44-51.
424 Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-*Fasciola hepatica*
425 antibody levels in bulk-tank milk samples and production parameters in dairy herds. Prev. Vet. Med. 78, 57-66.
426 Collett D (1999). Modelling Binary Data, Chapman & Hall/CRC, Boca Raton Florida, pp. 24.
427 Conceição, M.A.P., Durão, R.M., Costa, I.H., da Costa, J.M.C., 2002. Evaluation of a simple sedimentation method
428 (modified McMaster) for diagnosis of bovine fascioliosis. Vet. Parasitol. 105, 337-343.
429 Dalton, J.P., McGonigle, S., Rolph, T.P. and Andrews, S.J., 1996. Induction of protective immunity in cattle against
430 infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Inf. Immunity
431 64, 5066-5074.
432 Dorsman, W., 1956. Fluctuation within a day in the liver-fluke egg-count of the rectal contents of cattle. Vet. Rec. 68,
433 571-574.

434 Dorsman, W., 1960. The diagnosis of subclinical fascioliasis by means of faecal examination, and the control of liver
435 flukes (*Fasciola hepatica*). Bulletin de l'Office International des Epizooties 54, 502-508.
436 Elelu, N., Ambali, A., Coles, G.C., Eisler, M.C., 2016. Cross-sectional study of *Fasciola gigantica* and other trematode
437 infections of cattle in Edu Local Government Area, Kwara State, north-central Nigeria. Parasit. Vectors 9, 470.
438 Elliott, T., Kelley, J., Rawlin, G., Spithill, T., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against
439 *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Vet.
440 Parasitol. 209, 117-124.
441 Estuningsih, E., Spithill, T., Raadsma, H., Law, R., Adiwinata, G., Meeusen, E., Piedrafita., 2009. Development and
442 application of a fecal antigen diagnostic sandwich ELISA for estimating prevalence of *Fasciola gigantica* in cattle
443 in central Java, Indonesia. J. Parasitol. 95, 450-455.
444 George, S., Vanhoff, K., Baker, K., Lake, L., Rolfe, P., Seewald, W., Emery, D., 2017. Application of a coproantigen ELISA
445 as an indicator of efficacy against multiple life stages of *Fasciola hepatica* infections in sheep. Vet. Parasitol. 246,
446 60-69.
447 Gonzalez-Lanza, C., Manga-Gonzalez, Y., Del-Pozo-Carnero, P., Hidalgo-Argüello, R., 1989. Dynamics of elimination of
448 the eggs of *Fasciola hepatica* (Trematoda, Digenea) in the faeces of cattle in the Porma Basin, Spain. Vet.
449 Parasitol. 34, 35-43.
450 Gordon, D.K., Roberts, L.C.P., Lean, N., Zadoks, R.N., Sargison, N.D., Skuce, P.J., 2013. Identification of the rumen fluke,
451 Calicophoron daubneyi, in GB livestock: possible implications for liver fluke diagnosis. Veterinary Parasitology
452 195, 65-71.
453 Hagens, F., Over, H., 1966. Egg-Output and egg-count of *Fasciola hepática* in cattle. Tijdschrift voor Diergeneeskunde
454 91, 523-531.
455 Happich, F., Boray, J., 1969. Quantitative diagnosis of chronic fasciolosis. 1. Comparative studies on quantitative faecal
456 Examinations for chronic *Fasciola hepatica* infection in sheep. Aust. Vet. J. 45, 326-328.
457 Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica*
458 exposure in high-yielding dairy herds. Prev. Vet. Med. 121, 41-48.

459 Ibarra, F., Montenegro, N., Vera, Y., Boulard, C., Quiroz, H., Flores, J., Ochoa, P., 1998. Comparison of three ELISA tests
460 for seroepidemiology of bovine fascioliosis. Vet. Parasitol. 77, 229-236.
461 Kajugu, P.-E., Hanna, R., Edgar, H., McMahon, C., Cooper, M., Gordon, A., Barley, J., Malone, F., Brennan, G., Fairweather,
462 I., 2015. *Fasciola hepatica*: specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples
463 from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes
464 (paramphistomes), under field conditions. Vet. Parasitol. 212, 181-187.
465 Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole
466 resistance in *Fasciola hepatica*. Trends Parasitol. 32, 458-469.
467 Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of
468 the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria,
469 South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Vet.
470 Parasitol. 277, 109019.
471 Kuerpick, B., Conraths, F.J., Staubach, C., Froehlich, A., Schnieder, T., Strube, C., 2013. Seroprevalence and GIS-supported
472 risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. Parasitology 140, 1051-1060.
473 Malone, J., Craig, T., 1990. Cattle liver flukes: risk assessment and control. Compendium on Continuing Education for the
474 Practicing Veterinarian 12, 747-754.
475 Martínez-Sernández, V., Orbegozo-Medina, R.A., González-Warleta, M., Mezo, M., Ubeira, F.M., 2016. Rapid enhanced
476 MM3-COPRO ELISA for detection of *Fasciola* coproantigens. PLoS Neglected Tropical Diseases 10, e0004872.
477 Mazeri, S., Sargison, N., Kelly, R.F., Bronsvoort, B.M.D., Handel, I., 2016. Evaluation of the performance of five
478 diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard
479 approach. PloS one 11, e0161621.
480 McCann, C., Baylis, M., Williams, D., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy
481 herds in England and Wales. Vet. Rec. 166, 612-617.
482 Mezo, M., González-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola*
483 *hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). J. Parasitol. 90, 845-852.

484 Molloy, J., Anderson, G., Fletcher, T., Landmann, J., Knight, B., 2005. Evaluation of a commercially available enzyme-
485 linked immunosorbent assay for detecting antibodies to *Fasciola hepatica* and *Fasciola gigantica* in cattle, sheep
486 and buffaloes in Australia. Vet. Parasitol. 130, 207-212.
487 Morley, N.J., 2018. Paramphistomosis of ruminants: the role of free-living metacercariae. Trends Parasitol. 34, 97-98.
488 Novobilský, A., Averpil, H.B., Höglund, J., 2012. The field evaluation of albendazole and triclabendazole efficacy against
489 *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Vet. Parasitol. 190, 272-276.
490 Novobilský, A., Sollenberg, S., Höglund, J., 2015. Distribution of *Fasciola hepatica* in Swedish dairy cattle and associations
491 with pasture management factors. Geospatial Health 9, 293-300.
492 Palmer, D., Lyon, J., Palmer, M., Forshaw, D., 2014. Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica*
493 infection in sheep, cattle and horses. Aust. Vet. J. 92, 357-361.
494 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2020. nlme: Linear and nonlinear mixed effects models.
495 URL: http://CRAN.R-project.org/package=nlme.
496 Pritchard, G., Forbes, A., Williams, D., Salimi-Bejestani, M., Daniel, R., 2005. Emergence of fasciolosis in cattle in East
497 Anglia. Vet. Rec. 157, 578-582.
498 R Core Team, 2020. R: A language and environment for statistical computing. URL: http://www.R-project.org/.
499 Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the
500 true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic
501 test. Int. J. Parasitol. 36, 1153-1158.
502 Reichel, M., 2002. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke
503 (*Fasciola hepatica*) infection in sheep and cattle. Vet. Parasitol. 107, 65-72.
504 Salimi-Bejestani, M., Daniel, R., Felstead, S., Cripps, P., Mahmoody, H., Williams, D., 2005. Prevalence of *Fasciola*
505 *hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Vet. Rec.-English
506 Ed. 156, 729-731.

507 Sargison, N., Francis, E., Davison, C., Bronsvoort, B.M.d., Handel, I., Mazeri, S., 2016. Observations on the biology,
508 epidemiology and economic relevance of rumen flukes (Paramphistomidae) in cattle kept in a temperate
509 environment. Veterinary Parasitology 219, 7-16.
510 Sargison, N.D., Shahzad, K., Mazeri, S., Chaudhry, U., 2019. A high throughput deep amplicon sequencing method to
511 show the emergence and spread of Calicophoron daubneyi rumen fluke infection in United Kingdom cattle
512 herds. Veterinary Parasitology 268, 9-15.
513 Schweizer, G., Braun, U., Deplazes, P., Torgerson, P., 2005. Estimating the financial losses due to bovine fasciolosis in
514 Switzerland. Vet. Rec. 157, 188-193.
515 Selemetas, N., Phelan, P., O'Kiely, P., Waal, T., 2014. Weather and soil type affect incidence of fasciolosis in dairy cow
516 herds. Vet. Rec. 175, 371-371.
517 Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A.-S., Sengupta, M.E., Beesley, N.J.,
518 Hodgkinson, J., Williams, D., Thamsborg, S.M., 2018. Patterns of *Fasciola hepatica* infection in Danish dairy
519 cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasit. Vectors
520 11, 674.
521 Uberoi, E. 2020. UK Dairy Industry Statistics (http://www.parliament.uk/commons-library, House of Commons library).
522 Valero, M.A., Ubeira, F.M., Khoubbane, M., Artigas, P., Muiño, L., Mezo, M., Pérez-Crespo, I., Periago, M.V., Mas-Coma,
523 S., 2009. MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep
524 experimentally infected with *Fasciola hepatica* and *F. gigantica*. Vet. Parasitol. 159, 77-81.
525 Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold.
526 Vet. Parasitol. 98, 195-214.
527 Wijffels, G.L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J.,
528 Bowen, F., Friedel, T. and Spithill, T.W., 2014. Vaccination of sheep with purified cysteine proteases of *Fasciola*
529 *hepatica* decreases worm fecundity. Exp. Parasitol. 78, 132-148.
530 Willmott, S., Pester, F., 1952. Variations in faecal egg-counts in paramphistome infections as determined by a new
531 technique. J. Helminthol. 26, 147-156.

4.10 Tables

511

512 **Table 1.** Cows used in the study, including identification number, total fluke count and age.

Animal ID	412	536	650	836	844	1100	2300	3294	3491	4316
Age	11.1	4.6	8.6	6.0	5.7	3.0	10.0	8.1	3.1	2.9
TFC	14	11	9	54	12	27	56	10	37	72

513 TFC: total fluke count

514

515 **Table 2.** The controls added to the cELISA plates.

Control	Replicates	Range OD	Avg OD	Lower CI (95%)	Upper CI (95%)
Negative control (dilution buffer)	10	0.00-0.00	0.00	0.00	0.00
Kit positive control (Kit +ve)	10	1.34-1.83	1.56	1.45	1.67
High positive extract (High +ve)	10	1.49-2.04	1.80	1.67	1.92
Low positive extract (Low +ve)	10	1.22-1.67	1.41	1.32	1.50

516

517

518 **Table 3.** Variation in cELISA and LFEC values in individual cows sampled over 5 consecutive days.

	Cut-off	SE-AM	SE-PM	SE Total	CI 95%	Reference
Batch +ve OD	>1.3%	100%	100%	100%	100%, 100%	Brockwell et al., 2014
Batch +ve OD	>1.6%	100%	100%	100%	100%, 100%	Elliott et al., 2015
Batch +ve OD	>5.4%	100%	100%	100%	100%, 100%	Palmer et al., 2014
Batch +ve OD	>8.0%	96%	91%	93%	88%, 93%	Kit Cut-Off
High +ve Ext. OD	>1.3%	100%	100%	100%	100%, 100%	Brockwell et al., 2014
High +ve Ext. OD	>1.6%	100%	100%	100%	100%, 100%	Elliott et al., 2015
High +ve Ext. OD	>5.4%	95%	92%	93%	88%, 93%	Palmer et al., 2014
High +ve Ext. OD	>8.0%	85%	75%	80%	66%, 80%	Kit Cut-Off
Kit +ve OD	>1.3%	100%	100%	100%	100%, 100%	Brockwell et al., 2014
Kit +ve OD	>1.6%	100%	100%	100%	100%, 100%	Elliott et al., 2015
Kit +ve OD	>5.4%	98%	95%	97%	94%, 97%	Palmer et al., 2014
Kit +ve OD	>8.0%	89%	76%	83%	69%, 83%	Kit Cut-Off
OD	>0.014	100%	100%	100%	100%, 100%	Brockwell et al., 2013
OD	>0.030	100%	100%	100%	100%, 100%	Charlier et al., 2008
OD	>0.114	98%	97%	97%	86%, 97%	Mezo e al., 2004
OD	>0.084	98%	97%	97%	95%, 97%	Martinez-Sernandez et al., 2016

519 The various % cut offs were calculated using the OD values and converting the OD values to a percentage of

520 the Batch +ve control, the High +ve extract control or the Kit +ve control (Table 2).

27

Table 4. Variation in cELISA OD and LFEC values in individual cows sampled over 5 consecutive days.

Cow #	cELISA OD min	cELISA OD max	Fold change	LFEC min	LFEC max	Fold change
1100	0.58	1.80	3.1	1	11	11
2300	0.28	0.91	3.2	2	32	16
3294	0.07	0.62	8.9	0	5	5
3491	0.37	1.18	3.2	2	27	13.5
412	0.09	0.68	7.9	0	5	5
4316	0.60	1.69	2.8	1	12	12
536	0.39	1.61	4.1	0	7	7
650	0.07	0.36	5.0	0	1	1
836	0.50	1.31	2.6	2	29	14.5
844	0.07	0.42	5.6	1	10	10
Avg	**0.3027**	**1.058**	**3.5**	**0.9**	**13.9**	**9.5**

521

522

4.11 Figure descriptions

Figure captions

Figure 1. Scatterplot showing the cELISA OD as a function of cow age for AM and PM sampling events. Superimposed is a line of best fit to the data.

Figure 2. Scatterplot showing the cELISA OD as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

Figure 3. Scatterplot showing LFEC (ep2g) as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

Figure 4. Scatterplots showing cELISA OD as a function of sampling event over five consecutive days for each of the ten cows included in this study. Superimposed on each plot are the cELISA OD values predicted using the mixed-effects linear regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on day 4.

Figure 5. Box and whisker plots showing the distribution of (a) cELISA OD estimates at AM and PM sampling events for each of the ten cows included in this study, and (b) the percentage of the batch positive control for the same samplings. In plot (a) the four horizontal dashed lines represent the cut-off ODs described in: (i) Brockwell et al. (2013), 0.014, grey; (ii) Charlier et al. (2008), 0.030, gold; (iii) Martínez-Sernández et al. (2016), 0.084, blue; and (iv) Mezo et al. (2004), 0.114, pink. In plot (b) the four horizontal dashed lines represent the cut-off % positive values described in: (i) Brockwell et al. (2014), 1.3%, black; (ii) Elliott et al. (2015,) 1.6%, green; (iii) Palmer et al. (2014), 5.36%, red; (iv) the recommended kit-cut-off of 8%, purple.

Figure 6. Scatterplots showing LFEC (ep2g) as a function of sampling event for each of the ten cows included in this study. Superimposed on each plot, are the LFEC estimates predicted using the mixed-effects linear

564 regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on
565 day 4.

566 **Figure 7.** Box and whisker plots show the distribution of LFEC (ep2g) at AM and PM sampling events for each
567 of the ten cows included in this study. The horizontal dashed line shows the 5 ep2g cut-off for production loss
568 in cattle described by Malone and Craig (1990) and Vercruysse and Claerebout (2001).

569 **Figure 8.** Box and whisker plots show the distribution of PFEC (ep2g) on the log scale for each of the ten cows
570 included in this study. The horizontal dashed line shows the 10 ep2g cut-off, indicating approximately 143 to
571 145 adult paramphistomes in the rumen (Willmott and Pester, 1952).

572

Chapter 5 – *Fasciola hepatica* control practices on a sample of dairy farmers in Victoria, Australia

5.0 Preface

Results from my research in Chapter 3 and published studies described in Chapter 2 have determined a significant increase in TCBZ-resistant *F. hepatica* in Australia, particularly in the MID in Victoria. The discovery of drug resistance in the MID could significantly impact the dairy industry, as 25% of Australia's milk is produced in this region. In Chapter 4, I determined that the coproantigen ELISA (cELISA) and LFEC are robust tests for detecting cattle with burdens >10 flukes. This is important as a burden as low as ten flukes have been previously established in the literature to be the cut-off for production loss in dairy cattle (Charlier et.al., 2008). These diagnostic tools can be used to inform and assess the effectiveness of the IPM strategies discussed in Chapter 2. A crucial step in better managing *F. hepatica* in endemic regions, on dairy farms with a high prevalence, and those that have TCBZ resistance like those identified in Chapter 3 is understanding how farmers are currently controlling *F. hepatica.* In Victoria, very little is known about how dairy farmers control *F. hepatica* on their farms which prevents the development of comprehensive IPM plans.

The aim of Chapter 5 was to establish how Victorian dairy farmers are currently controlling *F. hepatica* on their farms to enable the development of IPM plans for individual farms and endemic regions.

To better help dairy farmers control liver fluke infection, I set out to determine the current liver fluke control practices used by dairy farmers across irrigated regions in Victoria. A survey was used to assessed how *F. hepatica* is currently controlled, how diagnostic tests are used, how flukicides are used and whether farmers used IPM. All dairy farmers who participated in the studies in Chapter 3 received a copy of the survey, and the survey was also made available online to other interested parties. Here I report the outcomes of this survey.

To access a higher quality copy of the Chapter, use the DOI link below.

5.1 Published article and contribution

Publication

This manuscript has been drafted for Frontiers in Veterinary Science and it was submitted on the 18 of February 2021 (Manuscript ID: 669117), the manuscript was accepted on the 28 April 2021 and a copy has been included in Chapter 7, section 7.2.

Kelley, J.M., Rawlin, G., Beddoe, T., Stevenson, M., and Spithill, T.W. (2021). *Fasciola hepatica* Control Practices on a Sample of Dairy Farms in Victoria, Australia. *Frontiers in veterinary science* 8**,** 540.https://doi.org/10.3389/fvets.2021.669117

Contribution

This paper was drafted and submitted as an original research article. J. Kelley designed the survey and questions with assistance from T. Elliott and M. Stevenson. J. Kelley distributed and promoted the survey. J. Kelley managed and analysed all the data generated from the survey. J. Kelley analysed the results with assistance from M. Stevenson and T. Spithill. J. Kelley wrote the paper with editing from all other authors.

J. Kelley contributed to approximately 90% of the production of this research article. She made a significant input in concept development, experimental design, analysis, and the writing of manuscript submission to Frontiers in Veterinary Science, under the supervision of Professor Terry W Spithill.

Fasciola hepatica control practices on a sample of dairy farms in Victoria, Australia

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4197 words

5.2 Abstract

5.3 Introduction

Abstract

In Australia, little is known about strategies used by farmers to control *Fasciola hepatica* (*F. hepatica*) infection in dairy cattle. Triclabendazole-resistant *F. hepatica* have recently been found on several dairy and beef properties in Australia. It is difficult to draw conclusions about how widespread resistance is in Australian dairy cattle because we have little information about flukicide usage, drug resistance testing and alternative flukicide usage on-farm. This study's objective was to determine how dairy farmers are currently controlling *F. hepatica* and identify knowledge gaps where *F. hepatica* control strategies need to be communicated to farmers to improve management. The survey was distributed online or by hardcopy, and 36 dairy farmers completed the survey. There were 34 questions that included closed, open-ended, multi-check box, demographic and text questions. Descriptive statistics were used to quantify each response. The survey results showed high use of clorsulon, limited rotation of flukicides and limited use of diagnostic tests to inform treatment options and timing. There was poor adherence to best management practice in determining the dose of flukicides administered to cattle, with farmers often relying on estimating body weights or average body weights, suggesting that underdosing of animals is likely to be prevalent. Most respondents in this study did not isolate, and quarantine treat newly returned or purchased animals before joining them with the main herd. The research identified four knowledge gaps where communication needs to be enhanced to improve control of *F. hepatica*: diagnostic testing to inform flukicide use, rotation of flukicide actives, flukicide administration and increased testing of replacement animals.

1. Introduction

Fasciola hepatica (*F. hepatica*) has been a problem in Australia since colonisation (Penny, 1869). Early outbreaks of fasciolosis had high mortality rates, and animals within irrigation regions were at higher risk (Watt, 1979; McCausland et al., 1980). In dairy cattle, *F. hepatica* infection reduces weight gain, milk production and conception rates (reviewed in Schweizer et al. (2005)). Naïve young cattle (calves and heifers) are more vulnerable to fasciolosis than adult stock, as they have no previous exposure to *F. hepatica* and therefore have no acquired immunity. Oakley et al. (1979) found that *F. hepatica* infection in heifers limited growth rate, impaired feed conversion, delayed puberty, lowered conception rates and reduced calf weight. The observed effects were more pronounced in animals that had a lower plane of nutrition.

In Victoria, replacement animals (<12 months) are isolated from adult stock in order to comply with the bovine Johne's disease program (Dairy Australia, 2015). The program prevents contact between adult stock and replacements, leading to replacements being consecutively reared on the same paddocks. These

53 paddocks tend to be more marginal and have a lower quality pasture base than grazed land provided to
54 the milking herd. In Australia, dairy cattle predominately graze outside year-round. The key driver of the
55 profit in pasture-based dairy farming in Australia is to increase milk produced per grazed hectare by growing
56 more pasture of a higher quality and increasing consumption (Moran et al., 2000). Watson and Watson
57 (2015) found that the stocking rate of dairy farms across Australia has increased over the last 15 years and
58 has reached more than two cows per hectare in some regions.

59 These intensive grazing strategies used on dairy farms in Australia increase pasture consumption per
60 hectare but also increase pasture contamination with faecal matter (Lean et al., 2008). It is a growing
61 concern that the dairy industry's intensification is increasing the development of parasite drug resistance
62 and subclinical production losses (Lean et al., 2008). Over the last 15 years, the average stocking density on
63 dairy farms has increased from 1.51 to 1.72/ha (Watson and Watson, 2015). However, the stocking rate in
64 high intensity irrigated pasture regions of Victoria is above the national average at 1.84/ha in the Loddon
65 Valley, Torrumbarry, Central Goulburn and Murray Valley and 2.34/ha in the Macalister Irrigation District
66 (MID). Triclabendazole (TCBZ) resistance has been confirmed on several dairy farms in these irrigated
67 regions in Victoria (Brockwell et al., 2014; Elliott et al., 2015; Kelley et al., 2020). Until the early 1980's fluke
68 control relied on fencing off the intermediate host habitat, draining wet areas and using flukicides of low
69 efficacy. After the release of TCBZ, extensive work was done to communicate *F. hepatica* control strategies
70 to farmers. Hort (1998) found that 51% of sheep farmers adhered to these best practice guidelines
71 published by the Departments of Agriculture in New South Wales and Victoria as described in Boray et al.
72 (1999). The program recommended treating in Autumn (April/May) and Spring (August/September) every
73 year, with an additional Summer (January) treatment for young animals and adults if they were located in
74 high-risk regions. Since 1998, there has been no tracking of the program's adherence or effectiveness. There
75 has also been no monitoring of how other flukicides have been used, how diagnostic tools have been
76 incorporated into *F. hepatica* control strategies by dairy farmers in Australia and whether there has been
77 an increase in the uptake of integrated parasite management strategies (IPM).

78 In Europe, a small number of parasite management surveys identified several knowledge gaps where *F.*
79 *hepatica* control could be optimised on dairy farms (Bloemhoff et al., 2014; Selemetas et al., 2015; Easton
80 et al., 2018). Bloemhoff et al. (2014) found that 3% of dairy farmers treating for *F. hepatica* used a product
81 unsuitable for purpose, and grazing management options were not effectively utilised on-farm. Selemetas
82 et al. (2015) found that pasture and grazing management options had to be carefully communicated to
83 avoid dairy farmers assuming they had a low risk of *F. hepatica* because they have good drainage. In
84 addition, Easton et al. (2018) found there was limited use of diagnostics to inform on-farm decision making,

3

5.4 Material and Methods

and anthelmintic purchasing behaviour was driven by factors relating to convenience. The only *F. hepatica* survey conducted in Australia was in sheep flocks and beef herds; however, only the sheep data were published (Hort, 1998). Hort (1998) identified two knowledge gaps in how Australian sheep farmers were managing *F. hepatica*. Firstly, a high proportion of farmers were unaware that their flock was infected with *F. hepatica* because of a lack of routine diagnostic testing. The second gap was that 10% of sheep farmers used products that had no efficacy against *F. hepatica*.

The complexity of the *F. hepatica* life cycle increases the difficulty in communicating how to use flukicides, diagnostic tools and management practices to control *F. hepatica* on-farm. It is a major hurdle in working with farmers as they need to control both the parasitic stage in cattle and snails as well as the free-living stage in waterways and on pasture, which is only possible if knowledge gaps are identified and addressed. The aim of this study was to determine the *F. hepatica* control strategies used in Victorian irrigated dairy regions. We investigated how dairy farmers control *F. hepatica*, looked for knowledge gaps in current *F. hepatica* control strategies, and identified what information needs to be communicated to farmers to improve *F. hepatica* management and reduce production losses in dairy cattle.

2. Methods

2.1 Ethical statement

All procedures and documentation used in this study were approved by the La Trobe University Science, Health and Engineering (SHE) College Human Ethics Sub-Committee (CHESC) under negligible risk project S17-068, which was in accordance with the ethical standards outlined by the National Statement on Ethical Conduct in Human Research (2007) and the Australian Code for the Responsible Conduct of Research (2007).

2.2. Survey distribution and questions

The survey was piloted in 2013 to 19 dairy farmers in the MID. After minor amendments, the survey was distributed to Victorian dairy farmers via hard copy and online (SurveyMonkey®) from 01 June 2017 to 30 December 2017 (Supplementary Table 1). The survey consisted of 34 questions split into five sections: section 1 location and research awareness; section 2 drainage and irrigation; section 3 stock details and diagnostics; section 4 flukicides; and section 5 drenching practices. The questionnaire was made up of 16 closed questions, eight multiple-choice, seven open-ended questions, two text questions and one demographic question. No individual identifying data was collected, and survey respondents were not required to complete all questions.

5.5 Results

116 **2.3 Survey respondents**

117 Those who responded to the survey were volunteers recruited both in-person and online. The survey was
118 advertised on the project website (www.flukecontrol.com), on dairy social media platforms and in-person
119 at dairy-specific events in Victoria. Respondents completed the survey during their own time. The survey
120 allowed respondents to skip questions and provide as much or as little information as they wanted to
121 provide. In total, 67 surveys were received, and 36 respondents were included in the analysis. The authors
122 note that recruitment took place during the 'Victorian dairy crisis', which saw large numbers of dairy
123 farmers leave the industry, sell-off stock, cut back on expenses and significantly lowered the confidence in
124 the industry (Economics Reference Committee, 2017; Dairy Australia, 2018; 2021).

125 **2.4 Analysis**

126 Online surveys were downloaded into a proprietary spreadsheet package (Microsoft Excel, Microsoft
127 Corporation, Redmond, USA), and hardcopy results were transcribed directly into the same spreadsheet.
128 Results for the closed and multiple-choice questions are presented as frequencies and percentages (%) of
129 the total number of survey respondents. Questions that received no responses have been included in the
130 analysis. Given the relatively small number of survey respondents, dependent variables could not be
131 grouped by independent variable categories such as irrigation region, calving-type and herd-size. Graphics
132 were produced using Prism (GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego,
133 California USA, www.graphpad.com)). Maps were developed using the Geographic Information System
134 Quantum GIS (QGIS Geographic Information System; QGIS Association. http://www.qgis.org) using data
135 obtained from the State of Victoria (2021) and State of Victoria (2018).

136 **3. Results**

137 Of the 67 survey responses, 31 submitted online were excluded because they were incomplete (i.e. no
138 answers were provided to any of the survey questions). In total, 36 surveys from Victorian dairy farmers
139 were analysed (Table 1). A response rate could not be determined as the survey was distributed online via
140 email, social media and e-newsletters, as well as hardcopies being handed out at industry events.

141 **3.1 Descriptive statistics of respondents and their dairy business**

142 Seventy-two per cent of the survey respondents were male, with the majority aged between 45 and 54
143 years (Table 1). The highest number of surveys were received from the central Goulburn Irrigation District
144 (n = 14), followed by the MID (n = 7). All other irrigation dairy regions were represented by at least one
145 respondent in this study (Figure 1). The average area of all dairy farms was 427 ha, milking an average of
146 457 cows and rearing an average of 138 heifers and 130 calves with a total stocking density of 1.7/ha (Table

5

147 2). Of the 36 farms, 75% were split calving, 22% seasonal calving and 3% (1 respondent) year-round calving
148 (Table 3). Ninety-two per cent of farms had an irrigated pasture base, and only one farm in the study was
149 identified as organic (Table 3). The most frequently used method of irrigation was flood. Flood was used
150 solely on 56% of farms and in combination with other types of irrigation methods on 35% of farms (Table
151 3). The second most common method of irrigation was centre pivot, followed by laterals, sprays, lineal
152 move, and one farm solely used a travelling gun (3% one respondent) (Table 3).

153 **3.2 Dairy farm management**

154 All but two survey respondents identified that their farms had problems with waterlogging (Table 4). The
155 highest proportion (53%) reported that between 1 to 19% of their farmland had problems with
156 waterlogging, and 78% stated that stock had access to these areas (Table 4). In addition, 61% of
157 respondents reported that stock had access to irrigation channels on their farms. Eighty-six per cent of
158 respondents (31/36) regularly conducted irrigation channel maintenance, often using a combination of
159 methods to improve water use efficiency. The most common methods were sprayed for weeds, fixed
160 leaking delvers and excavated irrigated channels (Table 4). Two respondents included other maintenance
161 practices: one grazed channels with stock, and the other replaced channels with pipes (Table 4).

162 **3.3 *F. hepatica* diagnostic testing**

163 The bulk tank milk ELISA (BTM ELISA) (Salimi-Bejestani et al., 2005) was used to detect *F. hepatica* on 33%
164 of farms and liver fluke faecal egg counts (LFEC) on 28% of farms (Figure 2a). No other *F. hepatica* diagnostic
165 tests were used (Figure 2a). The highest frequency of testing occurred in adult milkers (Figure 2b). Forty-
166 two per cent of respondents tested once per year, 6% tested twice per year, and one respondent tested
167 three times per year (Figure 2b). For heifers and calves, only two farms tested these stock categories (Figure
168 2b). Nineteen per cent of respondents reported that they had tested for *F. hepatica* drug resistance, of
169 which two stated to have worked with the lead author (Figure 2a).

170 **3.4 Flukicide use**

171 In 2015 - 2016, 72% of respondents treated their stock for *F. hepatica* (Table 5). TCBZ and clorsulon (CLOR)
172 were widely used across stock categories. The highest frequency of treatments occurred in milkers,
173 followed by calves and heifers, which received the least *F. hepatica* treatments per year (Figure 3). CLOR
174 was most frequently used by respondents to treat *F. hepatica,* followed by TCBZ (Figure 3). Only one
175 respondent used oxyclozanide (OXY) to treat all livestock categories (Figure 3). TCBZ and CLOR were used
176 once or twice per year, but some opted for a higher treatment frequency in younger stock (Figure 3). The
177 highest treatment frequency for CLOR was three times per year, whereas the highest frequency for TCBZ

was six (Figure 3). For the preceding five years, flukicide use showed that CLOR was still the preferred product for treating *F. hepatica* in dairy cattle (Figure 4a). Several respondents used multiple flukicides to treat *F. hepatica* (Figure 4b), but 41% solely relied on one flukicide chemical class for the five-year period. Of the respondents who reported they had either used an external calf rearer or purchased stock, only 3% (1 respondent) and 8% of respondents quarantine treated newly returned or purchased animals (Figure 5).

3.5 Flukicide administration

Survey respondents used a variety of methods to determine when to treat their animals for *F. hepatica*. The most frequent approach was to treat at dry-off (31%) (Figure 6a). Other methods involved using various options: at dry-off and during lactation (11%) and at dry-off and based on animals' appearance (11%). Only two respondents used diagnostics to inform treatment administration; one respondent solely relied on diagnostics, whereas the other used it in combination with other methods (Figure 6a). Treatment based on the animal's appearance was often used to determine when to treat (Figure 6a).

When purchasing a flukicide, respondents relied more heavily on a single method of selection (59%), which was often based on advice from a veterinarian (25%), previous use (14%), or a recommendation from a reseller (14%), friend or neighbour (3% one respondent) or farm advisor (3% one respondent) (Figure 6b). Twenty-five per cent of respondents who used multiple methods to determine what flukicide to purchase often included price, previous use and veterinarian advice as key criteria (Figure 6b).

Seventy-two per cent of respondents expressed an interest in receiving more information about *F. hepatica* drenching practices (Table 5). Sixty-nine per cent of respondents used a single method to determine the flukicide dose to be administered to their cattle (Figure 6c). A quarter of survey respondents weighed the heaviest to determine the dose for the mob, 19% used the average group body weight, 17% estimated the individual weight of animals, 6% weighed each animal, and 3% (1 respondent) estimated the weight of the heaviest animal (Figure 6c). Nineteen per cent of respondents used a combination of methods to determine the dose; one weighed the heaviest and used a weigh tape (Figure 6c). One respondent who reported other methods in Figure 6a and c was an organic farmer who did not utilise flukicides. Instead, they incorporated copper 3-4 times a year into the animal's diet; the dose used was determined by a nutritionist (data not shown).

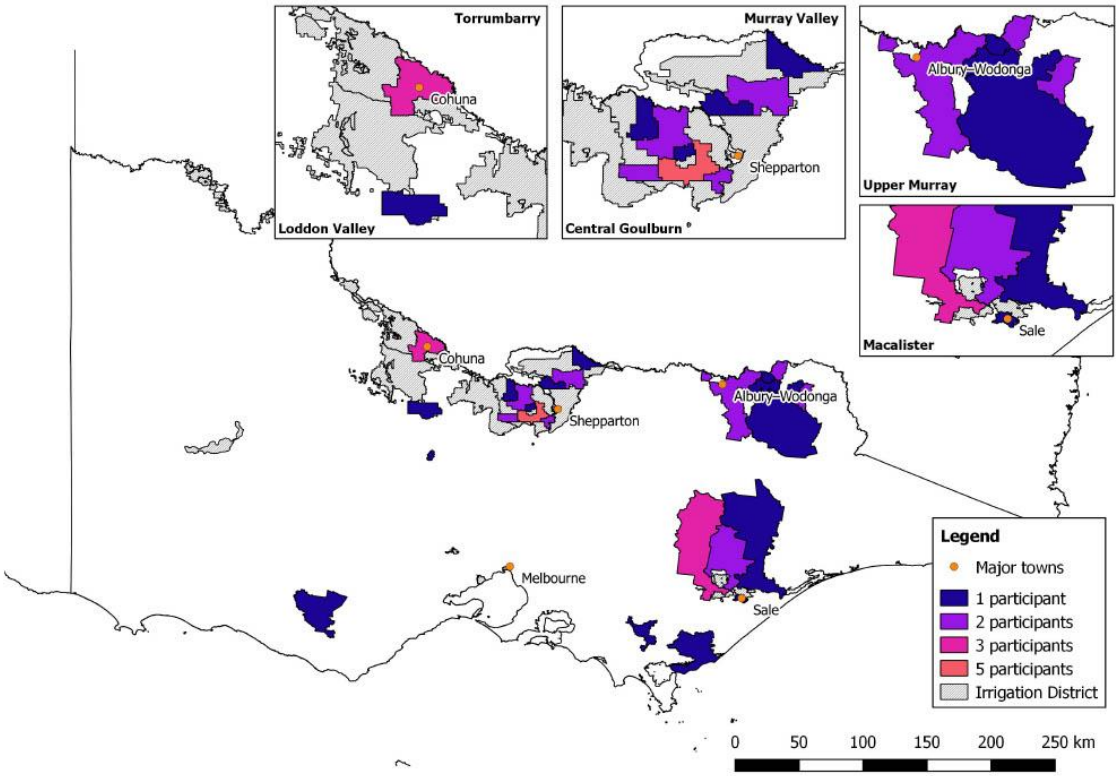

Figure 5.1. A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Map of Victoria showing the number of survey respondents by postcode area. Blue represents one respondent; purple: two respondents; pink: three respondents; orange: five respondents. Grey hashed lines represent irrigation regions within Victoria.

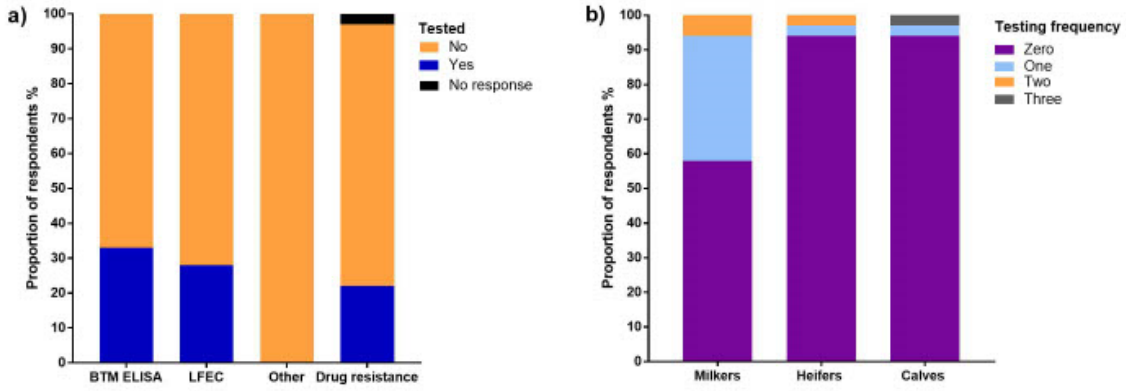

Figure 5.2. (A) The proportion of respondents using different types of *F. hepatica* diagnostic testing on-farm. (B) The proportion of respondents using a various frequency of diagnostic testing per year for each animal category.

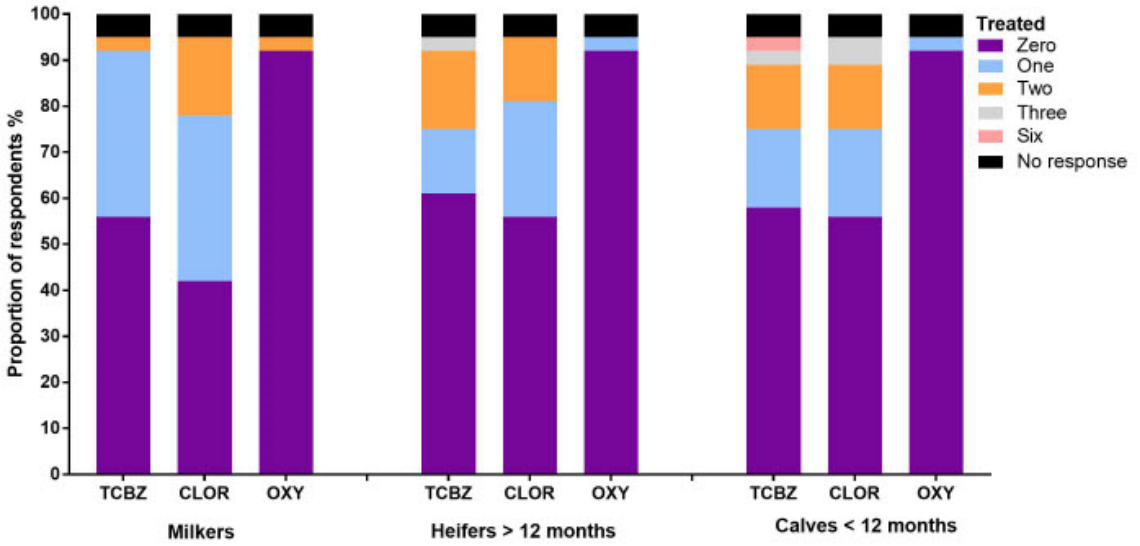

Figure 5.3. The proportion of respondents using various number of annual treatments with three different flukicides in each stock category (2015/2016 financial year).

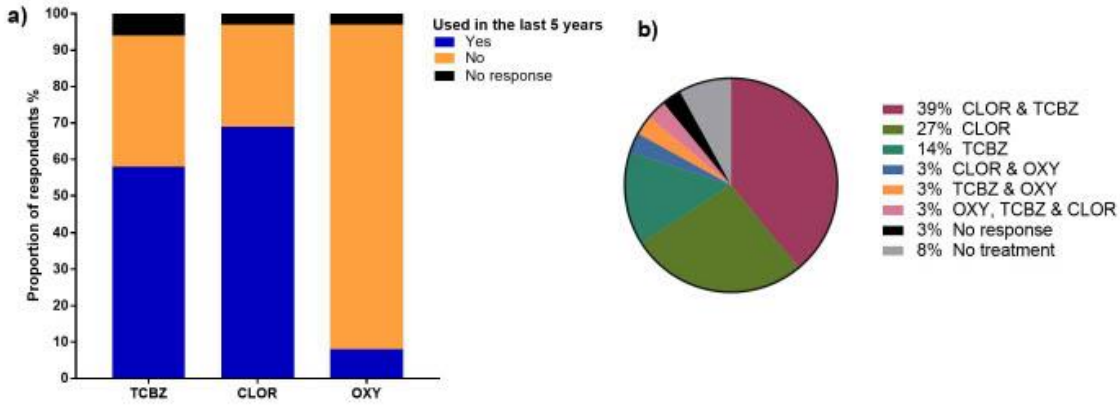

Figure 5.4. (A) The proportion of respondents using three different flukicides over the five years preceding the 2015/2016 financial year. (B) Proportion of respondents using single or multiple flukicides over the same time period.

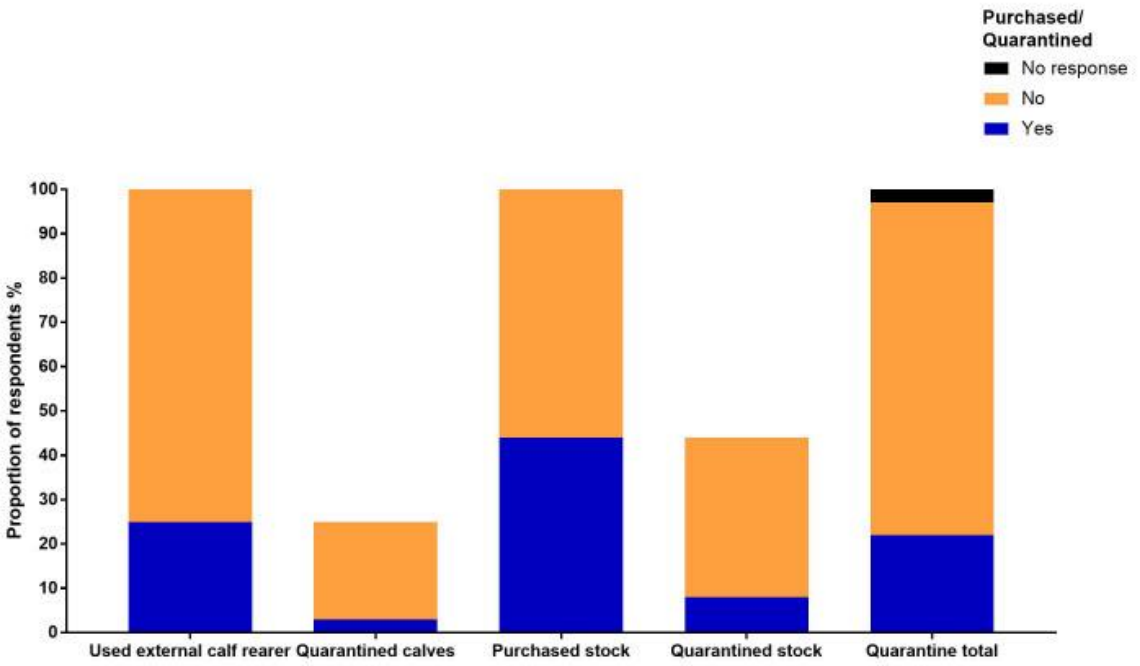

Figure 5.5. The proportion of respondents that applied quarantine treatments to calves reared or purchased externally.

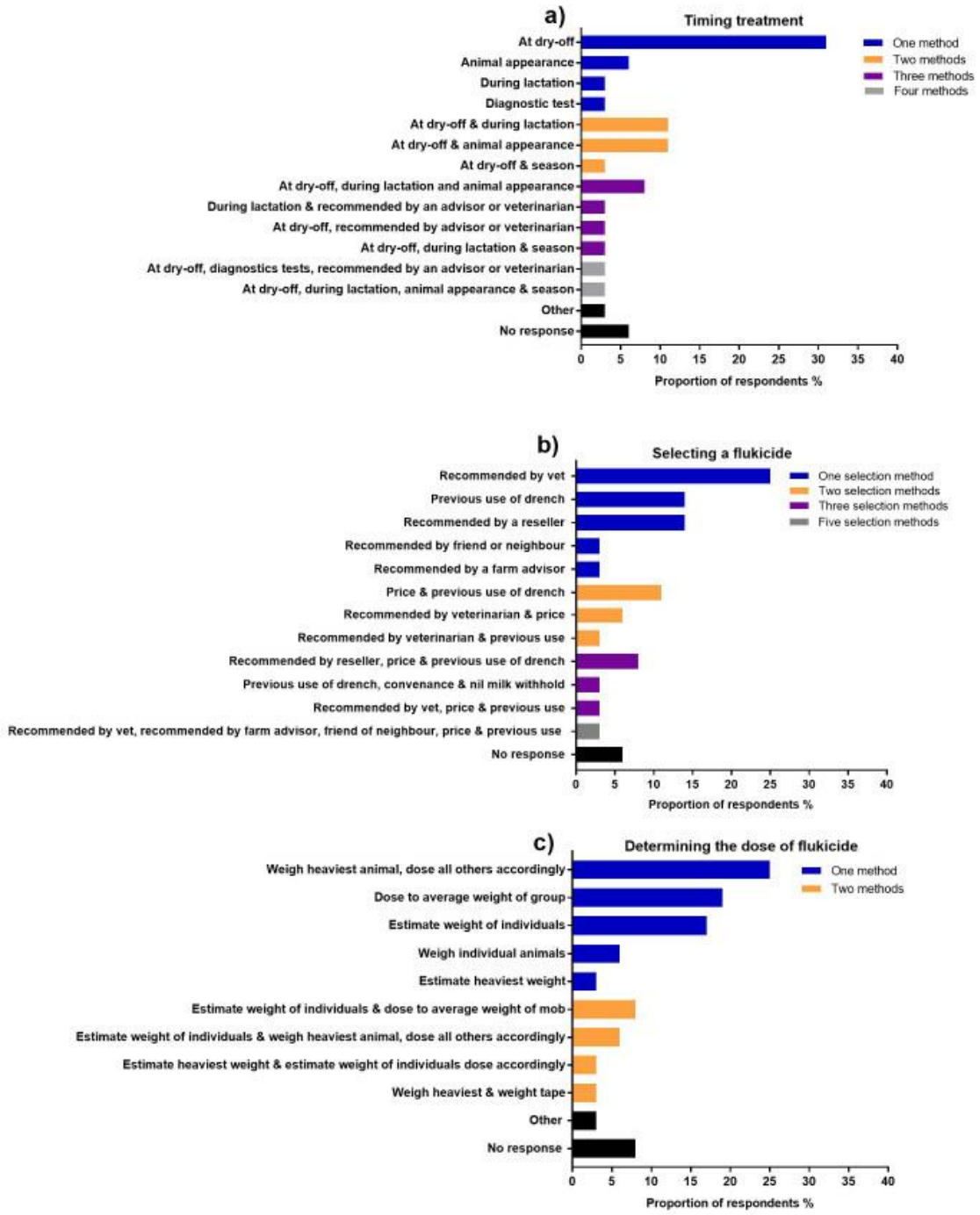

Figure 5.6. The proportion of respondents using various methods to treat animals with flukicides. (A) Method used to decide timing of treatment. (B) Method used to select a flukicide. (C) Method used to determine the dose of flukicide to administer to their animals.

5.6 Discussion

206 **Discussion**

207 **4.1 Survey response**

208 The aim of this study was to document current fluke management practices, fluke diagnostic test use and
209 flukicide use on irrigated dairy farms in Victoria. Recruitment of survey respondents during the 2017 dairy
210 crisis was difficult. The Commonwealth of Australia's Senate Economics Reference Committee (2017) noted
211 that during this time, the Australian dairy industry was facing an unprecedented crisis affecting the
212 livelihoods of 40% of the 6,000 dairy farmers in Australia. The response rate could not be determined as
213 the survey was distributed on multiple online platforms, and hard copies were handed out at industry
214 events. We note that three surveys were returned with a note stating the respondents had left the dairy
215 industry. The reduced participation numbers reflect the reduced confidence in the Australian dairy industry
216 future, which has been in decline since 2016 (75% to 45%), and the intention of 24% of dairy farmers to
217 leave the industry within five years (Schirmer et al., 2014; Dairy Australia, 2018). At the end of the
218 2015/2016 financial year, there were 4141 dairy farms in Victoria; it has since decreased to 3516 farms in
219 2018/2019 (Dairy Australia, 2021).

220 **4.2 Dairy farms and survey respondents**

221 Coverage error was present in this survey, reflected by limited geographical coverage, underrepresentation
222 of farms in Victoria and overrepresentation of farms and herds of larger size (Figure 1, Table 1). The
223 Department of Agriculture and Water Resources (2017) found that the average Victorian dairy farm was
224 252 ha, milked 345 cows and had a stocking density of 2.1 cows/ha. The overestimation of these variables
225 may also be a result of the phrasing of the survey question, which asked for total farm area, not total usable
226 or grazed area, which would have reduced the farms' size and increased the stocking density. Ninety-seven
227 per cent of farms had an irrigated pasture base (Table 3). The predominate method of water application
228 was using the border-check irrigation method (known commonly as flood) irrigation which is consistent
229 with Watson and Watson (2015) and Khan et al. (2010), who found that 50% to 60% of Victorian dairy
230 farmers solely used flood irrigation (Table 3). The descriptive statistics obtained from the 36 respondents
231 were consistent with work published by Schirmer et al. (2015), who found the highest proportion of dairy
232 farmers were aged between 45-54 years, and the majority of respondents were male (>60%) (Table 1).

233 **4.3 Integrated parasite management**

234 Non-chemical control options play a crucial role in reducing the reliance on flukicides to treat *F. hepatica*.
235 IPM strategies focus on reducing *F. hepatica* egg contamination of pasture, restricting host access to
236 intermediate host habitat and limiting host exposure to infective stages of *F. hepatica.* In this study, 42% of

8

237 respondents identified that more than >20% of their properties had waterlogging problems (Table 4). Host
238 proximity to waterlogged areas, irrigation channels, and naturally occurring water bodies increase the risk
239 of exposure and infection with *F. hepatica* (Overend and Bowen, 1995; Alves et al., 2011; Kuerpick et al.,
240 2013; Olsen et al., 2015). Researchers in New Zealand also identified that pugging caused by waterlogged
241 soils increased the intermediate host population (*Austropeplea tomentosa* and *Pseudosuccinea columella*)
242 within the pasture (Harris and Charleston, 1977). Given that in this study, stock on 78% of farms and 61%
243 of farms had access to waterlogged areas and irrigation channels, respectively, the risk of contamination
244 and exposure to either *F. hepatica* or the intermediate host is potentially high (Table 4). Fencing could play
245 a key role in reducing stock access to these high-risk areas, but Watson and Watson (2015) found that
246 fencing is typically planned over a long period and is dependent on-farm finances.

247 **4.4. *F. hepatica* diagnostics**

248 Our survey results suggest that we should be advocating for greater use of diagnostic tests as only 33% of
249 farms used BTM ELISA and 28% of farms used LFEC to inform decision making (Figure 2a). The frequency of
250 testing was highest in adult stock, whereas only two farms tested young animals (Figure 2b). Given that
251 young animals are generally reared on more marginal paddocks, they are more vulnerable to *F. hepatica,*
252 and infection can have flow-on effects that impact future animal fertility, suggesting that increased testing
253 should occur in these animals (Oakley et al., 1979; Takeuchi-Storm et al., 2017; Takeuchi-Storm et al., 2018).
254 Work by Mezo et al. (2008) in Spain found that only 15% of dairy farmers tested their cattle before flukicide
255 administration, and most were unaware of the herd's *F. hepatica* status. Farmers instead relied on blanket
256 preventative flukicide treatments. Kelley et al. (2020) identified the same trend in Victorian dairy farms as
257 several farmers were routinely treating their cattle with flukicides even though the animals were not
258 infected with *F. hepatica.* In the United Kingdom, Easton et al. (2018) found that the lowest use of diagnostic
259 and resistance tests to inform decision making was in the dairy industry. In this study, 19% of respondents
260 reported that they had tested for *F. hepatica* drug resistance (Figure 2a). Given that we did not ask the
261 farmers to explain their method for testing for resistance, it is difficult to ascertain if they followed best
262 practice guidelines or used appropriate tests to confirm resistance.

263 **4.5 Flukicide use**

264 The survey findings suggest that the use of TCBZ and frequency of flukicide treatments in dairy cattle has
265 decreased from the recommendations laid out by Boray et al. (1999). CLOR was more widely used in all
266 stock categories compared with TCBZ, and only one participant used OXY (Figure 3, 4a and b). The most
267 common approach was to treat all stock categories annually except for TCBZ in heifers, which were treated
268 twice per year (Figure 3). Forty-one per cent of respondents relied on single actives (CLOR or TCBZ) and, in

some cases, at a high frequency (Figure 3 and 4b). Given that dairy farmers in Australia are limited to using only TCBZ, CLOR and OXY to treat *F. hepatica*, this raises concerns about the increased selection pressures on these chemicals (Supplementary Table 2)(Hume, 2018). A large proportion of respondents relied on CLOR, which is only sold in combination with ivermectin (Figure 3) (Hume, 2018). Bullen (2016) found that on 15 of 20 dairy farms tested in the MID in Victoria, at least one nematode species was resistant to doramectin. Globally, there have been three reports of CLOR resistant *F. hepatica* (Kelley et al., 2016). It is challenging to assess flukicide efficacy if the product is only effective against adult *F. hepatica* (Elliott et al., 2015). However, given the high use of CLOR in Australia, a methodology for testing efficacy needs to be developed. The study found that only a small number of respondents were using OXY, which could be incorporated into flukicide rotations, particularly in areas where TCBZ resistance has been identified in Victoria (Brockwell et al., 2014; Elliott et al., 2015; Kelley et al., 2020). The United Kingdom and Ireland have successfully communicated that TCBZ resistance is a growing problem, leading to increased OXY use in dairy cattle (Bloemhoff et al., 2014; Selemetas et al., 2015). Another important component of IPM is to limit the introduction and spread of resistant parasites by quarantining newly purchased animals or animals returning to the farm. Most respondents in this study did not isolate and treat animals before joining them with the main herd; this breakdown in quarantine was also observed by Mezo et al. (2008) on dairy farms in Spain (Figure 5).

4.6 Flukicide administration

Boray et al. (1999) recommended treating based on the season, which only two respondents in this study used as a factor in their decision making. Instead, most respondents were treated at dry-off (Figure 6a). This is consistent with research in Ireland and the United Kingdom where Selemetas et al. (2015) found that 96% of farmers treated at dry-off and Bloemhoff et al. (2014) found that after the tightening of anthelmintic regulations, the proportion treating at dry-off increased from 59% to 81%. In most cases, it is more convenient to treat at dry-off as milk withholds restrict the use of flukicides in lactating cattle. In Australia, however, there are two registered products that can be used during lactation, allowing farmers greater freedom in when they treat. All products registered in Australia have been listed in supplementary table 2. Only two respondents in this study used diagnostics to inform treatment timing (Figure 6a). When purchasing a flukicide, respondents relied heavily on a single selection method (59%), of which 45% selected based on advice and 14% on previous use (Figure 6b). Cornelius et al. (2015) found that whomever sheep farmers sort advice from significantly influenced what other control methods were used on-farm. Farmers that relied on professionals (e.g. private veterinarians, government veterinarians or private consultants) were more likely to use diagnostics to inform decision making, test for resistance, drench less

5.7 Conclusion

and be aware of IPM. Given that veterinarians and advisors were used by many dairy farmers in selecting flukicides, one avenue for improving *F. hepatica* management would be to educate those professionals who work with dairy farmers (Figure 6b). This approach could then be extended to include rural resellers. Easton et al. (2016) in the United Kingdom surveyed prescribers of anthelmintics and identified several knowledge gaps, which were then addressed to improve advice given to farmers at the point of purchase. Another important IPM strategy is to avoid the underdosing of cattle which limits the selection pressure for resistance. Besier and Hopkins (1989) established that sheep farmers were poor estimators of live weight, leading to 85% of farmers underdosing their sheep for nematode control. Eighty-six per cent of cattle farmers also underestimated live weight but by a greater margin than in sheep, 47% compared to 18% underestimation (Besier and Hopkins, 1989; Machila et al., 2008). In this study, 50% of survey respondents estimated weight and used average weights to determine flukicide doses (Figure 6c). Underdosing is likely to be prevalent within the dairy industry, given that only 40% were weighing the heaviest animal, weighed each animal or used weigh tapes to determine dose volume (Figure 6c). Further work would be needed to be to confirm the impact of estimating weights on the dose administered to cattle.

Conclusion

Seventy-two per cent (26 farmers) of respondents who completed the survey wanted more information on *F. hepatica* control strategies. The evidence generated from this survey has identified several areas where *F. hepatica* management in Victoria could be optimised and has identified what IPM strategies need to be communicated to dairy farmers. Our key findings are: (1) that diagnostic tests are underutilised to inform flukicide timing and management of *F. hepatica* in replacement animals; (2) flukicide doses were not accurately determined, and underdosing is likely to be prevalent within the dairy industry; (3) there was an over-reliance on single flukicide actives, and OXY was rarely used to treat *F. hepatica*; and (4) non-chemical approaches were not effectively utilised and animals had considerable access to high-risk *F. hepatica* areas on-farms. Coyne et al. (2020) identified that the three biggest barriers to change on sheep farms with confirmed TCBZ resistance were overcoming habitual practices, economic feasibility and the increased complexity in implementing IPM strategies. The best way forward for the dairy industry in Victoria would be, firstly, to do a more extensive (regionally representative) survey to establish regional differences in the management of *F. hepatica* to generate the evidence base for a tailored extension and control program. Secondly, we recommend that an economic study be performed on the financial returns of implementing an IPM strategy on dairy farms in Victoria (Torres-Acosta et al., 2012). These steps will generate the evidence base needed to encourage dairy farmers to overcome the barriers to change and implement IPM strategies on their farms.

11

5.8 Acknowledgments

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5.9 References

References

Alves, D., Carneiro, M., Martins, I., Bernardo, C., Donatele, D., Pereira Júnior, O., et al. (2011). Distribution and factors associated with *Fasciola hepatica* infection in cattle in the south of Espírito Santo State, Brazil. *Journal of Venomous Animals and Toxins including Tropical Diseases* 17, 271-276.

Besier, R., and Hopkins, D. (1989). Farmers' estimations of sheep weights to calculate drench dose. *Journal of the Department of Agriculture, Western Australia, Series 4* 30, 120-121.

Bloemhoff, Y., Danaher, M., Forbes, A., Morgan, E., Mulcahy, G., Power, C., et al. (2014). Parasite control practices on pasture-based dairy farms in the Republic of Ireland. *Veterinary Parasitology* 204, 352-363. doi: 10.1016/j.vetpar.2014.05.029.

Boray, J.C., Hutchinson, G., and Love, S. (1999). *Liver fluke disease in sheep and cattle.* NSW Agriculture. http://www.wormboss.com.au/sheep-goats/files/pages/worms/flukes/liver-fluke/Prime_Fact_446_Liver_fluke_disease_in_sheep_and_cattle.pdf [Accessed 16/02/2021]

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., and Sangster, N.C. (2014). Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. *International Journal for Parasitology: Drugs and Drug Resistance* 4, 48-54. doi: https://doi.org/10.1016/j.ijpddr.2013.11.005.

Bullen, S. (2016). Anthelmintic resistance in gastrointestinal nematode parasites of dairy cattle in the Macalister Irrigation District of Victoria. Australian Veterinary Journal 94, 35-41. DOI: 10.1111/avj.12407

Cornelius, M., Jacobson, C., and Besier, R. (2015). Factors likely to influence the adoption of targeted selective treatment strategies by sheep farmers in Western Australia. Preventive Veterinary Medicine 121, 325-331. doi: 10.1016/j.prevetmed.2015.08.004

Coyne, L.A., Bellet, C., Latham, S.M., and Williams, D. (2020). Providing information about triclabendazole resistance status influences farmers to change liver fluke control practices. *Veterinary Record* 187, 357-357. DOI: 10.1136/vr.105890

Dairy Australia (2015). "Dairy farm guidelines for BJD control", in: *Best practice recommendations for managing the risk of BJD in Australian dairy herds.* https://www.dairyaustralia.com.au/resource-repository/2020/07/09/dairy-farm-guidelines-for-bjd-control#.YCTIQmgzYuU

Dairy Australia (2018). "Dairy Situation and Outlook June 2018", in: *National Dairy Farmer Survey.*). https://www.dairyaustralia.com.au/resource-repository/2020/09/01/dairy-australia-situation-and-outlook-june-2018#.YCtxdmgzbct [Accessed 07/02/2021]

Dairy Australia (2021). *Cow & Farms Data* https://www.dairyaustralia.com.au/en/industry-statistics/cow-and-farms-data#.YCrUf2gzbct [Accessed 16/02/2021 2021].

Department of Agriculture and Water Resources (2017). "Answers to questions on notice", in: *Budget Estimates May 2017.* (Estimates Committees: Commonwealth of Australia).

https://www.aph.gov.au/Parliamentary_Business/Senate_estimates/rratctte/estimates/bud1718/AG/index [Accessed 07/02/2021]

Easton, S., Bartley, D.J., Hotchkiss, E., Hodgkinson, J.E., Pinchbeck, G.L., and Matthews, J.B. (2016). Use of a multiple choice questionnaire to assess UK prescribing channels' knowledge of helminthology and best practice surrounding anthelmintic use in livestock and horses. *Preventive Veterinary Medicine* 128, 70-77. doi: 10.1016/j.prevetmed.2016.04.010.

Easton, S., Pinchbeck, G.L., Bartley, D.J., Hodgkinson, J.E., and Matthews, J.B. (2018). A survey of experiences of UK cattle and sheep farmers with anthelmintic prescribers; are best practice principles being deployed at farm level? *Preventive Veterinary Medicine* 155, 27-37. doi: 10.1016/j.prevetmed.2018.04.009

Economics Reference Committee (2017). "Australia's dairy industry: rebuilding trust and a fair market for farmers". (Senate Printing Unit, Parliament House, Canberra.: Commonwealth of Australia). https://www.aph.gov.au/Parliamentary_Business/Committees/Senate/Economics/Dairyindustry/Report [Accessed 07/02/2021]

Elliott, T.P., Kelley, J.M., Rawlin, G., and Spithill, T. W. (2015). High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. *Veterinary Parasitology* 209(1), 117-124. doi: 10.1016/j.vetpar.2015.02.014

Harris, R., and Charleston, W. (1977). An examination of the marsh microhabitats of *Lymnaea tomentosa and L. columella* (Mollusca: Gastropoda) by path analysis. *New Zealand Journal of Zoology* 4, 395-399.

Hort, C. (1998). "A survey of *Fasciola hepatica* infection of sheep and cattle in NSW and Victoria.", in: *Proceedings of the Australian Sheep Veterinary Society Conference: The Australian Veterinary Association*), 61-64.

Hume, W. (2018). A review of management strategies for control of *Fasciola hepatica* in cattle in South Eastern Australia. Honours thesis, La Trobe University.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., and Spithill, T.W. (2016). Current threat of triclabendazole resistance in *Fasciola hepatica*. *Trends in Parasitology* 32, 458-469. doi: 10.1016/j.pt.2016.03.002

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., et al. (2020). Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. *Veterinary Parasitology* 277, 109019. doi: 10.1016/j.vetpar.2019.109019

Khan, S., Abbas, A., Rana, T., and Carroll, J. (2010). Dairy water use in Australian dairy farms: past trends and future prospects. *Melbourne, VIC: CSIRO Water for a Healthy Country National Research Flagship*. https://doi.org/10.4225/08/58615ca753885 [Accessed 07/02/2021]

Kuerpick, B., Conraths, F.J., Staubach, C., Froehlich, A., Schnieder, T., and Strube, C. (2013). Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. *Parasitology* 140, 1051-1060. doi: 10.1017/S0031182013000395.

409 Lean, I., Westwood, C., and Playford, M. (2008). Livestock disease threats associated with intensification of pastoral
410 dairy farming. *New Zealand Veterinary Journal* 56, 261-269. doi: 10.1080/00480169.2008.36845
411 Machila, N., Fèvre, E.M., Maudlin, I., and Eisler, M.C. (2008). Farmer estimation of live bodyweight of cattle:
412 implications for veterinary drug dosing in East Africa. *Preventive Veterinary Medicine* 87, 394-403.
413 doi: 10.1016/j.prevetmed.2008.06.001.
414 McCausland, I., Vandegraaff, R., and Nugent, L. (1980). Fascioliasis in dairy cows on irrigated pasture. *Australian*
415 *Veterinary Journal* 56, 324-326. doi: 10.1111/j.1751-0813.1980.tb05739.x
416 Mezo, M., González-Warleta, M., Castro-Hermida, J.A., and Ubeira, F.M. (2008). Evaluation of the flukicide
417 treatment policy for dairy cattle in Galicia (NW Spain). *Veterinary Parasitology* 157, 235-243.
418 doi: 10.1016/j.vetpar.2008.07.032
419 Moran, J., Drysdale, G., Shambrook, D., and Markham, N. (2000). A study of key profit drivers in the Victorian dairy
420 industry. *Asian Australasian Journal of Animal Sciences* 13, 54-57.
421 Oakley, G., Owen, B., and Knapp, N. (1979). Production effects of subclinical liver fluke infection in growing dairy
422 heifers. *The Veterinary Record* 104, 503-507. doi: 10.1136/vr.104.22.503
423 Olsen, A., Frankena, K., Toft, N., Thamsborg, S.M., Enemark, H.L., and Halasa, T. (2015). Prevalence, risk factors and
424 spatial analysis of liver fluke infections in Danish cattle herds. *Parasites & Vectors* 8, 160. doi:
425 10.1186/s13071-015-0773-x.
426 Overend, D., and Bowen, F. (1995). Resistance of *Fasciola hepatica* to triclabendazole. *Australian Veterinary Journal*
427 72, 275-276. doi: 10.1111/j.1751-0813.1995.tb03546.x.
428 Penny, J. (1869). Report of Royal Commission on fluke. *Launceston Examiner (Tas. : 1842 - 1899)* 04 September
429 1869.
430 Salimi-Bejestani, M., Daniel, R., Felstead, S., Cripps, P., Mahmoody, H., and Williams, D. (2005). Prevalence of
431 *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk.
432 *Veterinary Record-English Edition* 156, 729-731. doi: 10.1136/vr.156.23.729
433 Schirmer, J., Yabsley, B., Peel, D., Mylek, M., Hanigan, I., and Brown, K. (2014)"Wellbeing of dairy farmers: Findings
434 from the 2014 Regional Wellbeing Survey Farmers and agriculture report ". (Centre for Research and Action
435 in Public Health: University of Canberra). https://www.canberra.edu.au/research/institutes/health-
436 research-institute/regional-wellbeing-survey/survey-results/reports#2014
437 [Accessed 07/02/2021]
438 Schirmer, J., Mylek, M. and Yabsley, B. (2015). "Wellbeing, community connections and resilience of dairy farmers:
439 Findings from the 2015 Regional Wellbeing Survey". (Centre for Research and Action in Public Health:
440 University of Canberra). https://www.canberra.edu.au/research/institutes/health-research-
441 institute/regional-wellbeing-survey/survey-results/reports#2014 [Accessed 07/02/2021]
442 Schweizer, G., Braun, U., Deplazes, P., and Torgerson, P. (2005). Estimating the financial losses due to bovine
443 fasciolosis in Switzerland. *Veterinary Record* 157, 188-193. doi: 10.1136/vr.157.7.188.

15

444 Selemetas, N., Phelan, P., O'Kiely, P., and de Waal, T. (2015). The effects of farm management practices on liver
445 fluke prevalence and the current internal parasite control measures employed on Irish dairy farms.
446 *Veterinary Parasitology* 207**,** 228-240. doi: 10.1016/j.vetpar.2014.12.010
447 State of Victoria (2018). *Irrigation district.* Department of Environment, Land, Water and Planning.
448 https://discover.data.vic.gov.au/dataset/irrigation-district [Accessed 30/01/2021]
449 State of Victoria (2021). *Postcode Boundaries.* Department of Environment, Land, Water and Planning.
450 https://discover.data.vic.gov.au/dataset/postcode-boundaries-polygon-vicmap-admin [Accessed
451 02/01/2021]
452 Takeuchi-Storm, N., Denwood, M., Hansen, T.V.A., Halasa, T., Rattenborg, E., Boes, J., et al. (2017). Farm-level risk
453 factors for *Fasciola hepatica* infection in Danish dairy cattle as evaluated by two diagnostic methods.
454 *Parasites & Vectors* 10**,** 555. doi: 10.1186/s13071-017-2504-y
455 Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A.-S., Sengupta, M.E., et al. (2018).
456 Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the
457 parasite based on different diagnostic methods. *Parasites & Vectors* 11**,** 674. doi: 10.1186/s13071-018-
458 3248-z
459 Torres-Acosta, J., Mendoza-de-Gives, P., Aguilar-Caballero, A., and Cuéllar-Ordaz, J. (2012). Anthelmintic resistance
460 in sheep farms: update of the situation in the American continent. *Veterinary Parasitology* 189**,** 89-96.
461 doi: 10.1016/j.vetpar.2012.03.037.
462 Watson, P., and Watson, D. (2015). "Sustainability Framework NRM Survey".
463 http://www.dairyingfortomorrow.com.au/wp-content/uploads/Australian-Dairy-Sustainability-Framework-
464 NRM-Survey-2015.pdf: Dairy Australia.
465 Watt, G. (Year). "An approach to determining the prevalence of liver-fluke in a large region", in: *Proceedings of the*
466 *2nd International Symposium on Veterinary Epidemiology and Economics, 7-11 May 1979, Canberra,*
467 *Australia.*: Australian Government Publishing Service, 152-155. Retrieved from An approach to determining
468 the prevalence of liver fluke in a large region | SciQuest

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5.10 Figure descriptions

Figure captions

Figure 1. A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Map of Victoria showing the number of survey respondents by postcode area. Blue represents one respondent; purple: 2 respondents; pink: 3 respondents; orange: 5 respondents. Grey hashed lines represent irrigation regions within Victoria.

Figure 2. (A) The proportion of respondents using different types of *F. hepatica* diagnostic testing on-farm. **(B)** The proportion of respondents using a various frequency of diagnostic testing per year for each animal category.

Figure 3. The proportion of respondents using various number of annual treatments with three different flukicides in each stock category (2015/2016 financial year).

Figure 4. (A) The proportion of respondents using three different flukicides over the five years preceding the 2015/2016 financial year. **(B)** Proportion of respondents using single or multiple flukicides over the same time period.

Figure 5. The proportion of respondents that applied quarantine treatments to calves reared or purchased externally.

Figure 6. The proportion of respondents using various methods to treat animals with flukicides. **(A)** Method used to decide timing of treatment. **(B)** Method used to select a flukicide. **(C)** Method used to determine the dose of flukicide to administer to their animals.

5.11 Tables

485 **Table 1.** A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Demographic details of
486 survey respondents.

Question	Number of respondents (%)
Irrigation region:	
Central Goulburn (CG)	14 (39)
Macalister Irrigation District (MID)	7 (19)
Murray Valley (MV)	4 (11)
Upper Murray (UM)	4 (11)
Torrumbarry (TIA)	3 (8)
South Gippsland	2 (6)
Loddon Valley (LV)	1 (3)
Western Victoria	1 (3)
Age (years):	
18 to 24	0 (0)
25 to 34	7 (19)
35 to 44	9 (25)
45 to 54	12 (33)
55 to 64	3 (8)
65 to 74	3 (8)
> 75	2 (6)
Education:	
Secondary	8 (22)
TAFE or Trade qualification	4 (11)
Associate degree or diploma	10 (28)
Bachelor's degree	10 (28)
Postgraduate or masters	3 (8)
No response	1 (3)
Gender:	
Male	26 (72)
Female	10 (28)

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489 **Table 2**. A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Descriptive statistics of
490 farm area and stock numbers on each of the farms managed by survey respondents.

Question	n	Mean (SD)	Median	Q1, Q3	Min, max
Farm area (ha)	36	427 (512)	250	150,521	40, 2400
No. adults	36	457 (356)	335	249,663	40, 2000
No. heifers > 12 months	36	138 (120)	120	65,180	6, 700
No. calves < 12 months	36	130 (102)	93	64,203	0, 500

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Table 3. A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Types of farms, details of irrigation methods and details of calving systems on each of the farms managed by survey respondents.

Question	Number of respondents (%)
Organic dairy system:	
Yes	1 (3)
No	35 (97)
Farm type:	
Irrigated pasture base	33 (92)
Dry-land pasture base	1 (3)
No response	2 (6)
Irrigation:	
Flood	20 (56)
Travelling gun	1 (3)
Flood and centre pivot	4 (11)
Flood and lineal move	1 (3)
Flood and laterals	2 (6)
Flood and spray	2 (6)
Flood, centre pivot and linear move	2 (6)
Flood, centre pivot and laterals	1 (3)
None	1 (3)
No response	2 (6)
Calving system:	
Year-round	1 (3)
Split calving	27 (75)
Seasonal calving	8 (22)

Table 4. A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Percentage of farm waterlogged at any time during the year, whether or not cattle have access to waterlogged areas and details of irrigation maintenance on each of the farms managed by survey respondents.

Question	Number of respondents (%)
Percentage of farm waterlogged:	
0	2 (6)
1 to 19	19 (53)
20 to 39	3 (8)
40 to 59	6 (17)
60 to 79	2 (6)
80 to 99	4 (11)
100	0 (0)
Cattle access to waterlogged areas:	
Yes	28 (78)
No	5 (14)
No response	3 (8)
Irrigation maintenance:	
Excavate	1 (3)
Spray weeds	3 (8)
Spray weeds and excavate	3 (8)
Graze with stock and excavate channels	1 (3)
Spray weeds and fixing leaking delvers	11 (31)
Spray weeds, fixing leaking delvers and excavate channels	11 (31)
Spray weeds, replace delvers with pipes and fix leaking delvers	1 (3)
No response	4 (11)
None	1 (3)
Access to irrigation channels?	
Yes	22 (61)
No	11 (31)
No response	3 (8)

504 **Table 5.** A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Whether or not fluke
505 treatment was carried out in 2015-2016, and whether or not respondents would be interested in receiving more
506 information about fluke.

Question	Number of respondents (%)
Treated for fluke in 2015 – 2016?	
Yes	26 (72)
No	10 (28)
More information about fluke?	
Yes	26 (72)
No	9 (25)
No response	1 (3)

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5.12 Supplementary

Supplementary 5.1. The *F. hepatica* control survey that was disseminated to dairy farmers in Victorian irrigations dairy regions.

SECTION 1: LOCATION AND RESEARCH AWARENESS

Q1 Please state your **Victorian** postcode below.

_____ _____ _____ _____

Q2 Please select the category that includes your age.

- ☐ 18 to 24
- ☐ 25 to 34
- ☐ 35 to 44
- ☐ 45 to 54
- ☐ 55 to 64
- ☐ 65 to 74
- ☐ 75 >
- ☐ Prefer not to state

Q3 Please select your highest level of education.

- ☐ Secondary
- ☐ TAFE or Trade qualification
- ☐ Associate degree or diploma
- ☐ Bachelor degree
- ☐ Post graduate or masters
- ☐ Prefer not to state
- ☐ Other (please specify) _____

Q4 Please select your gender.

- ☐ Male
- ☐ Female
- ☐ Prefer not to state

Q5 Did you treat for liver fluke in 2016?

- ☐ Yes
- ☐ No

Q6 Have you previously worked with **Jane Kelley** from La Trobe University on the liver fluke study?

- ☐ Yes
- ☐ No

Q7 Would you like to receive more information about liver fluke drenching practices?

- ☐ Yes
- ☐ No

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SECTION 2: DRAINAGE AND IRRIGATION

Q8 What is the total approximate area (ha) of your farm?

Total farm area: _____ha

Q9 What percentage of your farm do you estimate can suffer from waterlogging?

- ☐ 100% The entire farm can suffer from waterlogging
- ☐ 80% to 99%
- ☐ 60% to 79%
- ☐ 40% to 59%
- ☐ 20% to 39%
- ☐ 1% to 19%
- ☐ 0% No waterlogging occurs on any part of the farm

Q10 Do cattle on your farm have access to these waterlogged areas?

- ☐ Yes
- ☐ No

Q11 Please state the approximate irrigated area (ha) for each irrigation type.

None	**Please proceed to section Q14 by turning the page.**
Flood irrigation	_____ ha
Travelling gun	_____ ha
Centre pivot	_____ ha
Lineal move	_____ ha
Other (please specify)	_____ _____ ha

Q12 Please select what maintenance you carry out on your irrigation channels, delvers and drains.

- ☐ None
- ☐ Fixing leaking delvers
- ☐ Excavating channels
- ☐ Spraying weeds
- ☐ Other (please specify) _____

Q13 Do cattle on your farm have access to irrigation channels, delvers and drains?

- ☐ Yes
- ☐ No

SECTION 3: STOCK DETAILS AND DIAGNOSTICS

Q14 For each age group please state the number of stock.

Milkers _____

Heifers >12 months _____

Calves <12 months _____

Q15 Please select your calving system.

- ☐ Year-round
- ☐ Split calving
- ☐ Seasonal calving
- ☐ Other (please specify) _____

Q16 Have you ever used an external heifer rearer?

- ☐ Yes
- ☐ No

Q17 Did you purchase any stock in 2016?

- ☐ Yes
- ☐ No

Q18 In 2016, did you use the **milk test (ELISA)** to determine whether your milkers were infected with **liver fluke**?

- ☐ Yes
- ☐ No

Q19 In 2016, did you use **faecal egg counts (FECs)** to determine whether your stock were infected with liver fluke?

- ☐ Yes
- ☐ No

Q20 In 2016, did you use any other test to determine whether your stock were infected with liver fluke?

- ☐ Yes
- ☐ No

If yes, please specify below:

_____

Q21 For each age group please state how many times your stock were tested for liver fluke in 2016.

Milkers _____

Heifer > 12 months _____

Calves < 12 months _____

SECTION 4: FLUKICIDES

Q22	For each age group please state how many times you used **Triclabendazole** (Example: Fasinex™, Flukare™) to treat for liver fluke in 2016.
	Milkers _____ Heifer > 12 months _____ Calves < 12 months _____
Q23	Have you used any **Triclabendazole** (Example: Fasinex™, Flukare™) product in the past 5 years (2010 to 2015)?
	☐ Yes ☐ No
Q24	For each age group please state how many times you used **Clorsulon** (Example: Ivomec plus™, Virbamec Plus™, Bomectin F™) to treat for liver fluke in 2016.
	Milkers _____ Heifer > 12 months _____ Calves < 12 months _____
Q25	Have you used any **Clorsulon** (Example: Ivomec plus™, Virbamec Plus™, Bomectin F™) product in the past 5 years (2010 to 2015)?
	☐ Yes ☐ No
Q26	For each age group please state how many times you used **Oxyclozanide** (Example: Nilzan LV™) to treat for liver fluke in 2016.
	Milkers _____ Heifer > 12 months _____ Calves < 12 months _____
Q27	Have you used any **Oxyclozanide** (Example: Nilzan LV™) product in the past 5 years (2010-2015)?
	☐ Yes ☐ No
Q28	Did you use any other product in 2016 to treat for liver fluke? Please provide details below.
	_____

SECTION 5: DRENCHING PRACTICES

Q29 When do you drench for liver fluke? Select all that apply.

- ☐ At dry-off
- ☐ During lactation
- ☐ Only when recommended by a farm advisor or veterinarian
- ☐ Based on the appearance of animal or mob i.e. bottle jaw, ill thrift
- ☐ Based on diagnostic tests
- ☐ Other (please specify) _____

Q30 How do you determine what drench to buy? Select all that apply.

- ☐ Recommended by veterinarian
- ☐ Recommended by reseller
- ☐ Recommended by farm advisor
- ☐ Recommended by friend or neighbour
- ☐ Price
- ☐ Previous use of drench
- ☐ Other (please specify) _____

Q31 How do you determine the volume of drench to administer to your cattle? Select all that apply.

- ☐ Estimate weight of individuals and dose accordingly
- ☐ Weigh individual animals and dose accordingly
- ☐ Dose to average weight of group
- ☐ Weigh heaviest and dose all others according to this animal
- ☐ Other (please specify) _____

Q32 Do you quarantine drench? i.e. do you isolate newly purchased cattle or cattle from another property and treat them for liver fluke prior to joining them with the main herd?

- ☐ Yes
- ☐ No

Q33 Have you ever tested for **liver fluke drug resistance** on your dairy farm?

- ☐ Yes
- ☐ No

Q34 If you have any additional information you feel is relevant to your property's liver fluke control program, please list below.

Thank you for taking the time to answer all the questions, your input is greatly appreciated. Your answers will help us to develop liver fluke management guidelines for dairy farms in Victoria.

PLEASE RETURN THE SURVEY IN THE PRE-ADDRESSED REPLY PAID ENVELOPE TO JANE KELLEY AT LA TROBE UNIVERSITY

Supplementary 5.2. All registered flukicide products for the treatment of *F. hepatica* in dairy cattle in Australia (sourced from Hume, 2018).

Product	Company	Composition	Use	Age of fluke killed	Host	Meat WHP	Use in lactating cattle	Milk WHP
Exifluke 240	Bayer Australia Ltd	Triclabendazole 240 g/L	Oral	All stages	Dairy & Beef	14 days	Not during lactation	Do not use less than 21 days before calving. Milk from treated cows must not be used for human consumption or supplied for processing for 96 hours (eight milking's) after calving.
Fasimec Cattle Oral	Elanco Australasia Pty Ltd	Triclabendazole 120 g/L, ivermectin 2 g/L	Oral	All stages	Dairy & Beef	21 days	Not during lactation	Do not use in lactating cows or within 28 days of calving where milk or milk products may be used for human consumption.
Fasinex 240	Elanco Australasia Pty Ltd	Triclabendazole 240 g/L	Oral	All stages	Dairy, Beef & Sheep	21 days	Not during lactation	Do not use in lactating cows where milk and milk products from treated cows may be used for human consumption.
Flukare C Plus Selenium	Virbac Australia Pty Ltd	Triclabendazole 120 g/L, selenium (as sodium selenate) 1 g/L	Oral	All stages	Dairy, Beef, Sheep & Goats	21 days	Not during lactation	Do not use in animals which are producing milk or milk products for human consumption. Do not use less than 21 days before calving, lambing or kidding in cows, ewes or does where milk or milk products from treated

Product	Company	Composition	Use	Age of fluke killed	Host	Meat WHP	Use in lactating cattle	Milk WHP
								animals may be used for human consumption.
Flukazole C plus Selenium	Virbac Australia Pty Ltd	Triclabendazole 120 g/L, oxfendazole 45.3 g/L, selenium (as sodium selenate) 1 g/L	Oral	All stages	Dairy, Beef & Sheep	21 days	Not during lactation	Do not use in cows or sheep which are producing milk or milk products for human consumption. Do not use less than 21 days before calving in cows or lambing in ewes where milk and milk products from treated animals may be used for human consumption.
Genesis Ultra Injection	Boehringer Ingelheim Animal Health Australia Pty. Ltd.	Ivermectin 10 mg/mL, clorsulon 100 mg/mL	Injection	Adult Liver Fluke Only	Dairy & Beef	28 days	Heifers only before first mating	Do not use in dairy cattle (except replacement dairy heifers) that are producing or may in the future produce milk for human consumption or processing. Do not use in replacement dairy heifers after the first mating.
Baymec Gold Injection	Baymec Gold Injection	Ivermectin 10 g/L, clorsulon 100 g/L	Injection	Adult Liver Fluke Only	Dairy & Beef	42 days	Can be used during lactation	No WHP

Product	Company	Composition	Use	Age of fluke killed	Host	Meat WHP	Use in lactating cattle	Milk WHP
Ivomec Plus Antiparasitic Injection for Cattle	Boehringer Ingelheim Animal Health Australia Pty. Ltd.	Ivermectin 10 mg/mL, clorsulon 100 mg/mL	Injection	Adult Liver Fluke Only	Dairy & Beef	28 days	Can be used during lacation	No WHP
Nilzan LV	Coopers Animal Health	Levamisole 64 g/L (≡ levamisole hydrochloride 75 g/L), oxyclozanide 150 g/L	Oral	Adult Liver Fluke Only	Dairy, Beef & Sheep	14 days	Can be used during lactation	No WHP
Noromectin Plus Injection for Cattle	NORBROOK LABORATORIES AUSTRALIA PTY LTD	Ivermectin 10 mg/mL, clorsulon 100 mg/mL	Injection	Adult Liver Fluke Only	Dairy & Beef	28 days	Can be used during lactation	No WHP
Virbamec Plus Injection for Cattle	Virbac Australia Pty Ltd	Ivermectin 10 g/L, clorsulon 100 g/L	Injection	Adult Liver Fluke Only	Dairy & Beef	28 days	Can be used during lactation	No WHP

Chapter 6 – General discussion

6.1 Scope of the thesis

Since the release of triclabendazole (TCBZ) in Australia in the early 1980s (Boray et al., 1983), there have been relatively few reports on *F. hepatica* in Australian cattle. It was evident in Brockwell et al. (2014) and Elliott et al. (2015) that there has been limited on-farm monitoring of *F. hepatica* as both studies established that *F. hepatica* was resistant to TCBZ on several farms, and there was a high prevalence of *F. hepatica* in the Gippsland region of Victoria. These reports suggested that more needed to be done to establish where *F. hepatica* is endemic in cattle, to evaluate how diagnostic testing can be applied on-farm to inform control and to determine what strategies should be incorporated into an IPM plan that can be implemented on-farm to reduce the reliance on chemical control options and production losses due to fasciolosis.

Chapters 3, 4 and 5 have been presented as research papers. A summary of each Chapter is below, with a discussion of the findings and the gaps identified by the research.

Chapter 1 summarises the ecology, distribution and life cycle of *F. hepatica* in Australia. It also identifies research gaps within the literature that need to be investigated to improve the control of *F. hepatica* in Australia.

Chapter 2 gives a global overview of the use of TCBZ for treating *F. hepatica* in ruminants and summarises all reported cases of drug resistance in *F. hepatica* to 2016. The Chapter further discusses the long-term implications of drug resistance for the control of *F. hepatica*.

Chapter 3 establishes the prevalence of *F. hepatica* in Victorian irrigated dairy regions which was the first state-wide prevalence study since Watt (1979). The results indicate that *F. hepatica* is endemic in the Macalister (MID), the Upper Murray (UM) and Murray Valley (MV) irrigation districts and identifies three more cases of TCBZ-resistant *F. hepatica* on dairy farms in two of the endemic regions.

Chapter 4 demonstrates that both the coproantigen ELISA (cELISA) and LFEC are robust tools capable of detecting burdens >10 flukes in naturally infected cattle. It also establishes that LFEC is more accurate when faecal samples are collected at the morning (AM) milking. These findings provide insight into how the cELISA and LFEC can be best utilised on dairy farms to inform IPM strategies into the future.

Chapter 5 provides insights into how dairy farmers in Victoria currently control *F. hepatica* on their farms. The Chapter also identifies several issues that should be addressed to

improve current *F. hepatica* control methods. The key findings include; 1) stock on 78% of farms had access to waterlogged areas or irrigation channels; 2) only 33% of farmers used BTM ELISA, and 28% of farmers used LFEC to inform *F. hepatica* decision making; 3) 41% of farmers relied on single actives (CLOR and TCBZ) in some cases at a high frequency; 50% of farmers estimated and used average weights to determine flukicide dose.

6.2 Chapter 3 – Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties

In Chapter 3, a major prevalence study was conducted across irrigated dairy regions in Victoria involving 1669 cattle in 83 herds. The previous state-wide prevalence study was conducted by Watt (1979) using abattoir inspection data which is a less sensitive method and is known to miss about 30% of *F. hepatica* infected livers (Charlier et al., 2008); the sensitivity of the technique is between 63.2 and 68% (Rapsch et al., 2006; Mazeri et al., 2016). Our study used the methodology described in Elliott et al. (2015) and tested dairy cattle across the state using the cELISA and LFEC. The cELISA and LFEC diagnostic tests were highly sensitive methods for detecting *F. hepatica* in naturally infected dairy cattle, as discussed further in Chapter 4.

We found that *F. hepatica* was endemic in the MID, the UM and the MV in the Goulburn-Murray (GM) irrigation district. Since Watt (1979), the prevalence of *F. hepatica* has increased in the MID (from 51% to 72-73%), decreased in the GM irrigation districts (from 68% to 16-19%) and remained unchanged in the UM at 64%. The prevalence in these regions exceeded the 25% herd prevalence, which is the cut-off for economic loss for *F. hepatica* in cattle, as discussed in Vercruysse and Claerebout (2001). The MID had the highest within-herd prevalence, with 15 of the 20 farms tested in the region having a prevalence >90%, which is consistent with a smaller study conducted by Elliott et al. (2015). In contrast, in the UM, only four of the ten farms and, in the MV, only two of the nine farms had a within-herd prevalence of> 90%. The higher incidence of *F. hepatica* in the MID could be linked to stocking density which has been shown to increase pasture contamination with faeces and pugging, which creates ideal microhabitats within the pasture for *Austropeplea tomentosa* (*A. tomentosa*) (Harris and Charleston, 1977; Lean et al., 2008). In the MID, the stocking density is 2.34 cows/ha, and pugging is a problem on 47% of farms, whereas the stocking density is 1.84 cows/ha and pugging is a problem on only 25% of farms in the UM and MV (Watson and Watson, 2015).

The lower prevalence in the UM and GM is also likely related to irrigation practices in these regions. As discussed in Chapter 1, flood irrigation creates the ideal conditions for transmission of *F. hepatica* to cattle and the proliferation of *A. tomentosa*. In Chapter 5, it was established that

97% of dairy farmers who participated in the survey had an irrigated pasture base and more than 56% used flood irrigation on their farms. Farmers in the UM rely on rivers, groundwater and collected surface water for irrigation, whereas farms in the MID and MV rely on irrigation schemes to deliver water to their farms. The reduced prevalence in the UM could be because farms are not connected via irrigation infrastructure, and there are no channels for stock to access. However, the reduced prevalence of *F. hepatica* in the GM is not consistent with work published by Durr et al. (2005) and Boray et al. (1969). Both studies identified that irrigated regions and farms had a higher incidence of *F. hepatica* than non-irrigated farms. The reason for the lower prevalence of *F. hepatica* in the GM could not be determined in Chapter 3. However, we hypothesised it was linked to the irrigation infrastructure upgrades and salinity in the district. In the GM irrigation district, dirt irrigation channels have been replaced by pipes, or channels have been lined with shale. A similar strategy was used to eradicate schistosomiasis in Japan (Tanaka and Tsuji, 1997). Salinity has also been a problem for many years in the GM and often exceeds the level tolerated by *A. tomentosa* (Boray, 1964; Hart et al., 2020). Follow-up research would need to be conducted to test these hypotheses. Given that new irrigation infrastructure works are planned for the MID, there is an opportunity to evaluate whether similar upgrades will change the epidemiology of *F. hepatica* in the MID (McCormack et al., 2020).

Several on-farm management practices were also identified in Chapter 3 that could be changed to improve the control of *F. hepatica,* reduce the prevalence of the parasite and save farmers money. Several farms in the GM irrigation district were administering preventative flukicides to their herds, but the herds were found to be 'fluke free'. Findings from Chapter 5 found that only 2/36 farmers were using diagnostic tests to inform flukicide selection and timing. Farmers should be encouraged to use diagnostic tests to inform their decision making and only treat when cattle are positive for *F. hepatica*. Strategic flukicide drenching has been shown to reduce the frequency of treatments on-farm, as well as reduce the fluke burden within cattle and the infection level within intermediate host snails (Parr and Gray, 2000; Mezo et al., 2008).

Routine testing of stock will also help in the early detection of TCBZ resistance. In Chapter 3, a further three cases of TCBZ resistance were identified in replacement animals. On one farm in the MID, acute fasciolosis combined with undiagnosed TCBZ resistance caused the deaths of four young animals. Replacement cattle should be routinely tested with diagnostic tools like the cELISA and LFEC to assess whether treatment is needed and determine if there is reduced efficacy of TCBZ by retesting the treated cattle three weeks post administration of the drench. The lack of routine testing in cattle likely contributed to delayed detection of TCBZ resistance in *F. hepatica*. It took from the study published by Overend and Bowen (1995) to Brockwell et al. (2014) to confirm TCBZ resistance in cattle in Australia. In Chapters 3 and 5, it was noted that there had been a trend away

from using TCBZ in dairy cattle. Most farmers were using clorsulon (CLOR). Given the high frequency and prolonged use of CLOR discussed in Chapter 5, a methodology for testing for resistance in flukicides that only target the adult stage needs to be developed (Elliott et al., 2015). In 2016, only three CLOR resistance cases were reported globally, as reviewed in Chapter 2. Given the change in CLOR use in Australia, this may increase over the next decade.

Quarantining animals will be essential in preventing the introduction of new *F. hepatica* infections and drug-resistant strains to farms. Quarantine practices are used to limit the introduction of parasites and resistant strains to farms by; testing animals before joining with the main herd, if the animals are found to be positive they should be treated with dual actives, kept on dirty paddocks and tested three weeks after treatment administration to assess if they are still infected *F. hepatica* (Kelley et al. 2016). In Chapter 5, it was identified that only 22% of farmers quarantined newly purchased or returned animals. This was evident in Chapter 3, as one farm in the Torrumbarry Irrigation District was found to have a within-herd prevalence of 100%, in contrast to all other farms and animals in the region that we studied tested negative for *F. hepatica.* The most likely explanation for this finding is a breakdown in quarantine practices on that farm. In Chapter 3, prevalence data could not be combined with cattle movement data. Future prevalence studies in Australia should link findings with the National Livestock Identification System and Property Identification Codes to assess livestock movement's impact on *F. hepatica* prevalence on individual farms. This method was used by Innocent et al. (2017) in the UK to strategically direct resources into regions/farms that were identified as having a higher incidence of *F. hepatica* and generated the evidence base to inform regional control options.

The key to long-term control of *F. hepatica* in Victoria is determining where *F. hepatica* is endemic and how widespread fluke drug resistance is in cattle. Our results in Chapter 3 confirm a high prevalence in MID, UM and MV irrigation districts that TCBZ was present on three additional farms and that reduced production due to infection with *F. hepatica* cost farmers approximately $50,000 per farm in lost milk production (Kelley et al. 2020). However, opportunities to mitigate such losses were also identified. Strategies included: using diagnostic tools to determine whether it is necessary to treat, testing the efficacy of flukicides after treatment, and using quarantine practices to prevent the introduction of parasites and resistant fluke strains. These strategies can be combined to develop an IPM plan which then can be implemented on-farm.

6.3 Chapter 4 – Analysis of daily variation in the release of faecal eggs and coproantigen of *Fasciola hepatica* in naturally infected dairy cattle and the impact on diagnostic test sensitivity

Chapter 4 established how to use the cELISA and LFEC on-farm to inform strategic flukicide drenching and IPM strategies. Previously the cELISA and LFEC have been successfully used in cattle to assess whether *F. hepatica* was resistant to TCBZ and closantel, monitoring infections over consecutive months, and to determine the prevalence of *F. hepatica* in dairy regions in Victoria (Brockwell et al., 2014; Elliott et al., 2015; Kajugu et al., 2015; Novobilský and Höglund, 2015; George et al., 2019; Kelley et al., 2020). However, it has not been established whether the diagnostic sensitivity was affected by sample collection timing during the day, although some studies have shown that these tests are quantitative and can reflect the burden within the liver (Mezo et al., 2004; Charlier et al., 2008; Brockwell et al., 2013). In Chapter 4, we established that both the cELISA and LFEC are robust and sensitive tools that can accurately identify cattle with burdens exceeding 10 *F. hepatica,* which is the cut-off for production loss in naturally infected dairy cattle in Belgium; the optimal cut-off in Australia has yet to be determined (Charlier et al., 2008). The following section discusses Chapter 4 and how the findings can inform strategic flukicide treatments and IPM strategies.

The lack of routine testing was identified in Chapters 3 and 5 to be a problem. Farmers should strategically treat based on diagnostic outcomes, as the cost for testing ten cattle with the LFEC or cELISA is about AUD 250 compared to the AUD 1900 for drenching with flukicide plus labour costs (G Rawlin 2019, personal communications, 3 September). Strategic treatments informed by diagnostic tests reduce the number of flukicide treatments and limit the influence of convenience in the decision making process (Parr and Gray, 2000; Mezo et al., 2008; Easton et al., 2018). As discussed in Chapter 4, we established that the cELISA using the cut-offs described in Brockwell et al. (2013), Brockwell et al. (2014), Elliott et al. (2015) and Charlier et al. (2008) had a 100% sensitivity. The cELISA accurately detected all positive cattle at every sampling point, even in the lowest burden animal (infected with nine flukes). In contrast, using the commercial kit cut-off or the cut-offs described in Mezo et al. (2004), Palmer et al. (2014) and Martínez-Sernández et al. (2016) resulted in decreased sensitivity and false negatives. Sampling in the AM was found to have the highest correlation to fluke burden (R = 0.64) than R = 0.58 when sampling in the PM. The variation in the release of coproantigens was highest at the PM sampling time but did not greatly affect the overall correlation to the burden in the liver (R = 0.64 AM, R = 0.58 PM).

With the LFEC, sampling in the AM was also found to have the highest correlation to burden R = 0.78 than R = 0.66 when sampling in the PM. Chapter 4 determined that the sensitivity of the

FlukeFinder kit® was 88% which is the first time the sensitivity has been reported for this sedimentation technique first described in Malone and Craig (1990). All 71 false negatives were in animals with burdens <14 *F. hepatica.* However, 42 of the false negatives were from animal 650, which had the lowest burden (nine flukes), less than the production cut-off of 10 described in Charlier et al. (2008). Based on the findings in Chapter 4, we would advise sampling in the AM if using the LFEC or the cELISA and that both methods are semi-quantitative for estimating the fluke burden. The season in which sampling occurs should also be considered, as work published in Mazeri et al. (2016) found evidence to suggest that the sensitivity of the cELISA and the LFEC are affected by the season. The lowest sensitivity was observed in autumn for the LFEC because of the presence of immature *F. hepatica*, whereas the sensitivity was lowest in summer for the cELISA. However, the cELISA cut-off used in Mazeri et al. (2016) was the Palmer et al. (2014) cut-off, which we established to be one of the least sensitive methods. Further research would need to be done to assess if the sensitivity of the cELISA is affected by season when using a more accurate cut-off.

All ten animals tested in Chapter 4 were positive for paramphistomes. Previous work found that the cELISA does not cross-react with paramphistomes and the LFEC has a high specificity (Mezo et al., 2004; Rapsch et al., 2006; Kajugu et al., 2015; Mazeri et al., 2016). The paramphistome egg counts indicated that some cattle had burdens >140 adults in the rumen (Willmott and Pester, 1952). A high proportion of cattle were also observed to be-coinfected with rumen fluke in Chapter 3 (data not shown). Paramphistomes were thought to be relatively benign in Australia, but burdens this high have never previously been reported. Given the high counts and estimated burdens, research needs to confirm the impact of paramphistomes on the health of dairy cattle and production. The only chemical with efficacy against paramphistomes is oxyclozanide (OXY) which only kills the adult parasites (Rolfe and Boray, 1987). Suppose if paramphistomes are found to have an impact on the health and production of cattle. In that case, there is only one product registered with the Australian Pesticides and Veterinary Medicines Authority (APVMA) that contains OXY, but it no longer lists paramphistomes on the label (APVMA, 2021) (Supplementary 5.2). The use of OXY must be carefully managed as it reduces milk production for two days, causes scouring, and extreme care must be taken if treating ill, pregnant, or stressed animals as the product has a low safety margin (APVMA, 2021). OXY is also one of three products registered for the treatment of *F. hepatica* in dairy cattle. However, the side effects likely limit its appeal to dairy farmers even though it can be used during lactation (Supplementary 5.2).

We established in Chapter 4 that the cELISA and LFEC can detect burdens >10 flukes, and that the tests have a high sensitivity and specificity. Our data also suggest that sampling in the AM should become standard industry practice when using the cELISA or LFEC for fluke diagnosis. Therefore, both the cELISA and LFEC can be used to determine when to drench to avoid production losses

and limit pasture contamination. The LFEC can also be used to monitor the paramphistome burden within infected cattle.

6.4 Chapter 5 – *Fasciola hepatica* control practices on a sample of dairy farmers in Victoria, Australia

In Chapter 5, we established the practices used by 36 dairy farmers from across the major irrigated dairy regions in Victoria to determine what chemical and non-chemical approaches they used to control *F. hepatica.* This study was the first *F. hepatica* management survey conducted with dairy farmers in Australia. Here we discuss how *F. hepatica* is controlled on dairy farms in Victoria and assess how IPM could improve the control of *F. hepatica* and reduce production losses. The work in Chapter 5 established that there were four areas where *F. hepatica* control could be improved.

1) Pasture management

A key strategy in IPM is minimising the risk to cattle by preventing access to high-risk areas (i.e. irrigation channels, drains) or using grazing management to move stock to lower risk paddocks (i.e. cropped-land, paddocks with no waterbodies) during high-risk periods. In Chapter 5, we established that cattle on 61% of farms had access to waterlogged areas and irrigation channels which is consistent with work by Watson and Watson (2015), who found that only 35% of dairy farmers in Australia had completely fenced off water bodies to prevent cattle access. Osborne (1962) found that preventing access to water bodies reduced the burden of *F. hepatica* in sheep to 1 to 2 flukes if combined with improved drainage and pasture improvement. On some properties, it may not be possible to fence all water bodies if they are widespread or the size of the property is too large for it to be a financially viable strategy. In these situations, it is likely that only small discrete areas could be fenced. Advances in diagnostic testing soon may enable farmers to assess the infection risk of their pasture and water bodies, allowing the farmer to prioritise the highest risk areas for fencing or costly mechanical interventions like improving the drainage (Rathinasamy et al., 2021). Preventing cattle access to the higher risk areas or waterbodies could also be achieved through pasture rotation by strategically controlling when grazing occurs. Schweizer et al. (2007) found that if farms had many paddocks that could be rotated, pasture management was a cost-effective way of reducing the risk of *F. hepatica* transmission and the faecal contamination of snail habitats. When pasture rotation options were limited during high-risk periods, providing supplementary feed was beneficial in sheep (Osborne, 1962). In places like the MID and UM, where *F. hepatica* is endemic and resistant to TCBZ, there are high stocking rates, and the soil is prone to pugging, suggesting that feed-pads could be used during high-risk periods. Feed-pads are currently installed on 33% of Australian dairy farms and could minimise the risk to cattle and avoid the creation of snail habitats in the pasture during wet weather (Watson and

Watson, 2015). Similarly, some high-risk management strategies need to be stopped, including grazing irrigation channels and not quarantining incoming animals.

2) Diagnostic tests

In discussions with farmers in Chapter 3, the lead author noted that farmers did not perceive stock movement between properties owned by other family members, leased blocks or calves being reared off the farm as needing quarantining when returning to the main farm. Any stock movement without quarantining could introduce parasites or drug resistance to the main farm. Only 22% of farmers surveyed reportedly quarantined their animals. Diagnostics play a key role in informing when treatment should occur and what product should be used. However, only 36% of farmers conducted routine testing for *F. hepatica* in adult cows, and only two farmers tested their replacement animals. The bulk tank milk ELISA (BTM ELISA) (Salimi-Bejestani et al., 2005) was more widely used in Chapter 5 than LFECs, 33% and 28%, respectively. The under-utilisation of diagnostic tests was discussed in Chapter 3. A higher frequency of diagnostic testing in all stock categories would improve the management of *F. hepatica*, minimise the use of flukicides and prevent the long-term impact *F. hepatica* has on the fertility of replacement animals (Oakley et al., 1979; Parr and Gray, 2000; Mezo et al., 2008).

3) Flukicides in Australia

Flukicides were used by 72% of respondents in the 2015/2016 financial year, and the most widely used flukicide was CLOR which is consistent with findings in Chapter 3. A large proportion of these farmers solely relied on CLOR, which could be a problem for future control of *F. hepatica.* The overreliance and repeated use of a single active over time may prove to be a strong selector for the development of resistance (Kelley et al. 2016). A method for testing the efficacy of CLOR has been described by Elliott et al. (2015); however, the method has not been assessed and findings published. Based on the findings of this thesis, it is clear that a method needs to be developed and assessed, so the efficacy of CLOR can be monitored on dairy farms in Victoria. Dairy farmers are limited to only three flukicides to treat their cattle (Supplementary 5.2). With growing reports of TCBZ resistance on dairy farms in Victoria, the only product that has not been widely used is OXY, which only one farmer used in Chapter 5. (Brockwell et al., 2014; Elliott et al., 2015; Kelley et al., 2020). Dairy farmers should be encouraged to incorporate OXY into their flukicide rotation, which will also benefit the animals that are co-infected with paramphistomes, as discussed in Chapter 4.

4) Administration of flukicides

In addition, in Chapter 5, it was determined that underdosing of cattle was likely prevalent within the dairy industry, but further research is needed to confirm the incidence. Fifty per cent of the respondents estimated or used average weights to determine the flukicide dose to administer to

their stock. In a study by Machila et al. (2008), farmers were found to underestimate cattle's weight by, on average, 40%. Another contributing factor to the underdosing is drenching equipment, which needs to be calibrated at the start of drenching and during treatment administration to ensure the correct dose is administered. Questions about whether farmers calibrated their equipment were not investigated in the current survey but should be considered when implementing IPM strategies on-farm.

Combining non-chemical and chemical approaches to control *F. hepatica* will prolong the life of existing flukicides and minimise their use. The cELISA and LFEC should be routinely used to determine when to treat. Non-chemical approaches, including fencing, pasture rotation and feed-pads, should limit the exposure of cows to metacercariae and the faecal contamination of snail habitats. Work needs to be done to encourage farmers to implement IPM strategies to minimise the milk production losses estimated to be AUD 50,000 per farm per year (Kelley et al., 2020). An approach could be to assess the effectiveness of these IPM strategies on a representative dairy farm in each dairy region in Victoria so case study materials can be produced that will allow other farmers in the region to replicate the IPM plan on their farms.

6.6 Conclusion

In conclusion, the average state-wide prevalence of *F. hepatica* in dairy cattle was 39% by cELISA and LFEC and cost the Victorian dairy industry an estimated AUD 129 million per year in lost milk production or about 6% of total production in Victoria (Kelley et al., 2020). *F. hepatica* was shown to be endemic in the MID, UM and MV, with 21/39 farms in these regions having a within herd-level prevalence of > 90%. In addition, two more cases of TCBZ resistance were identified in the MID and one more case in the UM. In total, five dairy farms in Victoria have now been confirmed to have TCBZ-resistant fluke infections and all five farms are located in either the MID or UM (Brockwell et al., 2014; Elliott et al., 2015; Kelley et al., 2020).

There are only three registered chemical classes available to treat *F. hepatica* dairy cattle in Australia (Supplementary 5.2). CLOR was widely used in Chapter 3 and Chapter 5 by farmers to control *F. hepatica,* in contrast, only one farmer used OXY. Therefore farmers should be encouraged to include OXY in their flukicide rotations or in combination with other products to preserve the longevity of TCBZ and CLOR. Currently, there is no field methodology to test for resistance to a flukicide product that targets only the adult *F. hepatica.* One needs to be developed, so CLOR's efficacy can be monitored on dairy farms in Victoria.

The Republic of Ireland (ROI) recently established maximum residue limits in milk for many chemoprophylactic drugs (Bloemhoff et al., 2014). As a consequence, the use of several flukicides was restricted in the dairy industry. Bloemhoff et al. (2014) found that licensing restrictions had

wide-reaching impacts on flukicide use on dairy farms. If additional regulatory restrictions were imposed on the use of flukicides in Australia by the APVMA, it could significantly impact dairy production and severely handicap dairy farmers' ability to control *F. hepatica* in Victoria. Regulatory changes would have a more acute impact in the *F. hepatica* endemic regions identified in Chapter 3. In contrast, it would have little impact on *F. hepatica* control in the GM irrigation district. The GM irrigation district's findings provide a unique opportunity to investigate the impact of irrigation infrastructure upgrades on the epidemiology of *F. hepatica*. Such research could also shed light on how the planned irrigation upgrades in MID could affect the prevalence of *F. hepatica* in the region (McCormack et al., 2020).

Chapter 4 established that both the cELISA and LFEC were robust tools that can be used on farms to inform decision making when it comes to determining when to treat, what to treat with and if *F. hepatica* is TCBZ-resistant. The BTM ELISA and the LFEC were underutilised on-farm, and there was no use of the cELISA. Chapter 5 found that only 2/36 farmers used tests to inform when they treated their animals. Encouraging farmers to base their management decisions on diagnostic tests has been achieved in the dairy industry in relation to soil tests and fertiliser application (Watson and Watson, 2015). This strategy used by Dairy Australia to encourage increased use of soil tests could be adapted to parasite testing. However, a long-term strategic approach would be needed to ensure a change in the industry and increase the on-farm use of diagnostic tests. Forecasting 'high-risk *F. hepatica* periods' could simplify the process and identify approximately when farmers should test their herds. Given that OXY and CLOR can be used during lactation in Australia, strategic treatments are possible for the milking herd.

Chapter 5 also identified opportunities where new control practices could be implemented to minimise the impact of *F. hepatica* on the dairy industry in Victoria. These IPM strategies can be used in conjunction with chemical controls to maximise the longevity of the existing flukicides shown in supplementary 5.2. The results from the thesis suggest the following advice: dairy farmers should be encouraged to:

- Fence off high-risk areas and limit faecal contamination of *A. tomentosa* habitat.
- Manage pastures to allocate lower risk paddocks to more vulnerable animals and use feed-pads to avoid creating *A. tomentosa* habitats within the pasture during wet weather.
- Routinely test cattle with the BTM ELISA in the GM irrigation district and the LFEC and cELISA in endemic regions listed in Chapter 3.
- Quarantine test and treat all animals moving on and off farms to avoid the introduction of *F. hepatica* and resistance.

- Improve the accuracy of flukicide dose administration by weighing animals and calibrating drench equipment.

Implementing all these IPM strategies will not be easy and, in some cases, expensive. Given *F. hepatica* is estimated to cost farmers approximately AUD 50,000 per farm in lost milk production, a small economic study that assesses both the financial benefits and the effectiveness of implementing IPM strategies could be the easiest way to communicate and encourage change in the dairy industry (Kelley et al. 2020; Torres-Acosta et al., 2012). Due to the comparatively small size of the Australian dairy market and the complexity and expense of registering new flukicides or vaccines with the AVPMA; it is unlikely that a new product will be developed and released for use in the Australian dairy industry in the near future. Consequently, practical strategies need to be combined into an IPM plan and then implemented on-farm to preserve the efficacy of currently registered flukicides for future use in the dairy industry. With similar problems arising in dairy industries globally, the issues, research gaps and findings from the thesis are relevant beyond Australia and can be used to identify *F. hepatica* research priorities and inform *F. hepatica* control on dairy farms globally.

6.7 References

APVMA 2021. Coopers Nilzan LV oral drench (APVMA PubCRIS database, APVMA). Available: https://portal.apvma.gov.au/pubcris?p_auth=1KLhCxm9&p_p_id=pubcrisportlet_WAR_pubcrisportlet&p_p_lifecycle=1&p_p_state=normal&p_p_mode=view&p_p_col_id=column-1&p_p_col_pos=2&p_p_col_count=4&_pubcrisportlet_WAR_pubcrisportlet_id=36089&_pubcrisportlet_WAR_pubcrisportlet_javax.portlet.action=viewProduct [Accessed 25/02/ 2021].

Bloemhoff, Y., Danaher, M., Forbes, A., Morgan, E., Mulcahy, G., Power, C., Sayers, R., 2014. Parasite control practices on pasture-based dairy farms in the Republic of Ireland. Veterinary Parasitology 204, 352-363.

Boray, J., 1964. Studies on the ecology of *Lymnaea tomentosa*, the intermediate host of *Fasciola hepatica*. 1. History, geographical distribution, and environment. Australian Journal of Zoology 12, 217-230.

Boray, J., Crowfoot, P., Strong, M., Allison, J., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. The Veterinary Record 113, 315-317.

Boray, J., Happich, F., Andrews, J., 1969. The epidemiology of fasciolosis in two representative endemic regions of Australia. Australian Veterinary Journal 45, 549-553.

Brockwell, Y., Spithill, T., Anderson, G., Grillo, V., Sangster, N., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Veterinary Parasitology 196, 417-426.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. International Journal for Parasitology: Drugs and Drug Resistance 4, 48-54.

Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Veterinary Parasitology 153, 44-51.

Durr, P., Tait, N., Lawson, A., 2005. Bayesian hierarchical modelling to enhance the epidemiological value of abattoir surveys for bovine fasciolosis. Preventive Veterinary Medicine 71, 157-172.

Easton, S., Pinchbeck, G.L., Bartley, D.J., Hodgkinson, J.E., Matthews, J.B., 2018. A survey of experiences of UK cattle and sheep farmers with anthelmintic prescribers; are best practice principles being deployed at farm level? Preventive Veterinary Medicine 155, 27-37.

Elliott, T., Kelley, J., Rawlin, G., Spithill, T., 2015. High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Veterinary Parasitology 209, 117-124.

George, S., George, A., Rolfe, P., Emery, D., 2019. Comparative assessment of faecal diagnostics for detection and predictive modelling of endemic *Fasciola hepatica* infection in sheep and cattle on Australian farms. Veterinary Parasitology: X 1, 100001.

Harris, R., Charleston, W., 1977. An examination of the marsh microhabitats of *Lymnaea tomentosa and L. columella* (Mollusca: Gastropoda) by path analysis. New Zealand Journal of Zoology 4, 395-399.

Hart, B., Walker, G., Katupitiya, A., Doolan, J., 2020. Salinity Management in the Murray–Darling Basin, Australia. Water 12, 1829.

Innocent, G.T., Gilbert, L., Jones, E.O., McLeod, J.E., Gunn, G., McKendrick, I.J., Albon, S.D., 2017. Combining slaughterhouse surveillance data with cattle tracing scheme and environmental data to quantify environmental risk factors for liver fluke in cattle. Frontiers in Veterinary Science 4, 65.

Kajugu, P.-E., Hanna, R., Edgar, H., McMahon, C., Cooper, M., Gordon, A., Barley, J., Malone, F., Brennan, G., Fairweather, I., 2015. *Fasciola hepatica*: Specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Veterinary Parasitology 212, 181-187.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in Fasciola hepatica. Trends in parasitology 32, 458-469.

Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Veterinary Parasitology 277, 109019.

Lean, I., Westwood, C., Playford, M., 2008. Livestock disease threats associated with intensification of pastoral dairy farming. New Zealand Veterinary Journal 56, 261-269.

Machila, N., Fèvre, E.M., Maudlin, I., Eisler, M.C., 2008. Farmer estimation of live bodyweight of cattle: implications for veterinary drug dosing in East Africa. Preventive Veterinary Medicine 87, 394-403.

Malone, J., Craig, T., 1990. Cattle liver flukes: risk assessment and control. Compendium on Continuing Education for the Practicing Veterinarian 12, 747-754.

Martínez-Sernández, V., Orbegozo-Medina, R.A., González-Warleta, M., Mezo, M., Ubeira, F.M., 2016. Rapid enhanced MM3-COPRO ELISA for detection of *Fasciola* coproantigens. PLoS Neglected Tropical Diseases 10, e0004872.

Mazeri, S., Sargison, N., Kelly, R.F., Bronsvoort, B.M.d., Handel, I., 2016. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. PloS one 11.

McCormack, M., Chester, D., Neville, L., Garrett, J. 2020. More water for Gippsland with start of Phase 2 of Macalister Irrigation District project. In Press release (https://minister.infrastructure.gov.au/mccormack/media-release/more-water-gippsland-start-phase-2-macalister-irrigation-district-project, National Water Grid).

Mezo, M., González-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). Journal of Parasitology 90, 845-852.

Mezo, M., González-Warleta, M., Castro-Hermida, J.A., Ubeira, F.M., 2008. Evaluation of the flukicide treatment policy for dairy cattle in Galicia (NW Spain). Veterinary Parasitology 157, 235-243.

Novobilský, A., Höglund, J., 2015. First report of closantel treatment failure against *Fasciola hepatica* in cattle. International Journal for Parasitology: Drugs and Drug Resistance 5, 172-177.

Oakley, G., Owen, B., Knapp, N., 1979. Production effects of subclinical liver fluke infection in growing dairy heifers. The Veterinary Record 104, 503-507.

Osborne, H., 1962. Fascioliasis and black disease in sheep in New England, NSW. Australian Veterinary Journal 38, 1-7.

Overend, D., Bowen, F., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Australian Veterinary Journal 72, 275-276.

Palmer, D., Lyon, J., Palmer, M., Forshaw, D., 2014. Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses. Australian Veterinary Journal 92, 357-361.

Parr, S.L., Gray, J.S., 2000. A strategic dosing scheme for the control of fasciolosis in cattle and sheep in Ireland. Veterinary Parasitology 88, 187-197.

Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. International Journal for Parasitology 36, 1153-1158.

Rathinasamy, V., Tran, L., Swan, J., Kelley, J., Hosking, C., Williamson, G., Knowles, M., Elliott, T., Rawlin, G., Spithill, T.W., Beddoe, T., 2021. Towards understanding the liver fluke transmission dynamics on farms: Detection of liver fluke transmitting snail and liver fluke-specific environmental DNA in water samples from an irrigated dairy farm in Southeast Australia. Veterinary Parasitology 291, 109373.Rolfe, P., Boray, J., 1987. Chemotherapy of paramphistomosis in cattle. Australian Veterinary Journal 64, 328-332.

Salimi-Bejestani, M., Daniel, R., Felstead, S., Cripps, P., Mahmoody, H., Williams, D., 2005. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Veterinary Record-English Edition 156, 729-731.

Schweizer, G., Meli, M.L., Torgerson, P.R., Lutz, H., Deplazes, P., Braun, U., 2007. Prevalence of *Fasciola hepatica* in the intermediate host *Lymnaea truncatula* detected by real time TaqMan PCR in populations from 70 Swiss farms with cattle husbandry. Veterinary Parasitology 150, 164-169.

Tanaka, H., Tsuji, M., 1997. From discovery to eradication of schistosomiasis in Japan: 1847–1996. International Journal for Parasitology 27, 1465-1480.

Torres-Acosta, J., Mendoza-de-Gives, P., Aguilar-Caballero, A., Cuéllar-Ordaz, J., 2012. Anthelmintic resistance in sheep farms: update of the situation in the American continent. Veterinary Parasitology 189, 89-96.

Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. Veterinary Parasitology 98, 195-214.

Watson, P., Watson, D., 2015. Sustainability Framework NRM Survey (http://www.dairyingfortomorrow.com.au/wp-content/uploads/Australian-Dairy-Sustainability-Framework-NRM-Survey-2015.pdf, Dairy Australia), pp. 1-64.

Watt, G., 1979. An approach to determining the prevalence of liver-fluke in a large region. In: Proceedings of the 2nd International Symposium on Veterinary Epidemiology and Economics, 7-11 May 1979, Canberra, Australia., pp. 152-155.

Willmott, S., Pester, F., 1952. Variations in faecal egg-counts in paramphistome infections as determined by a new technique. Journal of Helminthology 26, 147-156.

Chapter 7 – Appendix

7.1 Accepted Manuscript – Analysis of Daily Variation in the Release of Faecal Eggs and Coproantigen of *Fasciola Hepatica* in Naturally Infected Dairy Cattle and the Impact on Diagnostic Test Sensitivity

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Research paper

Analysis of daily variation in the release of faecal eggs and coproantigen of *Fasciola hepatica* in naturally infected dairy cattle and the impact on diagnostic test sensitivity

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ABSTRACT

The liver fluke, *Fasciola hepatica (F. hepatica)* is a widespread parasite infection in dairy cattle in Victoria, South-eastern Australia. Robust diagnosis of fluke infection is needed in dairy cattle to identify sub-clinical infections which often go unnoticed, causing significant production losses. We tested the coproantigen ELISA (cELISA) and the FlukePinder faecal egg count kit® on naturally infected cows in a fluke endemic region of Victoria. The aim of the study was to investigate the variation in the release of coproantigens and eggs into faeces over a 5-day period, at the morning (AM) and afternoon (PM) milkings, and to assess the impact of the timing of faecal sample collection on diagnostic test sensitivity. Ten cows were enrolled into the study based on positive *F. hepatica* faecal egg counts (LFEC) and faecal samples from the ten cows were collected twice daily, at the 7–9 AM and 4–6 PM milking, for five consecutive days. At the conclusion of the sampling period, the cows were euthanized and *F. hepatica* burden determined at necropsy. A moderate negative correlation between cow age and cELISA optical density (OD) was observed using data from all samples (R -0.63; 95 % CI -0.68 to -0.57). Over the 5-day sampling period, we observed within-animal variation between days for both the cELISA OD (2.6–8.9 fold) and LFEC (5–16 fold), with more variation in values observed in the PM samples for both tests. The correlation with total fluke burden was higher in the AM sampling using both the cELISA and LFEC (R 0.64 and 0.78, respectively). The sensitivity was 100 % for the cELISA using various cut offs from the literature (0.014 OD, 0.030 OD, and 1.3 % or 1.6 % of the positive control). The sensitivity of the FlukePinder kit® (based on 588 faecal samples and not accounting for lack of independence in the data) was 88 % (95 % CI 85 %–90 %). Seventy one false negatives were recorded from the 588 LFEC tests all of which were observed in the cows with fluke burdens <14 flukes; 42 of the 71 false negative LFECs occurred in one individual cow which had the lowest burden of nine flukes. In dairy cows, the cut-off for production losses due to fasciolosis is estimated at> 10 fluke. Both the cELISA and the LFEC identified all cows that had burdens equal to or greater than this cut-off. Five of the ten cows also exhibited relatively high paramphistome egg counts.

1. Introduction

Fasciola hepatica (*F. hepatica*), more commonly known as liver fluke, has serious production-limiting impacts in dairy cattle, affecting milk production, milk quality, weight gain and fertility (Schweizer et al., 2005). In the UK the economic cost of reduced production due to fluke infections is estimated at £300 million per year (Williams et al., 2014). In Australia in 2020, reduced milk production due to *F. hepatica* infection was estimated to cost the Victorian dairy industry USD 101 million per year (Kelley et al., 2020). A robust quantitative diagnostic test that can identify *F. hepatica*-infected individuals and herds would allow for prompt intervention and treatment of cattle with appropriate flukicides. Europe has moved towards screening dairy herds with an ELISA detecting antibodies in bulk tank milk (BTM) samples which are readily

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available and easy to collect (Pritchard et al., 2005; Salimi-Bejestani et al., 2005; Charlier et al., 2007; Bennema et al., 2009; McCann et al., 2010; Kuerpick et al., 2013; Selemetas et al., 2014; Bloemhoff et al., 2015; Howell et al., 2015; Novobilský et al., 2015). The bulk tank milk sELISA (BTM sELISA) was first described by Salimi-Bejestani et al. (2005): this assay has a high sensitivity (96 %), and moderate specificity (80 %) and can accurately identify herds that are incurring production losses when herd prevalence is in excess of 25 % (Charlier et al., 2007). However, the BTM ELISA has its drawbacks as anti-*Fasciola* antibodies in milk can persist for up to six months even after successful treatment with a flukicide (Salimi-Bejestani et al., 2005). In Australia, most dairy herds use a split calving system, which means that flukicide treatments occur at different times of the year for individual herds. As a result, antibodies found in milk could arise from treated and untreated cattle which complicates the interpretation of a positive BTM ELISA test.

As a consequence, a different approach has been used to screen dairy cattle in Australia. Brockwell et al. (2014), Elliott et al. (2015) and Kelley et al. (2020) each used the commercial coproantigen ELISA (BIO K 201 kit, Bio X Diagnostics) test to screen multiple herds for *F. hepatica* as coproantigen release ceases 7 days after effective treatment with a flukicide (Brockwell et al., 2013). The coproantigen ELISA (cELISA) detects infection in cattle from >6 weeks post-infection (PI) and has a high sensitivity 77 %–100 % and specificity >99 % (Mezo et al., 2004; Brockwell et al., 2013; Mazeri et al., 2016). In addition, correlations between *F. hepatica* burden and OD were observed in cattle by Charlier et al. (2008) (R 0.60) and Brockwell et al. (2013) (R^2 0.8718) although recent work by Martínez-Sernández et al. (2016) found a somewhat weaker correlation (R^2 0.2998). However, Brockwell et al. (2013) observed a 2–6 fold variation in coproantigen release from cattle over a 5 day period. To address the variable release of coproantigens the cELISA kit was modified by Martínez-Sernández et al. (2016) increasing the sensitivity from 0.60 ng/mL to 0.15 ng/mL; however, the variability in the cELISA in daily samples increased by 6–12 fold (Mezo et al., 2004). There is a consensus in the literature that the cELISA kit-cut off recommended by the commercial manufacturer is too high to accurately distinguish between positive and negative cattle. As a result, studies have used various ELISA OD cut-offs for detecting *F. hepatica* infections in cattle: 0.114 OD (Mezo et al., 2004) 0.030 OD (Charlier et al., 2008), 0.014 OD (Brockwell et al., 2013), kit cut off × 0.67 (Palmer et al., 2014), 0.084 OD (Martínez-Sernández et al., 2016) as well as 1.3 % (Brockwell et al., 2014) or 1.6 % (Elliott et al., 2015) of the OD value of the positive control. The lack of consistency between reports makes it difficult to determine the sensitivity of the cELISA, but several studies in cattle have reported that the assay can detect as few as 1, 2, and 15 flukes in the liver (Mezo et al., 2004; Brockwell et al., 2013; Martínez-Sernández et al., 2016).

Similar problems occur when using *F. hepatica* faecal egg counts (LFEC). In cattle with low *F. hepatica* burdens (<10 flukes) false negatives frequently occur (Martínez-Sernández et al., 2016). There are many variations on the LFEC technique, but sedimentation has been found to be the most accurate in cattle (Happich and Boray, 1969; Kajugu et al., 2015). Two studies reported correlations between LFEC and *F. hepatica* burden. In cattle, the correlation was R^2 0.836 and in sheep R^2 0.571 (Brockwell et al., 2013; George et al., 2017). LFECs are highly specific >97.5 % (97.5–100%) and egg shedding does not persist after treatment with an effective flukicide (Ibarra et al., 1998; Anderson et al., 1999; Rapsch et al., 2006; Brockwell et al., 2013; Mazeri et al., 2016). However, weekly, daily and hourly variation *in F. hepatica* egg shedding has been observed in several studies in cattle (Dorsman, 1956, 1960; Hagens and Over, 1966; Brockwell et al., 2013). Based on hourly faecal sampling in cattle, Dorsman (1956) proposed that faecal collection should occur at 1:30 p.m. when the highest egg release was more likely to represent the burden of *F. hepatica* within the liver. Hagens and Over (1966) reached the same conclusion observing the peak release of eggs between 12:00 p.m.–8:00 p.m., similarly suggesting that this was the most suitable time for sampling cattle. However, the sensitivity of the LFEC is affected by the volume of faeces sampled, the faecal output by the animal, the burden of *F. hepatica* within the animal, the experience of the technician and the duration of the *F. hepatica* infection as the test only detects *F. hepatica* from > 8 PI weeks in cattle (Boray, 1969; Conceição et al., 2002; Rapsch et al., 2006; Charlier et al., 2008; Brockwell et al., 2013; Martínez-Sernández et al., 2016).

1.1. Objectives

Previous studies have investigated the level of variability in coproantigen shedding in animals between weeks and on consecutive days, but not variations within a day. Monitoring of *F. hepatica* egg shedding variation has been extensive. However, it has never been determined if peak egg shedding from 12:00 p.m. - 8:00 p.m. actually correlates with *F. hepatica* burden in the liver and is therefore a better time to collect faecal samples from cattle. In this study, recognising the variable release of both coproantigens and eggs, we investigated the sensitivity of two sample points in the morning (AM) and afternoon (PM) milking, the variation in coproantigen and LFEC shedding over a consecutive five-day period and the correlation of coproantigen levels and LFEC with *F. hepatica* burden in ten naturally infected dairy cows.

2. Methods

2.1. Study design

One pasture-fed, split calving dairy herd in Victoria, Australia identified by Kelley et al. (2020) was purposively selected for this study based on the herd owner's willingness to participate. Thirty cows were screened using the FlukeFinder® kit to determine if they were infected with *F. hepatica*. Ten cows were selected based on positive LFEC and purchased from the owner. The age of the cows ranged from 2.9 to 11.1 years and the predominant breed was Holstein. Following purchase, the ten selected cows remained on the farm and were kept separately from the main milking herd but grazed pasture and received grain and concentrate at milking as for the main milking herd. The study group was milked twice daily after the main herd: a morning milking (AM) between 7:00 a.m. and 9:00 a.m. and an afternoon milking (PM) between 4:00 p.m. and 6:00 p.m.. Sample collection over 5 consecutive days began on the 30 March 2017 and concluded on 03 April 2017. At the AM and PM milkings on each of the five sampling days 50 g faecal samples were collected rectally from each of the ten study group cows; a new rectal examination sleeve was used for each cow. On day six (04 April 2017) nine of the ten cows were euthanised. One cow (#3491) was unable to be yarded on day six and was instead euthanised on day 14 (12 April 2017). Cows were euthanised in two groups: group 1 between 10:00 a.m. and 11:00 a.m. and group 2 between 1:00 p.m. and 2:00 p.m. Livers and gall bladders were removed within 30 min of death and livers were stored at 4 °C until they could be sectioned.

This study was approved by the La Trobe University Ethics Committee, AEC16-62 and ran in conjunction with the State Government of Victoria, Department of Jobs, Precincts and Regions pathology and quarantine training program for veterinarians and animal health officers.

2.2. Faecal sample collection over 5 days

Faecal samples from each cow at the AM and PM milkings were split into two replicates. Replicate A and B each weighed approximately 25 g. Faecal matter was mixed thoroughly from each replicate and then two 2 g samples were weighed for each cow; aliquot 1 for the cELISA, and aliquot 2 for the LFEC. cELISA replicates were stored at 4 °C until aliquots were weighed, the aliquots were then frozen at −20 °C within 72 h of collection. LFEC aliquots were weighed and stored at 4 °C until counts were completed. On two occasions, faecal samples were unable to be collected: cow #3294 on day 4 PM and cow # 844 on day 5 PM. Faecal

samples collected on day 6 (the day of euthanasia) were not included in the analyses presented in this paper because faecal samples were collected outside of the time window for AM and PM milkings and were not replicates.

2.3. Total fluke count

The total liver fluke counts (TFC) were completed following the guidelines outlined by Reichel (2002) and Brockwell et al. (2013). Before removing the liver from the abdominal cavity, the small intestines were tied off using cable ties approximately 30 cm either side of the gallbladder, ensuring the gallbladder was left intact. The livers were removed and stored in individual ice boxes on ice. The livers were then moved to a 4 °C fridge and remained there until sectioned which occurred within 72 h. The livers were then cut into 5–10 mm strips and squeezed to remove *F. hepatica* from the bile ducts. Once the liver was sectioned, the slices of liver were soaked in PBS (1 × PBS including 16 mM Na_2HPO_4, 5 mM NaH_2PO_4.H2O, 120 mM NaCl; pH 7.4) and left at room temperature overnight. The following morning individual livers slices were soaked in a tub of warm water. The PBS and water were sieved, and all containers were inspected for *F. hepatica*. The TFC was determined by counting whole intact *F. hepatica*, plus the highest count for either head or tails from partially recovered *F. hepatica*. No noticeable scarring was present in nine of the ten livers. Animal 4316 had severe scarring, bile duct thickening and a pale appearance (data not shown). The researcher conducting the diagnostic testing remained blind to the results of the TFC until completion of all LFEC and cELISA.

2.4. cELISA

Faecal aliquots were stored at −20 °C until analysis using the cELISA Faecal BIO K 201 kit from Bio X Diagnostics, Belgium. The method used is described in Kelley et al. (2020) which includes details of modifications based on Brockwell et al. (2013). The cELISA plates were loaded with samples from one cow per strip (column): two duplicates of replicate A and B were added. The cELISA plate was also loaded with the following antigen controls: negative control (dilution buffer); kit positive control (Kit + ve) (the positive control reference antigen provided by the manufacturer with the kit batch, no dilution); a pooled high positive antigen extract (High + ve) sourced from a pool of faeces from known infected animals with a cELISA OD > 1.0; and a pooled low positive antigen extract (Low + ve) sourced from a pool of faeces from known infected animals with a cELISA OD < 0.8. These High + ve and Low + ve extracts were sourced from several artificially infected cattle from a separate study conducted by our laboratory (data not shown). All values are presented as either raw OD (450 nm) or the raw OD expressed as a percentage of the OD observed with various positive control antigens: the Kit + ve control, the High + ve control, or the Batch positive control (Batch + ve). The Batch + ve control is the OD provided with each kit batch by the manufacturer. All cELISA plates were from the same batch FASA16I07 and the Batch positive control OD value was 1.815.

2.5. F. hepatica and paramphistome faecal egg counts

The FlukeFinder® kit was used to determine the LFEC and paramphistome faecal egg count (PFEC) as high paramphistome egg counts were observed. The sedimentation method was used as described by Kelley et al. (2020). LFEC and PFEC are reported as the number of eggs per 2 g of faeces (ep2 g). Each LFEC sample was counted three times to assess technician accuracy given the high numbers of paramphistome eggs present in the samples. PFEC were not recorded for the following cows: cow #536 (day 4 AM replicate A), cow #836 (day 5 PM replicate A), cow #844 (day 1 PM replicate A and day 5 PM), cow #1100 (day 1 AM replicate A and day 5 AM replicate A), cow #3294 (day 4 PM, replicate A and B) and cow #3491 (day 5 AM replicate B).

2.6. Statistical analyses

Correlations between age, TFC and AM and PM cELISA OD and AM and PM LFECs were quantified using Spearman's rank correlation (R). Scatterplots were constructed to show cELISA OD estimates for each AM and PM as a function of sampling day and time. To quantify the difference between cELISA OD estimates for AM and PM sampling, accounting for lack of independence in the data arising from repeated cELISA OD estimates from the same cow over the 5 day study period, we used a mixed-effects linear regression model with sampling time (a categorical variable comprised of two levels: AM and PM) as a fixed effect, sampling day as a random slope and cow identity as a random intercept term. A similar approach was taken for the LFEC estimates. Scatterplots were constructed to show LFEC counts for each AM and PM as a function of sampling day. To quantify the difference between LFEC estimates for AM and PM sampling, a mixed-effects linear regression model was used with Box-Cox transformed LFECs (Box and Cox, 1964) as the outcome variable, sampling time (AM or PM) as a fixed effect, sampling day as a random slope and cow identity as a random intercept term. Analyses were conducted using the contributed nlme package (Bates et al., 2015; Pinheiro et al., 2020) in R (R Core Team, 2020). The presence of TFCs provided a rare opportunity to quantify the diagnostic sensitivity of LFEC for AM and PM samplings. To allow our results to be compared with other, similar studies where faecal samples were collected from individual cows on multiple occasions, diagnostic sensitivity of the LFEC was calculated without accounting for lack of independence in the data. Confidence intervals for diagnostic sensitivity were calculated using the exact method (Collett, 1999). The sensitivity was calculated using the method in Estuningsih et al. (2009).

3. Results

3.1. Summary correlation statistics

All ten cows in this study were *F. hepatica* positive with the number of flukes in the liver ranging from 9 to 72 (Table 1). No correlation was observed between age and TFC (R −0.32; 95 % CI −0.86 to −0.41) or between age and LFEC (R −0.23; 95 % CI −0.30 to −0.15). However, a moderate negative correlation between age and the cELISA OD was observed using data from both AM and PM samples (R −0.63; 95 % CI −0.68 to −0.57) (Fig. 1). At the AM and PM milkings there was a higher positive correlation between the cELISA and TFC at the AM milking (R 0.64; 95 % CI 0.54–0.73) relative to the PM milking (R 0.58; 95 % CI 0.44–0.67) (Fig. 2). A higher positive correlation was also observed between TFC and LFEC at the AM milking (R 0.78; 95 % CI 0.73–0.82) relative to the PM milking (R 0.66; 95 % CI 0.59–0.72) (Fig. 3).

3.2. cELISA controls and sensitivity

Three true positive controls (the kit positive control, a high positive faecal extract control and a low positive faecal extract control) and one negative control were included on all cELISA plates (Table 2). The high and low control extracts were sourced from local artificially infected cattle with a known TFC. The sensitivity of the cELISA was calculated using all the published cut offs based on the raw OD alone. In addition, the raw OD was converted to a percentage of the OD observed with the cELISA batch positive control (Batch + ve), the OD observed with the pooled high extract positive antigen control (High + ve) and the OD observed with the positive antigen control supplied with the kit (Kit + ve control) (Table 3). The highest sensitivity (100 %) was obtained using a number of cut offs and positive controls (Table 3). Of the 392 samples tested using the cELISA all samples were positive using an OD cut-off of 0.014 OD (Brockwell et al., 2013), 0.030 OD (Charlier et al., 2008) or a % cutoff of 1.3 % (Brockwell et al., 2014) and 1.6 % (Elliott et al., 2015): lower sensitivity was observed when the kit cut off of >8 % was used or using the >5.4 % cut-off of Palmer et al. (2014) (Table 3).

Table 1
Cows used in the study, including identification number, total fluke count and age.

Animal ID	412	536	650	836	844	1100	2300	3294	3491	4316
Age	11.1	4.6	8.6	6.0	5.7	3.0	10.0	8.1	3.1	2.9
TFC[a]	14	11	9	54	12	27	56	10	37	72

[a] TFC: total fluke count.

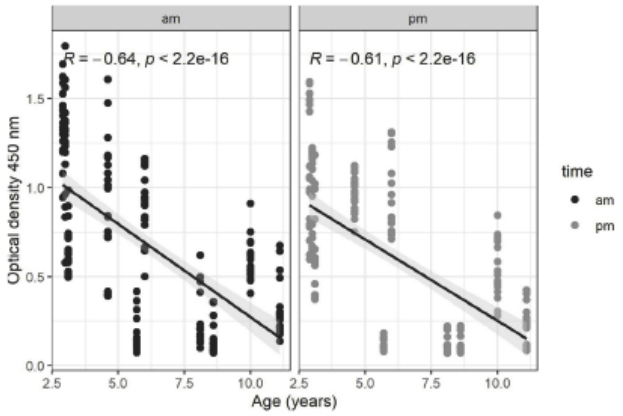

Fig. 1. Scatterplot showing the cELISA OD as a function of cow age for AM and PM sampling events. Superimposed is a line of best fit to the data.

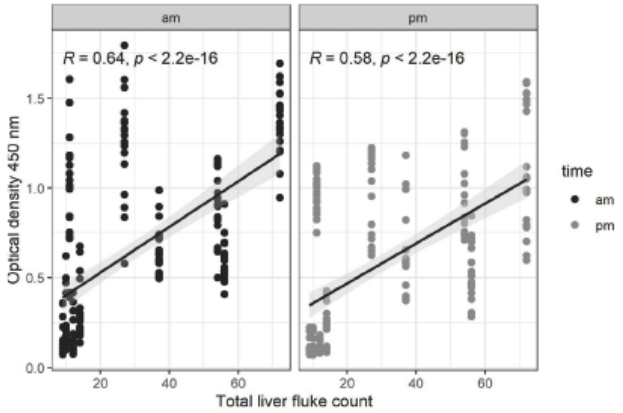

Fig. 2. Scatterplot showing the cELISA OD as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

3.3. cELISA OD variability at AM and PM sampling over 5 days

The kinetics of the cELISA OD values over the 5-day sampling period for each cow are shown in Fig. 4. PM cELISA OD values were, on average, 0.21 (95 % CI 0.04–0.12) OD units less than AM cELISA OD values. There was a 2.6–8.9 fold variation in the OD values for the cELISA over the 5 days with the greatest variation observed in cow #412, #650, #844 and #3294 (Table 4). In general, there was more variation in the PM samples (Fig. 5). Sensitivity of the cELISA was higher using the AM samples (Table 3).

3.4. LFEC counts and sensitivity

In the case of LFEC, 588 samples were counted and all ten cows were found to be positive at necropsy for *F. hepatica* with the LFEC ranging from 0 to 32 ep2 g (Figs. 3 and 6). On 189 occasions the LFEC exceeded the 5 ep2 g cut-off for production loss in cattle described in Malone and Craig (1990) and Vercruysse and Claerebout (2001). The sensitivity of the LFEC (based on 588 faecal samples and not accounting for lack of independence in the data) was 88 % (95 % CI 85 %–90 %) with a total of 71 false negative results recorded: 13 for cow #412; 11 for cow #536; 42

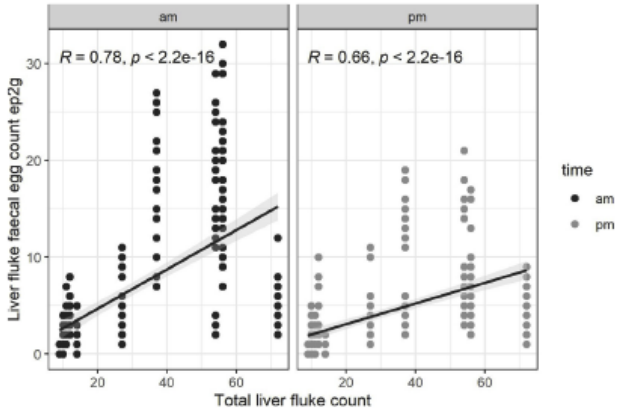

Fig. 3. Scatterplot showing LFEC (ep2g) as a function of total liver fluke count for AM and PM sampling events. Superimposed on each plot is a line of best fit to the data.

Table 2
The range of cELISA OD values obtained over several assays using one negative control and several positive control antigens added to the cELISA plates.

Control	Replicates	Range OD	Avg OD	Lower CI (95 %)	Upper CI (95 %)
Negative control (dilution buffer)[a]	10	0.00–0.00	0.00	0.00	0.00
Kit positive control (Kit + ve)[b]	10	1.34–1.83	1.56	1.45	1.67
High positive extract (High + ve)[c]	10	1.49–2.04	1.80	1.67	1.92
Low positive extract (Low + ve)[d]	10	1.22–1.67	1.41	1.32	1.50

All OD values are presented as OD (450 nm).
[a] Negative control: dilution buffer only.
[b] Kit + ve control: the positive control antigen provided by the manufacturer with the kit batch, no dilution.
[c] High + ve control: the pooled high positive antigen extract sourced from a pool of faeces from known infected animals with a cELISA OD > 1.0.
[d] Low + ve control: the pooled low positive antigen extract sourced from a pool of faeces from known infected animals with a cELISA OD < 0.8.

for cow #650 which had the lowest *F. hepatica* burden (n = 9); and five for cow #3294. Thirty of the false negative samples occurred at the AM sampling and 41 false negatives occurred at the PM sampling.

3.5. LFEC output variability at AM and PM sampling over 5 days

There was a 5–16 fold variation in LFECs over the 5-day sampling period with the greatest variation in cows #836, #1100, #2300, #3491 and #4316 (Table 4; Fig. 7). PM LFEC estimates were, on average, 0.81 (95 % CI 0.62–1.01) ep2g less than AM LFEC estimates. In general, there was more variation in the PM samples (Fig. 7).

3.6. PFEC

In total, 190 PFECs were performed and all ten cows were found to be positive for paramphistomes (Fig. 8). The PFEC ranged from 0 to 664 ep2g with the highest counts in cow #836. Cow #4316 was positive on only one occasion of the 20 samples counted. Cows #1100, #3294, and #3491 each recorded false negatives. Adult paramphistomes were collected from cows #844 and #836. DNA from four paramphistomes were sequenced by the Moredun Research Institute and each were found to be *Calicophoron calicophorum*. At every collection point cows #412, #650, #836, and #844 exceeded the 10 ep2g cut-off described in Willmott and Pester (1952) which represents a paramphistome burden of >140 adults (Fig. 8).

4. Discussion

In this study, we investigated the sensitivity of two fluke diagnostic tests (cELISA and LFEC) in naturally infected dairy cows using faecal samples collected at the AM and PM milking in order to determine the daily variation in coproantigen and egg shedding over 5 consecutive days, to assess the correlation between coproantigen and LFEC levels with *F. hepatica* burden and determine the impact of this variation on test sensitivity.

4.1. Animal age vs TFC, cELISA OD and LFEC

The TFC is commonly used for determining the true liver fluke burden in animals and validating diagnostic tests with a sensitivity of 99 % and specificity of 98 % (Mazeri et al., 2016). We used the TFC in this study since it is the most reliable method for assessing the sensitivity and on-farm application of the cELISA and the FlukeFinder® kit under Australian farm management practices. The lack of a correlation of animal age with the TFC and LFEC in this study is in contradiction to research published by Gonzalez-Lanza et al. (1989) who found that the rate of *F. hepatica* egg release and parasite prevalence generally increased with animal age. The reason for the differences is likely a result of the study design, Gonzalez-Lanza et al. (1989) tested 1301 animals compared to the ten cows in this study. A moderate negative correlation (R -0.63) was observed between animal age and cELISA OD (Fig. 1). This is consistent with findings reported by Takeuchi-Storm et al. (2018) who found on all but one farm that the cELISA OD, serum ELISA OD and LFEC peaked in cows at 2–4 years of age and declined from that point as cows got older.

4.2. cELISA OD daily variation and sensitivity

Fluctuations in the cELISA OD estimates were observed in *F. hepatica*

Table 3
Variation in the sensitivity of the cELISA values at AM and PM samplings in ten individual cows sampled over five consecutive days. The sensitivity of the cELISA was determined for all published cELISA cut-offs using both the raw OD cut offs and % of positive control cut offs, as referenced below. The sensitivity data are grouped together as follows: Raw OD: the SE calculated using the raw OD values of the animals; % Batch + ve OD: the SE calculated using the raw OD converted to a percentage of the OD observed with the cELISA Batch positive antigen control; % High + ve OD: the SE calculated using the raw OD converted to a percentage of the OD observed with the High extract positive antigen control; % Kit + ve OD: the SE calculated using the raw OD converted to a percentage of the OD observed with the Kit positive antigen control.

Data	Cut-off	SE%-AM [a]	SE%-PM [a]	SE% Total[a]	CI 95 %	Reference
Raw OD	>0.014	100	100	100	100, 100	Brockwell et al. (2013)
Raw OD	>0.030	100	100	100	100, 100	Charlier et al. (2008)
Raw OD	>0.114	98	97	97	86, 97	Mezo et al. (2004)
Raw OD	>0.084	98	97	97	95, 97	Martínez-Sernández et al. (2016)
% Batch + ve OD[b]	>1.3 %	100	100	100	100, 100	Brockwell et al. (2014)
% Batch + ve OD[b]	>1.6 %	100	100	100	100, 100	Elliott et al. (2015)
% Batch + ve OD[b]	>5.4 %	100	100	100	100, 100	Palmer et al. (2014)
% Batch + ve OD[b]	>8.0 %	96	91	93	88, 93	Kit Cut-Off
% High + ve OD[c]	>1.3 %	100	100	100	100, 100	Brockwell et al. (2014)
% High + ve OD[c]	>1.6 %	100	100	100	100, 100	Elliott et al. (2015)
% High + ve OD[c]	>5.4 %	95	92	93	88, 93	Palmer et al. (2014)
% High + ve OD[c]	>8.0 %	85	75	80	66, 80	Kit Cut-Off
% Kit + ve OD[d]	>1.3 %	100	100	100	100, 100	Brockwell et al. (2014)
% Kit + ve OD[d]	>1.6 %	100	100	100	100, 100	Elliott et al. (2015)
% Kit + ve OD[d]	>5.4 %	98	95	97	94, 97	Palmer et al. (2014)
% Kit + ve OD[d]	>8.0 %	89	76	83	69, 83	Kit Cut-Off

[a] SE: % sensitivity.
[b] Batch + ve: the Batch + ve control is the positive control OD value provided with each kit batch by the manufacturer and the Batch positive control OD value was 1.815.
[c] High + ve: a pooled high positive antigen extract sourced from a pool of faeces from known infected animals with a cELISA OD > 1.0.
[d] Kit + ve: the positive control reference antigen provided by the manufacturer with the kit batch.

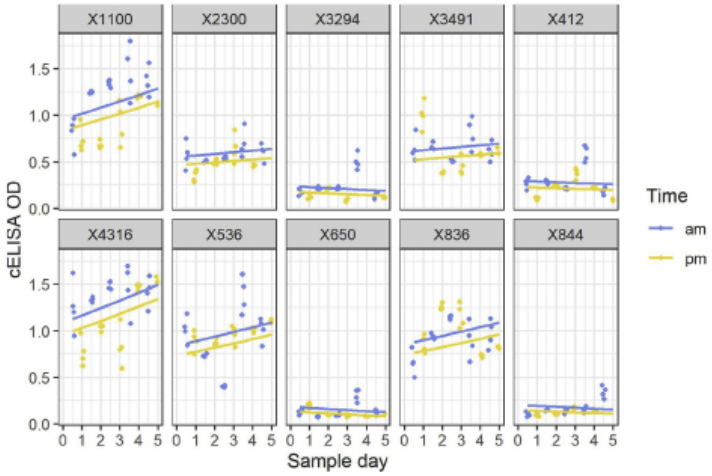

Fig. 4. Scatterplots showing cELISA OD as a function of sampling event over 5 consecutive days for each of the 10 cows included in this study. Superimposed on each plot are the cELISA OD values predicted using the mixed-effects linear regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on day 4.

infections in sheep by Mezo et al. (2004) and Valero et al. (2009) and in cattle by Brockwell et al. (2013). As a result, the commercial cELISA kit was modified by increasing the analytical sensitivity from 0.6 ng/mL to 0.15 ng/mL (Mezo et al., 2004; Martínez-Sernández et al., 2016). However, changing the detection limit of the cELISA kit increased the OD variability in cattle from a factor of 2–6 (Brockwell et al., 2013) to 6–12 (Martínez-Sernández et al., 2016). We observed a 2.6–8.9 fold variation in cELISA values in the ten cows in this study with the greatest variation seen in the PM samples (Table 4; Fig. 2). Mazeri et al. (2016), using the modified cut-off described in Palmer et al. (2014), found there were seasonal differences in the sensitivity of the cELISA: summer 80 %, winter 85 % and autumn 87 %. Given the cows used in this study were naturally infected and sampling took place in autumn, some of the variability we observed could be explained by the presence of immature *F. hepatica* in the cows. In sheep, a marked increase in coproantigen release occurred as *F. hepatica* reached patency (Valero et al., 2009).

We established the sensitivity of the cELISA assay to be 100 % using several published cut offs of 1.3 % (Brockwell et al., 2014), 1.6 % (Elliott et al., 2015), 0.014 OD (Brockwell et al., 2013) and 0.030 OD (Charlier et al., 2008). Our findings are consistent with four other studies that

Table 4
Variation in cELISA OD and LFEC values in the ten individual cows sampled over the five consecutive days.

	cELISA OD		Fold change	LFEC ep2g		Fold change
Cow #	min	max		min	max	
1100	0.58	1.80	3.1	1	11	11
2300	0.28	0.91	3.2	2	32	16
3294	0.07	0.62	8.9	0	5	5
3491	0.37	1.18	3.2	2	27	13.5
412	0.09	0.68	7.9	0	5	5
4316	0.60	1.69	2.8	1	12	12
536	0.39	1.61	4.1	0	7	7
650	0.07	0.36	5.0	0	1	1
836	0.50	1.31	2.6	2	29	14.5
844	0.07	0.42	5.6	1	10	10
Avg	0.3027	1.058	3.5	0.9	13.9	9.5

found the manufacturer's cut-off for the cELISA (8 % of the positive control) is too high, resulting in a high incidence of false negatives in low burden cattle (< 10 flukes) (Charlier et al., 2008; Novobilský et al., 2012; Brockwell et al., 2013; Palmer et al., 2014). False positives have also been observed in sheep after treatment with a flukicide (George et al., 2017). The impact of applying all published cut-offs on the sensitivity of cELISA using our dataset can be seen in Table 3 where sensitivity varies from 75 to 100 %. The specificity of the cELISA was not determined in this study as it has been found to be >99 % (Mezo et al., 2004; Kajugu et al., 2015). Moreover, it has been established by Brockwell et al. (2013), Kajugu et al. (2015) and Mazeri et al. (2016) that the cELISA does not cross-react with paramphistomes which were present in the ten cows tested (Fig. 8).

4.3. cELISA OD daily variation and correlation with TFC

The cause of the higher variation in cELISA OD at the PM sampling time relative to the AM sampling time is not clear. However, it is likely to be a result of a combination of biological processes as well as cELISA plate variability, given the variability in OD values observed with the positive controls between the plates obtained from a single commercial batch (Table 2). Brockwell et al. (2013) suggested that some of the biological causes were the intermittent release of coproantigens by *F. hepatica*, pathological changes to the bile ducts or gall bladder that may impact egg release and liver as well as variation in faecal consistency. Kajugu et al. (2015) also suggested that expulsion of coproantigens from the fluke gut is likely to be episodic which would impact the level of coproantigen in faeces over time. The observed correlations between the cELISA OD and TFC at the AM and PM samplings are similar to those reported by Charlier et al. (2008) in cattle (R 0.6) but lower than that reported by Brockwell et al. (2013) (R^2 0.8368). More recently, Martínez-Sernández et al. (2016) observed the correlation to be R 0.2998 which is less than our findings but that correlation was only calculated in cattle that had less than ten adult *F. hepatica*.

4.4. cELISA detection limit

Before the modification of the cELISA (BIO X kit), the original kit could detect burdens in cattle with more than two flukes, and 2/7 cattle that had one fluke (Mezo et al., 2004). In this study, the new version of the cELISA detected the lowest TFC of nine fluke (cow #650) at every sampling point. All samples collected for cows # 412 (14 fluke), # 536 (11 fluke), # 844 (12 fluke) and # 3294 (10 fluke) were also positive at every sampling point. Given the lowest reported cut-off for production loss in dairy cattle is 10 flukes, the data indicate that the cELISA kit is able to consistently identify individual cattle as fluke positive before production losses occur (Charlier et al., 2008). The aim of the Martínez-Sernández et al. (2016) study was to reduce false negatives by increasing the assay's sensitivity. Given that the old version of the kit was already capable of detecting 1–2 fluke, which is less than the production cut-off of ten *F. hepatica*, the reports of false negatives in the literature were likely a result of the cut-off used not the detection limit of the cELISA kit.

The value of the cELISA is the ability to detect low (≤10) and moderate to high (≥11) fluke burdens in animals as well as detecting *F. hepatica* that survive flukicide treatment (Kelley et al., 2016). The most critical outcome for practically managing *F. hepatica* in cattle is identifying whether the herd is incurring production losses, to determine whether the *F. hepatica* present are drug resistant as well as managing the herd to reduce the level of pasture contamination. The cELISA appears to be a robust tool to help achieve these outcomes.

4.5. LFEC sensitivity

The sensitivity of the LFEC was 88 % but it should be noted that our sample collection took place in autumn which has been found to decrease the sensitivity of the LFEC due to the fact that cattle were infected with pre-patent *F. hepatica* that had not reached sexual maturity (Mazeri et al., 2016). The presence of immature *F. hepatica* could have contributed to elevating the coproantigen level without increasing the LFEC (Mezo et al., 2004). Due to time constraints, the size of *F. hepatica* recovered were not measured, but smaller fluke (<12 mm) consistent with the size of immature fluke were observed in some cows. Future studies should investigate whether increasing the volume of faeces from 2 g affects the sensitivity of the FlukeFinder® sedimentation method. Conceição et al. (2002) and Rapsch et al. (2006) both established that the sensitivity of the LFEC can be increased by repeat sampling or by increasing the volume of faecal matter used. Conversely, due to the higher amount of debris in the sample, some studies have observed that an increased sample volume decreased the sensitivity and specificity of the LFEC because of the increased difficulty in identifying *F. hepatica* eggs (Conceição et al., 2002; Charlier et al., 2008; Daniel et al., 2012).

4.6. LFEC daily variation

Happich and Boray (1969) established that the faecal sedimentation technique is the most suitable for quantitative diagnosis in cattle, particularly in low burden infections (<10 flukes). Previous work by Brockwell et al. (2013) observed a 2–4 fold variation in egg output over a 5 day sampling period in cattle which is not consistent with our findings of a 5–16 fold variation. A relatively low egg output was observed for cow #4316 (Fig. 3; Fig. 6) even though it had the highest burden of *F. hepatica* ($n = 72$) (Table 1). Happich and Boray (1969) found that there is a negative association between the number of *F. hepatica* in the liver and egg-shedding capacity suggesting that a crowding effect could occur in cattle. The variation in egg output in cow #4316 may have been due to crowding effects as well as mechanical barriers in the liver caused by scarring and fibrosis that were observed when dissecting that liver (data not shown), which may have hampered egg release. The over-dispersion of *F. hepatica* in cattle and the considerable variation in egg shedding with fluke burden intensity can also increase the variability in *F. hepatica* egg numbers in faecal matter. In addition, the host immune response could also affect egg shedding but there has been limited research to investigate this effect. Vaccination of cattle and sheep with fluke antigens has been shown to reduce the egg counts, egg viability and egg maturation in some studies but the basis for the vaccine-induced effect is not clear (Wijffels et al., 2014; Dalton et al., 1996).

4.7. LFEC daily variation and TFC

Our findings show that sampling at AM has a greater correlation of LFEC with fluke burden (Fig. 3) where the observed correlation between LFEC and TFC was R 0.78 (AM) and R 0.66 (PM). This finding is not consistent with correlations of R^2 0.84 reported by Brockwell et al.

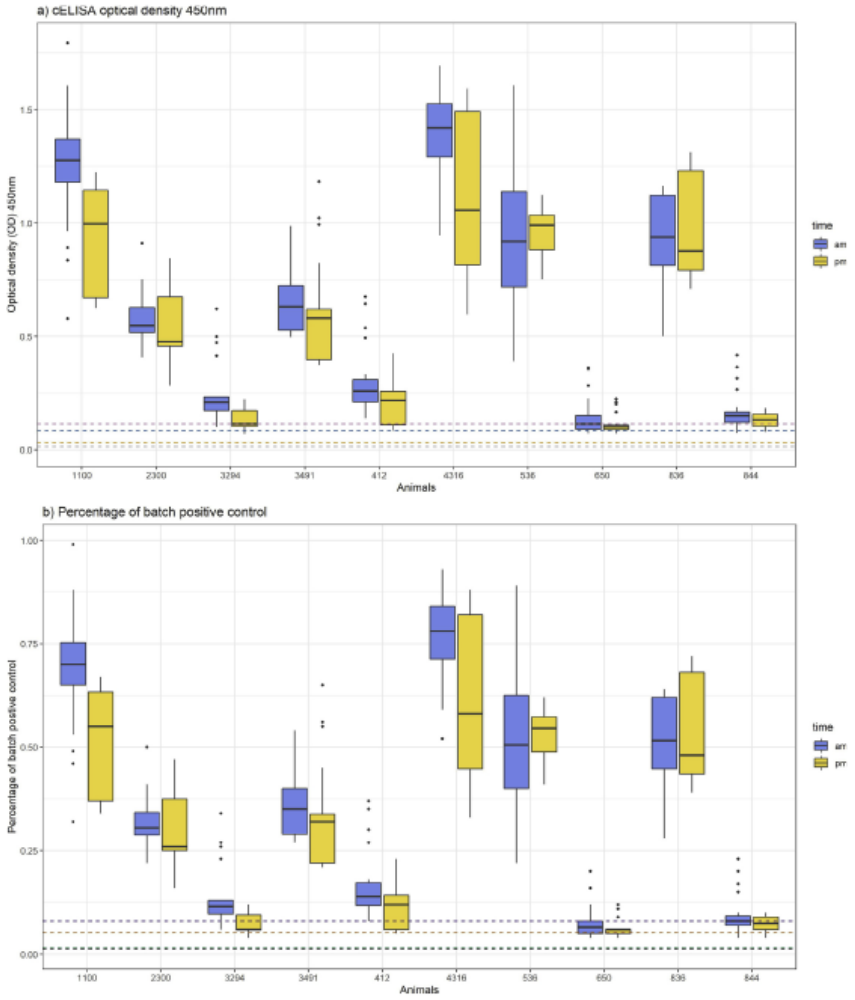

Fig. 5. Box and whisker plots showing the distribution of: (a) cELISA OD estimates at AM and PM sampling events for each of the ten cows included in this study; and (b) the percentage of the batch positive control for the same samplings. In plot (a) the four horizontal dashed lines represent the cut-off ODs described in: (i) Brockwell et al. (2013), 0.014, grey; (ii) Charlier et al. (2008), 0.030, gold; (iii) Martínez-Sernández et al. (2016), 0.084, blue; and (iv) Meso et al. (2004), 0.114, pink. In plot (b) the four horizontal dashed lines represent the cut-off % positive values described in: (i) Brockwell et al. (2014), 1.3 %, black; (ii) Elliott et al. (2015) 1.6 %, green; (iii) Palmer et al. (2014), 5.36 %, red; (iv) the recommended kit cut-off of 8 %, purple (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(2013) in cattle. The differences in the correlation observed may be due to differences in the study designs. Brockwell et al. (2013) used naive artificially infected cattle of the same age whereas our cows were naturally infected and of various ages. Our data also differ from the findings in three previous studies observing daily fluke egg shedding trends in cattle. These studies concluded that the highest egg output occurs between 12:00 p.m. to 12:00 a.m. (Dorsman, 1956, 1960; Hagens and Over, 1966). Based on these observations, Dorsman (1956) and Hagens and Over (1966) proposed that faecal sample collection should take place in the PM, assuming that the highest egg output reflects the fluke burden within the animal but the basis for this difference with our findings is not clear. Our study's findings are more consistent with work in sheep by George et al. (2017) who observed a correlation of R^2 0.571 between LFEC and TFC.

4.8. Paramphistomes

In this study, every cow was positive for paramphistomes. In Fig. 8,

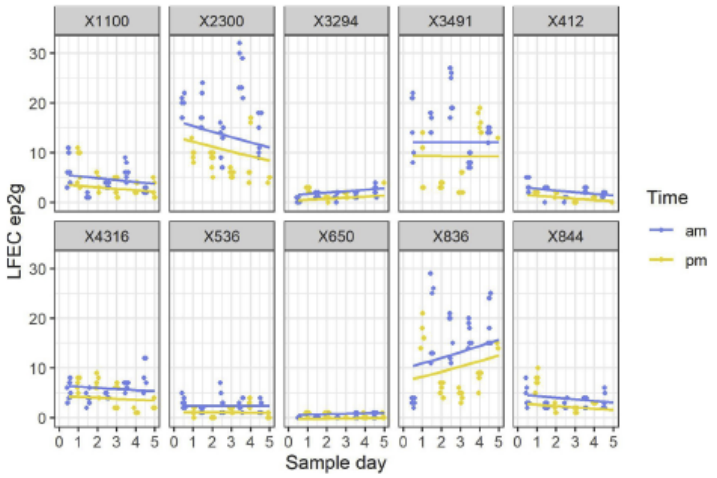

Fig. 6. Scatterplots showing LFEC (ep2g) as a function of sampling event for each of the ten cows included in this study. Superimposed on each plot are the LFEC estimates predicted using the mixed-effects linear regression model described in the text. Samples could not be collected from cow #3294 at the PM milking on day 4.

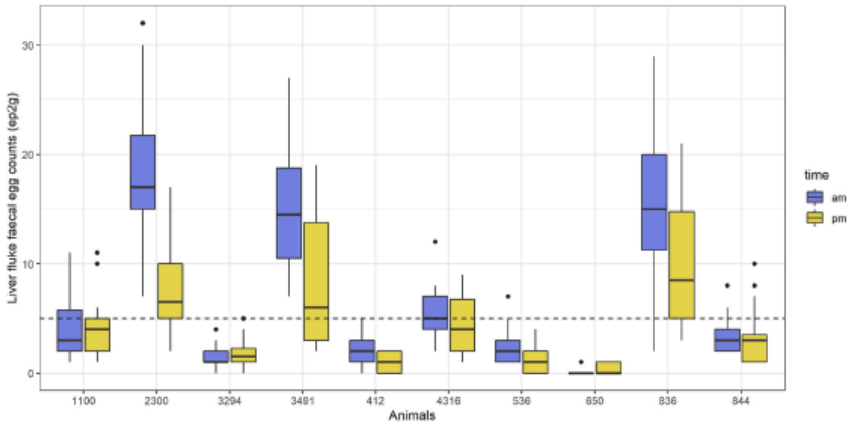

Fig. 7. Box and whisker plots showing the distribution of LFEC (ep2g) at AM and PM sampling events for each of the ten cows included in this study. The horizontal dashed line shows the 5 ep2g cut-off for production loss in cattle described by Malone and Craig (1990) and Vercruysse and Claerebout (2001).

four cows (#412, #650, #836 and #844) exceeded the 10 ep2g PFEC cut-off described by Willmott and Pester (1952). A PFEC of 30–40 ep6g (10–13 ep2g) indicates that there are approximately 143–145 adult paramphistomes in the rumen (Willmott and Pester, 1952). Paramphistomes were collected from both cows # 836 and 844: the Moredun Research Institute sequenced genomic DNA from four paramphistomes and each sample was found to be *C. calicophorum*. A high incidence of paramphistomes has been observed in two studies in Australia. Co-infections with paramphistomes were common in Kelley et al. (2020) (data not shown) and Molloy et al. (2005) determined that the prevalence of paramphistomes in cattle in South-eastern Queensland was 46.2 %. In recent years Europe has seen a dramatic increase in paramphistome prevalence in cattle and now considers it an emerging disease (Morley, 2018). Paramphistomes have previously been considered to be a relatively benign parasite in Australia, but given the high counts observed in this study and previous studies indicating a high incidence further work is needed in Australia to assess their impact on production.

4.9. Conclusion

In conclusion, the cELISA and the LFEC were found to be robust diagnostic tools in naturally infected dairy cows. Variation in the daily cELISA and LFEC were observed within cows over the 5-day faecal sampling period with both tests showing a higher correlation to TFC when samples were collected at the AM milking. Both tests accurately detected cows with burdens >10 fluke which is the production cut-off in cattle. In addition to these findings, each of the study group cows were infected with *C. calicophorum*, with some animals showing PFEC counts

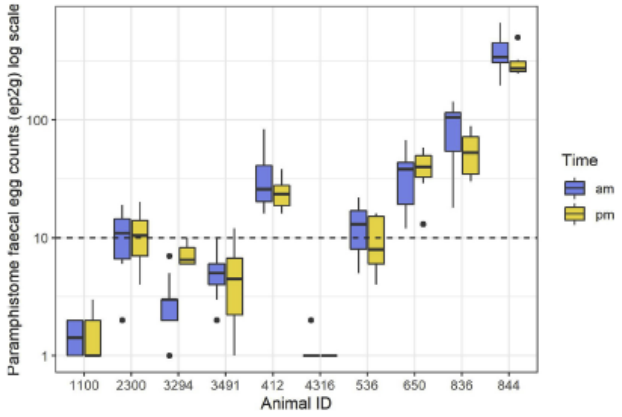

Fig. 8. Box and whisker plots showing the distribution of PFEC (ep2g) on the log scale for each of the ten cows included in this study. The horizontal dashed line shows the 10 ep2g cut-off indicating that there are approximately 143 to 145 adult paramphistomes in the rumen (Willmott and Pester, 1952).

higher than that reported previously in Australia, warranting further investigation to determine the effect of high intensity infections of *C. calicophorum* on productivity in dairy cattle.

CRediT authorship contribution statement

Jane M. Kelley: Methodology, Formal analysis, Investigation, Methodology, Validation, Visualization, Resources, Writing - original draft, Writing - review & editing. **Mark A. Stevenson:** Formal analysis, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Vignesh Rathinasamy:** Investigation, Validation, Writing - original draft. **Grant Rawlin:** Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing - original draft, Writing - review & editing. **Travis Beddoe:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Terry W. Spithill:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Anderson, N., Luong, T., Vo, N., Bui, K., Smooker, P.M., Spithill, T.W., 1999. The sensitivity and specificity of two methods for detecting *Fasciola* infections in cattle. Vet. Parasitol. 83, 15–24.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. lme4: linear mixed-effects models using Eigen and S4. J. Stat. Softw. 67, 1–48.

Bennema, S., Vercruysse, J., Claerebout, E., Schnieder, T., Strube, C., Ducheyne, E., Hendrickx, G., Charlier, J., 2009. The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). Vet. Parasitol. 165, 51–57.

Bloemhoff, Y., Forbes, A., Danaher, M., Good, B., Morgan, E., Mulcahy, G., Sekiya, M., Sayers, R., 2015. Determining the prevalence and seasonality of *Fasciola hepatica* in pasture-based dairy herds in Ireland using a bulk tank milk ELISA. Irish Vet. J. 68, 16–25.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv. Parasitol. 7, 95–210.

Box, G., Cox, D., 1964. An analysis of transformations (with discussion). J. R. Stat. Soc. Ser. B 26, 211–252.

Brockwell, Y., Spithill, T., Anderson, G., Grillo, V., Sangster, N., 2013. Comparative kinetics of serological and coproantigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Vet. Parasitol. 196, 417–426.

Brockwell, Y.M., Elliott, T.P., Anderson, G.R., Stanton, R., Spithill, T.W., Sangster, N.C., 2014. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. Int. J. Parasitol.: Drugs Drug Res. 4, 48–54.

Charlier, J., Duchateau, L., Claerebout, E., Williams, D., Vercruysse, J., 2007. Associations between anti-*Fasciola hepatica* antibody levels in bulk-tank milk samples and production parameters in dairy herds. Prev. Vet. Med. 78, 57–66.

Charlier, J., De Meulemeester, L., Claerebout, E., Williams, D., Vercruysse, J., 2008. Qualitative and quantitative evaluation of coprological and serological techniques for the diagnosis of fasciolosis in cattle. Vet. Parasitol. 153, 44–51.

Collett, D., 1999. Modelling Binary Data. Chapman & Hall/CRC, Boca Raton Florida, p. 24.

Conceição, M.A.P., Durão, R.M., Costa, I.H., da Costa, J.M.C., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Vet. Parasitol. 105, 337–343.

Dalton, J.P., McGonigle, S., Rolph, T.P., Andrews, S.J., 1996. Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Infect. Immun. 64, 5066–5074.

Daniel, R., Van Dijk, J., Jenkins, T., Akca, A., Mearns, R., Williams, D., 2012. Composite faecal egg count reduction test to detect resistance to triclabendazole in *Fasciola hepatica*. Vet. Rec. 171, 153-153.

Dorsman, W., 1956. Fluctuation within a day in the liver-fluke egg-count of the rectal contents of cattle. Vet. Rec. 68, 571–574.

Dorsman, W., 1960. The diagnosis of subclinical fascioliasis by means of faecal examination, and the control of liver flukes (*Fasciola hepatica*). Bulletin de l'Office International des Epizooties 54, 502–508.

Elliott, T., Kelley, J., Rawlin, G., Spithill, T., 2015. High prevalence of fascioliosis and evaluation of drug efficacy against *Fasciola hepatica*in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. Vet. Parasitol. 209, 117–124.

Estuningsih, E., Spithill, T., Raadsma, H., Law, R., Adiwinata, G., Meeusen, E., Piedrafita, 2009. Development and application of a fecal antigen diagnostic sandwich ELISA for estimating prevalence of *Fasciola gigantica* in cattle in central Java, Indonesia. J. Parasitol. 95, 450–455.

George, S., Vanhoff, K., Baker, K., Lake, L., Rolfe, P., Seewald, W., Emery, D., 2017. Application of a coproantigen ELISA as an indicator of efficacy against multiple life stages of *Fasciola hepatica* infections in sheep. Vet. Parasitol. 246, 60–69.

Gonzalez-Lanza, C., Manga-Gonzalez, Y., Del-Pozo-Carnero, P., Hidalgo-Argüello, R., 1989. Dynamics of elimination of the eggs of *Fasciola hepatica* (Trematoda, Digenea) in the faeces of cattle in the Porma Basin, Spain. Vet. Parasitol. 34, 35–43.

Hagens, F., Over, H., 1966. Egg-Output and egg-count of *Fasciola hepática* in cattle. Tijdschrift voor Diergeneeskunde 91, 523–531.

Happich, F., Boray, J., 1969. Quantitative diagnosis of chronic fasciolosis. 1. Comparative studies on quantitative faecal Examinations for chronic *Fasciola hepatica* infection in sheep. Aust. Vet. J. 45, 326–328.
Howell, A., Baylis, M., Smith, R., Pinchbeck, G., Williams, D., 2015. Epidemiology and impact of *Fasciola hepatica* exposure in high-yielding dairy herds. Prev. Vet. Med. 121, 41–48.
Ibarra, F., Montenegro, N., Vera, Y., Boulard, C., Quiroz, H., Flores, J., Ochoa, P., 1996. Comparison of three ELISA tests for seroepidemiology of bovine fascioliosis. Vet. Parasitol. 77, 229–236.
Kajugu, P.-E., Hanna, R., Edgar, H., McMahon, C., Cooper, M., Gordon, A., Barley, J., Malone, F., Brennan, G., Fairweather, I., 2015. *Fasciola hepatica*: specificity of a coproantigen ELISA test for diagnosis of fasciolosis in faecal samples from cattle and sheep concurrently infected with gastrointestinal nematodes, coccidians and/or rumen flukes (paramphistomes), under field conditions. Vet. Parasitol. 212, 181–187.
Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 32, 458–469.
Kelley, J.M., Rathinasamy, V., Elliott, T.P., Rawlin, G., Beddoe, T., Stevenson, M.A., Spithill, T.W., 2020. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. Vet. Parasitol. 277, 109019.
Kuerpick, B., Conraths, F.J., Staubach, C., Froehlich, A., Schnieder, T., Strube, C., 2013. Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. Parasitology 140, 1051–1060.
Malone, J., Craig, T., 1990. Cattle liver flukes: risk assessment and control. Comp. Cont. Educ. Pract. Vet. 12, 747–754.
Martínez-Sernández, V., Orbegozo-Medina, R.A., González-Warleta, M., Mezo, M., Ubeira, F.M., 2016. Rapid enhanced MM3-COPRO ELISA for detection of *Fasciola* coproantigens. PLoS Negl. Trop. Dis. 10, e0004872.
Mazeri, S., Sargison, N., Kelly, R.F., Bronsvoort, B.M.D., Handel, I., 2016. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. PLoS One 11, e0161621.
McCann, C., Baylis, M., Williams, D., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. Vet. Rec. 166, 612–617.
Mezo, M., González-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). J. Parasitol. 90, 845–852.
Molloy, J., Anderson, G., Fletcher, T., Landmann, J., Knight, B., 2005. Evaluation of a commercially available enzyme-linked immunosorbent assay for detecting antibodies to *Fasciola hepatica* and *Fasciola gigantica* in cattle, sheep and buffaloes in Australia. Vet. Parasitol. 130, 207–212.
Morley, N.J., 2018. Paramphistomosis of ruminants: the role of free-living metacercariae. Trends Parasitol. 34, 97–98.
Novobilský, A., Averpil, H.B., Höglund, J., 2012. The field evaluation of albendazole and triclabendazole efficacy against *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Vet. Parasitol. 190, 272–276.
Novobilský, A., Sollenberg, S., Höglund, J., 2015. Distribution of *Fasciola hepatica* in Swedish dairy cattle and associations with pasture management factors. Geospat. Health 9, 293–300.
Palmer, D., Lyon, J., Palmer, M., Forshaw, D., 2014. Evaluation of a copro-antigen ELISA to detect *Fasciola hepatica* infection in sheep, cattle and horses. Aust. Vet. J. 92, 357–361.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2020. nlme: Linear and Nonlinear Mixed Effects Models. URL. http://CRAN.R-project.org/package=nlme.
Pritchard, G., Forbes, A., Williams, D., Salimi-Bejestani, M., Daniel, R., 2005. Emergence of fasciolosis in cattle in East Anglia. Vet. Rec. 157, 578–582.
R Core Team, 2020. R: a Language and Environment for Statistical Computing. URL. http://www.R-project.org/.
Rapsch, C., Schweizer, G., Grimm, F., Kohler, L., Bauer, C., Deplazes, P., Braun, U., Torgerson, P.R., 2006. Estimating the true prevalence of *Fasciola hepatica* in cattle slaughtered in Switzerland in the absence of an absolute diagnostic test. Int. J. Parasitol. 36, 1153–1159.
Reichel, M., 2002. Performance characteristics of an enzyme-linked immunosorbent assay for the detection of liver fluke (*Fasciola hepatica*) infection in sheep and cattle. Vet. Parasitol. 107, 65–72.
Salimi-Bejestani, M., Daniel, R., Pelstead, S., Cripps, P., Mahmoody, H., Williams, D., 2005. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. Vet. Rec.-Engl. Ed. 156, 729–731.
Schweizer, G., Braun, U., Deplazes, P., Torgerson, P., 2005. Estimating the financial losses due to bovine fasciolosis in Switzerland. Vet. Rec. 157, 188–193.
Selemetas, N., Phelan, P., O'Kiely, P., Waal, T., 2014. Weather and soil type affect incidence of fasciolosis in dairy cow herds. Vet. Rec. 175, 371-371.
Takeuchi-Storm, N., Denwood, M., Petersen, H.H., Enemark, H.L., Stensgaard, A.-S., Sengupta, M.E., Beesley, N.J., Hodgkinson, J., Williams, D., Thamsborg, S.M., 2018. Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. Parasit. Vectors 11, 674.
Valero, M.A., Ubeira, F.M., Khoubbane, M., Artigas, P., Muiño, L., Mezo, M., Pérez-Crespo, I., Periago, M.V., Mas-Coma, S., 2009. MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with*Fasciola hepatica* and *F. gigantica*. Vet. Parasitol. 159, 77–81.
Vercruysse, J., Claerebout, E., 2001. Treatment vs non-treatment of helminth infections in cattle: defining the threshold. Vet. Parasitol. 98, 195–214.
Wijffels, G.L., Salvatore, L., Dosen, M., Wadlington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T., Spithill, T.W., 2014. Vaccination of sheep with purified cysteine proteases of *Fasciola hepatica* decreases worm fecundity. Exp. Parasitol. 78, 132–148.
Williams, D.J., Howell, A., Graham-Brown, J., Kamaludeen, J., Smith, D., 2014. Liver fluke–an overview for practitioners. Cattle Pract. 22, 238–244.
Willmott, S., Pester, F., 1952. Variations in faecal egg-counts in paramphistome infections as determined by a new technique. J. Helminthol. 26, 147–156.

7.2 Accepted Manuscript – *Fasciola hepatica* Control Practices on a Sample of Dairy Farms in Victoria, Australia

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Fasciola hepatica Control Practices on a Sample of Dairy Farms in Victoria, Australia

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In Australia, little is known about the strategies used by farmers to control *Fasciola hepatica* (*F. hepatica*) infection in dairy cattle. Triclabendazole-resistant *F. hepatica* have recently been found on several dairy and beef properties in Australia. It is difficult to draw conclusions about how widespread resistance is in Australian dairy cattle because we have little information about flukicide usage, drug resistance testing, and alternative flukicide usage on-farm. The study objectives were to determine how dairy farmers are currently controlling *F. hepatica* and to identify knowledge gaps where *F. hepatica* control strategies need to be communicated to farmers to improve management. The survey was distributed online or by hard copy and 36 dairy farmers completed the survey. There were 34 questions including closed, open-ended, multicheck box, demographic, and text questions. Descriptive statistics were used to quantify each response. The survey results showed high use of clorsulon, limited rotation of flukicides, and limited use of diagnostic tests to inform treatment options and timing. There was poor adherence to best management practice in determining the dose of flukicides administered to cattle, with farmers often relying on estimating body weights or average body weights, suggesting that underdosing of animals is likely to be prevalent. Most respondents in this study did not isolate and quarantine treated and newly returned or purchased animals before joining them with the main herd. The research identified four knowledge gaps where communication needs to be enhanced to improve control of *F. hepatica*: diagnostic testing to inform flukicide use, rotation of flukicide actives, flukicide administration, and increased testing of replacement animals.

Keywords: *Fasciola hepatica*, triclabendazole, clorsulon, dairy cattle, survey, control strategies, diagnostic tests, farm management

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INTRODUCTION

Fasciola hepatica (*F. hepatica*) has been a problem in Australia since colonization (1). Early outbreaks of fasciolosis had high mortality rates and animals within irrigation regions were at higher risk (2, 3). In dairy cattle, *F. hepatica* infection reduces weight gain, milk production, and conception rates [reviewed in (4)]. Naive young cattle (calves and heifers) are more vulnerable to fasciolosis than adult stock, as they have no

previous exposure to *F. hepatica* and, therefore, have no acquired immunity. Oakley et al. (5) found that *F. hepatica* infection in heifers limited growth rate, impaired feed conversion, delayed puberty, lowered conception rates, and reduced calf weight. The observed effects were more pronounced in animals that had a lower plane of nutrition.

In Victoria, replacement animals (<12 months) are isolated from adult stock in order to comply with the bovine Johne's disease program (BJD) (6). The program prevents contact between adult stock and replacements, leading to replacements being consecutively reared on the same paddocks. These paddocks tend to be more marginal and have a lower quality pasture base than grazed land provided to the milking herd. In Australia, dairy cattle predominately graze outside year-round. The key driver of the profit in pasture-based dairy farming in Australia is to increase milk produced per grazed hectare by growing more pasture of a higher quality and increasing consumption (7). Watson and Watson (8) found that the stocking rate of dairy farms across Australia has increased over the last 15 years and has reached more than two cows per hectare in some regions.

These intensive grazing strategies used on dairy farms in Australia increase pasture consumption per hectare but also increase pasture contamination with fecal matter (9). It is a growing concern that the dairy industry's intensification is increasing the development of parasite drug resistance and subclinical production losses (9). Over the last 15 years, average stocking density on dairy farms has increased from 1.51 to 1.72/ha (8). However, the stocking rate in high-intensity irrigated pasture regions of Victoria is above the national average at 1.84/ha in Loddon Valley, Torrumbarry, Central Goulburn, and Murray Valley and 2.34/ha in Macalister Irrigation District (MID). Triclabendazole (TCBZ) resistance has been confirmed on several dairy farms in these irrigated regions in Victoria (10–12). Until the early 1980s, fluke control relied on fencing off the intermediate host habitat, draining wet areas, and using flukicides of low efficacy. After the release of TCBZ, extensive work was done to communicate *F. hepatica* control strategies to farmers. Hort (13) found that 51% of sheep farmers adhered to these best practice guidelines published by the Departments of Agriculture in New South Wales and Victoria as described in Boray et al. (14). The program recommended treating in autumn (April/May) and spring (August/September) every year, with an additional summer (January) treatment for young animals and adults if they were located in high-risk regions. Since 1998, there has been no tracking of the program's adherence or effectiveness. There are only three chemical classes of flukicides registered for use in dairy cattle in Australia: TCBZ, clorsulon (CLOR), and oxyclozanide (OXY). There has also been no monitoring of how these three flukicides have been used, how diagnostic tools have been incorporated into *F. hepatica* control strategies by dairy farmers in Australia, and whether there has been an increase in the uptake of integrated parasite management strategies (IPM).

In Europe, a small number of parasite management surveys identified several knowledge gaps where *F. hepatica* control could be optimized on dairy farms (15–17). Bloemhoff et al. (15) found that 3% of dairy farmers treating for *F. hepatica* used a product unsuitable for the purpose, and grazing management options were not effectively utilized on-farm. Selemetas et al. (16) found that pasture and grazing management options had to be carefully communicated to avoid dairy farmers assuming they had a low risk of *F. hepatica* because they have good drainage. In addition, Easton et al. (17) found that there was limited use of diagnostics to inform on-farm decision-making and anthelmintic purchasing behavior was driven by factors relating to convenience. The only *F. hepatica* survey conducted in Australia was in sheep flocks and beef herds; however, only the sheep data were published (13). Hort (13) identified two knowledge gaps in how Australian sheep farmers were managing *F. hepatica*. Firstly, a high proportion of farmers were unaware that their flock was infected with *F. hepatica* because of a lack of routine diagnostic testing. The second gap was that 10% of sheep farmers used products that had no efficacy against *F. hepatica*.

The complexity of the *F. hepatica* life cycle increases the difficulty in communicating how to use flukicides, diagnostic tools, and management practices to control *F. hepatica* on-farm. It is a major hurdle in working with farmers as they need to control both the parasitic stage in cattle and snails as well as the free-living stage in waterways and on pasture, which is only possible if knowledge gaps are identified and addressed. The aim of this study was to determine the *F. hepatica* control strategies used in Victorian irrigated dairy regions. We investigated how dairy farmers control *F. hepatica*, looked for knowledge gaps in current *F. hepatica* control strategies, and identified what information needs to be communicated to farmers to improve *F. hepatica* management and reduce production losses in dairy cattle.

METHODS

Ethical Statement

All procedures and documentation used in this study were approved by the La Trobe University Science, Health and Engineering (SHE) College Human Ethics Sub-Committee (CHESC) under negligible risk project S17-068, which was in accordance with the ethical standards outlined by the National Statement on Ethical Conduct in Human Research (2007) and the Australian Code for the Responsible Conduct of Research (2007).

Survey Distribution and Questions

The survey was piloted in 2013 to 19 dairy farmers in the MID. After minor amendments, the survey was distributed to Victorian dairy farmers *via* hard copy and online (SurveyMonkey®) from June 1, 2017, to December 30, 2017 (**Supplementary Datasheet 1**). The survey consisted of 34 questions split into five sections: section 1: location and research awareness, section 2: drainage and irrigation, section 3: stock details and diagnostics, section 4: flukicides, and section 5: drenching practices. The questionnaire was made up of 16 closed questions, eight multiple choice, seven open-ended questions, two text questions, and one demographic question. No individual identifying data were collected and survey respondents were not required to complete all questions.

TABLE 1 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia: demographic details of survey respondents.

Question	Number of respondents (%)
Irrigation region	
Central Goulburn (CG)	14 (39)
Macalister Irrigation District (MID)	7 (19)
Murray Valley (MV)	4 (11)
Upper Murray (UM)	4 (11)
Torrumbarry (TIA)	3 (8)
South Gippsland	2 (6)
Loddon Valley (LV)	1 (3)
Western Victoria	1 (3)
Age (years)	
18–24	0 (0)
25–34	7 (19)
35–44	9 (25)
45–54	12 (33)
55–64	3 (8)
65–74	3 (8)
>75	2 (6)
Education	
Secondary	8 (22)
TAFE or Trade qualification	4 (11)
Associate degree or diploma	10 (28)
Bachelor's degree	10 (28)
Postgraduate or master's	3 (8)
No response	1 (3)
Gender	
Male	26 (72)
Female	10 (28)

Survey Respondents

Those who responded to the survey were volunteers recruited both in-person and online. The survey was advertised on the project website (www.flukecontrol.com), on dairy social media platforms, and in-person at dairy-specific events in Victoria. Respondents completed the survey during their own time. The survey allowed respondents to skip questions and provide as much or as little information as they wanted to provide. In total, 67 surveys were received and 36 respondents were included in the analysis. The authors note that recruitment took place during the "Victorian dairy crisis," which saw large numbers of dairy farmers leave the industry, sell-off stock, and cut back on expenses and significantly lowered the confidence in the industry (18–20).

Analysis

Online surveys were downloaded into a proprietary spreadsheet package (Microsoft Excel, Microsoft Corporation, Redmond, USA) and hard copy results were transcribed directly into the same spreadsheet. Results for the closed and multiple choice questions are presented as frequencies and percentages (%) of the total number of survey respondents. Questions that received no responses have been included in the analysis. Given the relatively small number of survey respondents, dependent variables could not be grouped by independent variable categories such as irrigation region, calving type, and herd size. Graphics were produced using Prism (GraphPad Prism version 7.03 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com). Maps were developed using the Geographic Information System Quantum GIS (QGIS Geographic Information System; QGIS Association, http://www.qgis.org) using data obtained from the State of Victoria (21) and State of Victoria (22).

RESULTS

Of the 67 survey responses, 31 that were submitted online were excluded because they were incomplete (i.e., no answers were provided to any of the survey questions). In total, 36 surveys from Victorian dairy farmers were analyzed (**Table 1**), representing about 4.2% of the ∼854 irrigated farms that are exposed to *F. hepatica* based on the known prevalence of 39% (8, 12). A response rate could not be determined as the survey was distributed online *via* email, social media, and e-newsletters as well as hard copies being handed out at industry events.

Descriptive Statistics of Respondents and Their Dairy Business

Seventy-two percent of the survey respondents were male, with the majority aged between 45 and 54 years (**Table 1**). The highest number of surveys was received from the central Goulburn Irrigation District ($n = 14$), followed by the MID ($n = 7$). All other irrigation dairy regions were represented by at least one respondent in this study (**Figure 1**). The average area of all dairy farms was 427 ha, milking an average of 457 cows and rearing an average of 138 heifers and 130 calves with a total stocking density of 1.7/ha (**Table 2**). Of the 36 farms, 75% were split calving, 22% seasonal calving, and 3% year-round calving (**Table 3**). Ninety-two percent of farms had an irrigated pasture base and only one farm in the study was identified as organic (**Table 3**). The most frequently used method of irrigation was flood. Flood was used solely on 56% of the farms and in combination with other types of irrigation methods on 35% of the farms (**Table 3**). The second most common method of irrigation was center pivot, followed by laterals, sprays, and lineal move and one farm solely used a traveling gun (3%) (**Table 3**).

Dairy Farm Management

All but two survey respondents identified that their farms had problems with waterlogging (**Table 4**). The highest proportion (53%) reported that between 1 and 19% of their farmland had problems with waterlogging and 78% stated that stock had access to these areas (**Table 4**). In addition, 61% of the respondents reported that stock had access to irrigation channels on their farms. Eighty-six percent of the respondents (31/36) regularly conducted irrigation channel maintenance, often using a combination of methods to improve water use efficiency. The most common methods were spraying for weeds, fixing leaking delvers, and excavating irrigated channels (**Table 4**). Two respondents included other maintenance practices: one grazed

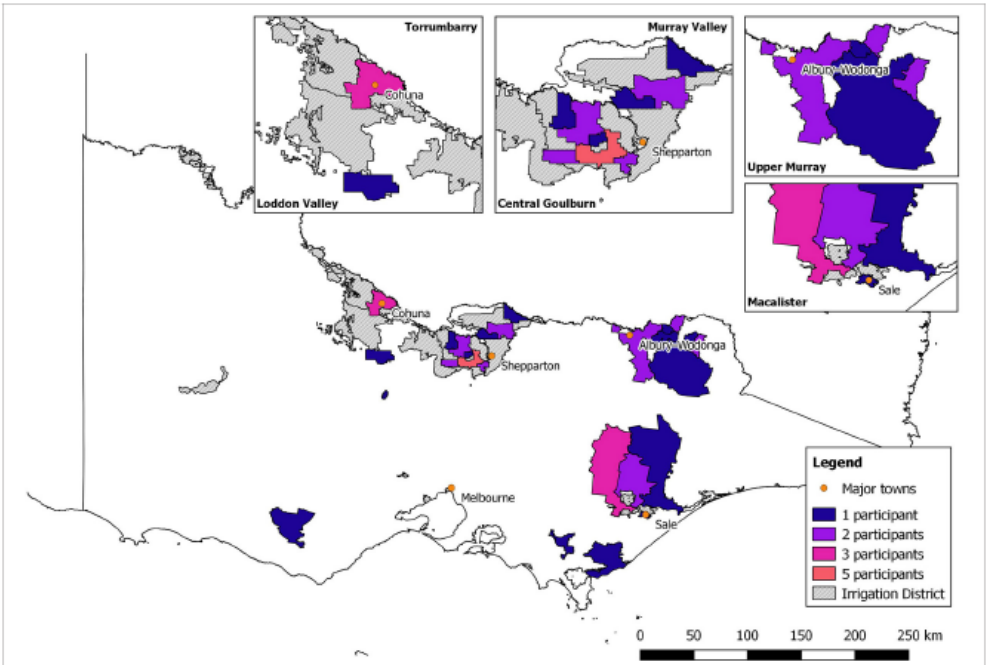

FIGURE 1 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia. Map of Victoria showing the number of survey respondents by postcode area. Blue represents one respondent; purple: two respondents; pink: three respondents; and orange: five respondents. Gray hashed lines represent irrigation regions within Victoria.

TABLE 2 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia: descriptive statistics of farm area and stock numbers on each of the farms managed by the survey respondents.

Question	*n*	Mean (SD)	Median	Q1, Q3	Min, max
Farm area (ha)	36	427 (512)	250	150, 521	40, 2,400
No. of adults	36	457 (356)	335	249, 663	40, 2,000
No. of heifers > 12 months	36	138 (120)	120	65, 180	6, 700
No. of calves < 12 months	36	130 (102)	93	64, 203	0, 500

channels with stock and the other replaced channels with pipes (**Table 4**).

F. hepatica Diagnostic Testing

The bulk tank milk ELISA (BTM ELISA) (23) was used to detect *F. hepatica* on 33% of farms and liver fluke fecal egg counts (LFEC) on 28% of farms (**Figure 2A**). No other *F. hepatica* diagnostic tests were used (**Figure 2A**). The highest frequency of testing occurred in adult milkers (**Figure 2B**). Forty-two percent of the respondents tested once per year, 6% tested twice per year, and one respondent tested three times per year (**Figure 2B**). For heifers and calves, only two farms tested these stock categories (**Figure 2B**). Nineteen percent of the respondents reported that they had tested for *F. hepatica* drug resistance, of which two stated to have worked with the lead author (**Figure 2A**).

Flukicide Use

In 2015–2016, 72% of the respondents treated their stock for *F. hepatica* (**Table 5**). TCBZ and CLOR were widely used across stock categories. The highest frequency of treatments occurred in milkers, followed by calves and heifers which received the least *F. hepatica* treatments per year (**Figure 3**). CLOR was most

TABLE 3 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia: types of farms, details of irrigation methods, and details of calving systems on each of the farms managed by the survey respondents.

Question	Number of respondents (%)
Organic dairy system	
Yes	1 (3)
No	35 (97)
Farm type	
Irrigated pasture base	33 (92)
Dryland pasture base	1 (3)
No response	2 (6)
Irrigation	
Flood	20 (56)
Traveling gun	1 (3)
Flood and center pivot	4 (11)
Flood and lineal move	1 (3)
Flood and laterals	2 (6)
Flood and spray	2 (6)
Flood, center pivot, and linear move	2 (6)
Flood, center pivot, and laterals	1 (3)
None	1 (3)
No response	2 (6)
Calving system	
Year-round	1 (3)
Split calving	27 (75)
Seasonal calving	8 (22)

TABLE 4 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia: percentage of farm waterlogged at any time during the year, whether or not cattle have access to waterlogged areas, and details of irrigation maintenance on each of the farms managed by the survey respondents.

Question	Number of respondents (%)
Percentage of farm waterlogged	
0	2 (6)
1–19	19 (53)
20–39	3 (8)
40–59	6 (17)
60–79	2 (6)
80–99	4 (11)
100	0 (0)
Cattle access to waterlogged areas	
Yes	28 (78)
No	5 (14)
No response	3 (8)
Irrigation maintenance	
Excavate	1 (3)
Spray weeds	3 (8)
Spray weeds and excavate	3 (8)
Graze with stock and excavate channels	1 (3)
Spray weeds and fix leaking delvers	11 (31)
Spray weeds, fix leaking delvers, and excavate channels	11 (31)
Spray weeds, replace delvers with pipes, and fix leaking delvers	1 (3)
No response	4 (11)
None	1 (3)
Access to irrigation channels?	
Yes	22 (61)
No	11 (31)
No response	3 (8)

frequently used by the respondents to treat *F. hepatica*, followed by TCBZ (**Figure 3**). Only one respondent used OXY to treat all livestock categories (**Figure 3**). TCBZ and CLOR were used once or twice per year, but some opted for a higher treatment frequency in younger stock (**Figure 3**). The highest treatment frequency for CLOR was three times per year, whereas the highest frequency for TCBZ was six (**Figure 3**). For the preceding 5 years, flukicide use showed that CLOR was still the preferred product for treating *F. hepatica* in dairy cattle (**Figure 4A**). Several respondents used multiple flukicides to treat *F. hepatica* (**Figure 4B**), but 41% solely relied on one flukicide chemical class for the 5-year period. Of the respondents who reported they had either used an external calf rearer or purchased stock, only 3 and 8% of the respondents quarantine treated and newly returned or purchased animals (**Figure 5**).

Flukicide Administration

Survey respondents used a variety of methods to determine when to treat their animals for *F. hepatica*. The most frequent approach was to treat at dry-off (31%) (**Figure 6A**). Other methods involved using various options: at dry-off and during lactation (11%) and at dry-off and based on animals' appearance (11%). Only two respondents used diagnostics to inform treatment administration; one respondent solely relied on diagnostics, whereas the other used it in combination with other methods (**Figure 6A**). Treatment based on the animal's appearance was often used to determine when to treat (**Figure 6A**).

When purchasing a flukicide, respondents relied more heavily on a single method of selection (59%), which was often based on advice from a veterinarian (25%), previous use (14%), or a recommendation from a reseller (14%), friend or neighbor (3%), or farm advisor (3%) (**Figure 6B**). Twenty-five percent of the respondents who used multiple methods to determine what flukicide to purchase often included price, previous use, and veterinarian advice as key criteria (**Figure 6B**).

Seventy-two percent of the respondents expressed an interest in receiving more information about *F. hepatica* drenching practices (**Table 5**). Sixty-nine percent of the respondents used a single method to determine the flukicide dose to be administered to their cattle (**Figure 6C**). A quarter of survey respondents weighed the heaviest to determine the dose for the mob, 19% used the average group body weight, 17% estimated the individual weight of animals, 6% weighed each animal, and 3% estimated the weight of the heaviest animal (**Figure 6C**). Nineteen percent of the respondents used a combination of methods to determine the dose; one weighed the heaviest and

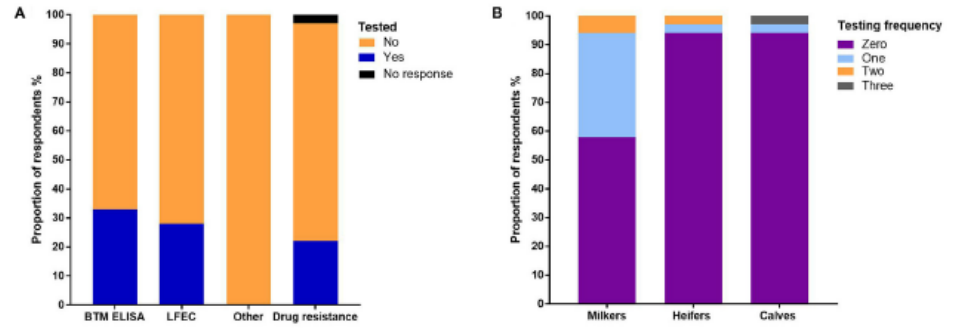

FIGURE 2 | (A) The proportion of respondents using different types of *F. hepatica* diagnostic testing on-farm. **(B)** The proportion of respondents using various frequencies of diagnostic testing per year for each animal category.

TABLE 5 | A survey of *Fasciola hepatica* control practices on dairy farms in Victoria, Australia: whether or not fluke treatment was carried out in 2015–2016 and whether or not respondents would be interested in receiving more information about fluke.

Question	Number of respondents (%)
Treated for fluke in 2015–2016?	
Yes	26 (72)
No	10 (28)
More information about fluke?	
Yes	26 (72)
No	9 (25)
No response	1 (3)

used a weigh tape (**Figure 6C**). One respondent who reported other methods in **Figures 6A,C** was an organic farmer who did not utilize flukicides. Instead, they incorporated copper three to four times a year into the animal's diet; the dose used was determined by a nutritionist (data not shown).

DISCUSSION

Survey Response

The aim of this study was to document current fluke management practices, fluke diagnostic test use, and flukicide use on irrigated dairy farms in Victoria. Recruitment of survey respondents during the 2017 dairy crisis was difficult. The Commonwealth of Australia's Senate Economics Reference Committee (18) noted that during this time, the Australian dairy industry was facing an unprecedented crisis affecting the livelihoods of 40% of the 6,000 dairy farmers in Australia. The response rate could not be determined as the survey was distributed on multiple online platforms and hard copies were handed out at industry events. We note that three surveys were returned with a note stating the respondents had left the dairy industry. The reduced participation numbers reflect the reduced confidence in the Australian dairy industry future, which has been in decline since 2016 (75–45%) and the intention of 24% of dairy farmers to leave the industry within 5 years (19, 24). At the end of the 2015/2016 financial year, there were 4,141 dairy farms in Victoria; it has since decreased to 3,516 farms in 2018/2019 (20).

Dairy Farms and Survey Respondents

Coverage error was present in this survey, reflected by limited geographical coverage, underrepresentation of farms in Victoria, and overrepresentation of farms and herds of larger size (**Figure 1**, **Table 1**). The Department of Agriculture and Water Resources (25) found that the average Victorian dairy farm was 252 ha, milked 345 cows, and had a stocking density of 2.1 cows/ha. The overestimation of these variables may also be a result of the phrasing of the survey question which asked for total farm area, not total usable or grazed area, which would have reduced the farms' size and increased the stocking density. Ninety-seven percent of farms had an irrigated pasture base (**Table 3**). The predominate method of water application was using the border-check irrigation method (known commonly as flood irrigation), which is consistent with Watson and Watson (8) and Khan et al. (26) who found 50–60% of Victorian dairy farmers solely used flood irrigation (**Table 3**). The descriptive statistics obtained from the 36 respondents were consistent with the work published by Schirmer et al. (27) who found the highest proportion of dairy farmers were aged between 45 and 54 years and the majority of respondents were male (>60%) (**Table 1**).

Integrated Parasite Management

Non-chemical control options play a crucial role in reducing the reliance on flukicides to treat *F. hepatica*. IPM strategies focus on reducing *F. hepatica* egg contamination of pasture, restricting host access to intermediate host habitat and limiting host exposure to infective stages of *F. hepatica*. In this study, 42% of the respondents identified that more than >20% of their properties had waterlogging problems (**Table 4**). Host proximity to waterlogged areas, irrigation channels, and naturally occurring

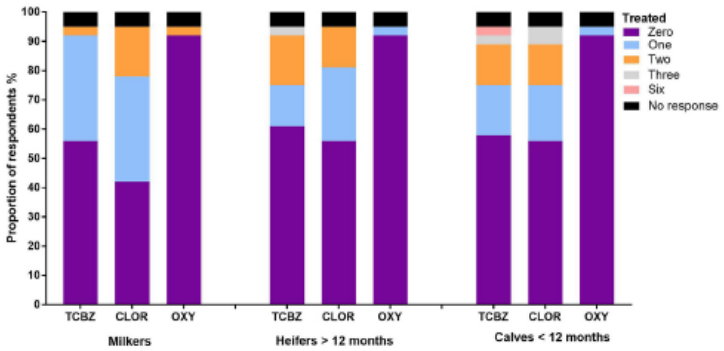

FIGURE 3 | The proportion of respondents using various numbers of annual treatments with three different flukicides in each stock category (2015/2016 financial year).

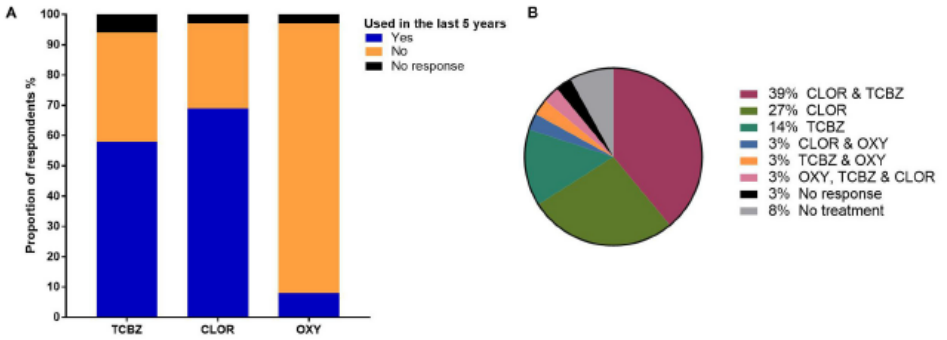

FIGURE 4 | (A) The proportion of respondents using three different flukicides over the 5 years preceding the 2015/2016 financial year. **(B)** Proportion of respondents using single or multiple flukicides over the same time period.

water bodies increases the risk of exposure and infection with *F. hepatica* (28–31). Researchers in New Zealand also identified that pugging caused by waterlogged soils increased intermediate host population (*Austropeplea tomentosa* and *Pseudosuccinea columella*) within the pasture (32). Given that in this study, stock on 78% of farms and 61% of farms had access to waterlogged areas and irrigation channels, respectively, the risk of contamination and exposure to either *F. hepatica* or the intermediate host is potentially high (**Table 4**). Fencing could play a key role in reducing stock access to these high-risk areas, but Watson and Watson (8) found that fencing is typically planned over a long period and is dependent on farm finances.

F. hepatica Diagnostics

Our survey results suggest that we should be advocating for greater use of diagnostic tests as only 33% of farms used BTM ELISA and 28% of farms used LFEC to inform decision-making (**Figure 2A**). The frequency of testing was the highest in adult stock, whereas only two farms tested young animals (**Figure 2B**). Given that young animals are generally reared on more marginal paddocks, they are more vulnerable to *F. hepatica* and infection can have flow-on effects that impact future animal fertility, suggesting that increased testing should occur in these animals (5, 33, 34). The work by Mezo et al. (35) in Spain found that only 15% of dairy farmers tested their cattle before flukicide administration and most were unaware of the herd's *F. hepatica* status. Farmers instead relied on blanket preventative flukicide treatments. Kelley et al. (12) identified the same trend in Victorian dairy farms as several farmers were routinely treating their cattle with flukicides even though the animals were not infected with *F. hepatica*. In the United Kingdom, Easton et al. (17) found that the lowest use of diagnostic and resistance tests to inform decision-making was in the dairy industry. In this study, 19% of the respondents reported that they had tested for *F. hepatica* drug resistance

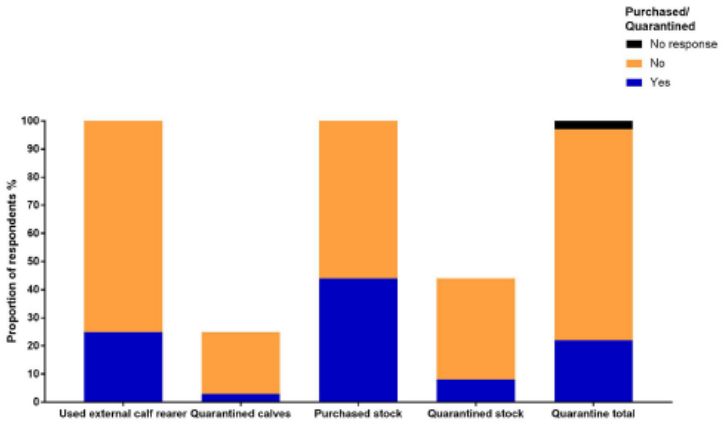

FIGURE 5 | The proportion of respondents that applied quarantine treatments to calves reared or purchased externally.

(**Figure 2A**). Given we did not ask the farmers to explain their method for testing for resistance, it is difficult to ascertain if they followed best practice guidelines or used appropriate tests to confirm resistance.

Flukicide Use

The survey findings suggest that the use of TCBZ and the frequency of flukicide treatments in dairy cattle have decreased from the recommendations laid out by Boray et al. (14). CLOR was more widely used in all stock categories compared with TCBZ and only one participant used OXY (**Figures 3**, **4A,B**). The most common approach was to treat all stock categories annually except for TCBZ in heifers which were treated twice per year (**Figure 3**). Forty-one percent of the respondents relied on single actives (CLOR or TCBZ) and, in some cases, at a high frequency (**Figures 3**, **4B**). Given that dairy farmers in Australia are limited to using only TCBZ, CLOR, and OXY to treat *F. hepatica*, this raises concerns about the increased selection pressures on these chemicals (**Supplementary Datasheet 2**) (36). A large proportion of respondents relied on CLOR, which is only sold in combination with ivermectin (**Figure 3**) (36). Bullen (37) found that on 15 of 20 dairy farms tested in the MID in Victoria, at least one nematode species was resistant to doramectin. Globally, there have been three reports of CLOR-resistant *F. hepatica* (38). It is challenging to assess flukicide efficacy if the product is only effective against adult *F. hepatica* (11). However, given the high use of CLOR in Australia, a methodology for testing efficacy needs to be developed. The study found that only a small number of respondents were using OXY which could be incorporated into flukicide rotations particularly in areas where TCBZ resistance has been identified in Victoria (10–12). The United Kingdom and Ireland have successfully communicated that TCBZ resistance is a growing problem, leading to increased OXY use in dairy cattle (15, 16). Another important component of IPM is to limit the introduction and spread of resistant parasites by quarantining newly purchased animals or animals returning to the farm. Most respondents in this study did not isolate and treat animals before joining them with the main herd; this breakdown in quarantine was also observed by Mezo et al. (35) on dairy farms in Spain (**Figure 5**).

Flukicide Administration

Boray et al. (14) recommended treating based on the season, which only two respondents in this study used as a factor in their decision-making. Instead, most of the respondents treated at dry-off (**Figure 6A**). This is consistent with research in Ireland and the United Kingdom where Selemetas et al. (16) found that 96% of farmers treated at dry-off and Bloemhoff et al. (15) found that after the tightening of anthelmintic regulations, the proportion treating at dry-off increased from 59 to 81%. Only two respondents in this study used diagnostics to inform treatment timing (**Figure 6A**). When purchasing a flukicide, respondents relied heavily on a single selection method (59%), of which 45% selected based on advice and 14% on previous use (**Figure 6B**). Cornelius et al. (39) found that whoever sheep farmers sort advice from significantly influenced what other control methods were used on-farm. Farmers who relied on professionals (e.g., private veterinarians, government veterinarians, or private consultants) were more likely to use diagnostics to inform decision-making, test for resistance, drench less, and be aware of IPM. Given that veterinarians and advisors were used by many dairy farmers in selecting flukicides, one avenue for improving *F. hepatica* management would be to educate those professionals who work with dairy farmers (**Figure 6B**). This approach could then be extended to include rural resellers. Easton et al. (40) in the

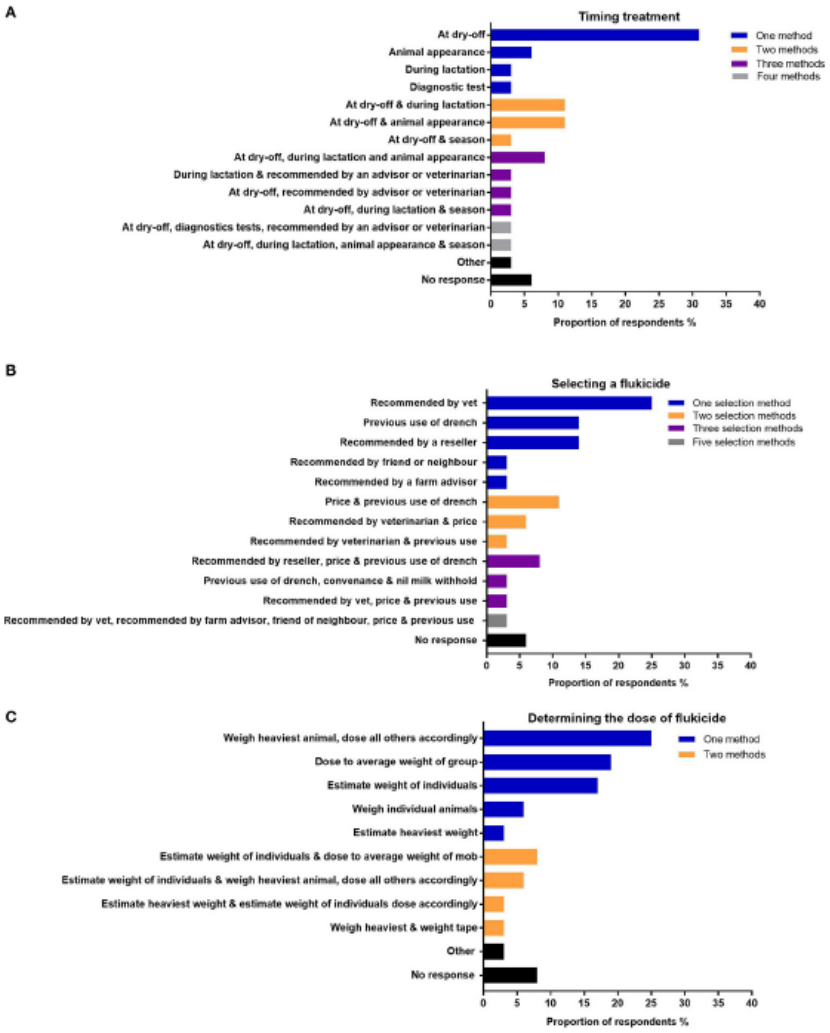

FIGURE 6 | The proportion of respondents using various methods to treat animals with flukicides. **(A)** Method used to decide timing of treatment. **(B)** Method used to select a flukicide. **(C)** Method used to determine the dose of flukicide to administer to their animals.

United Kingdom surveyed prescribers of anthelmintics and identified several knowledge gaps which were then addressed to improve advice given to farmers at point of purchase. Another important IPM strategy is to avoid the underdosing of cattle which limits the selection pressure for resistance. Besier and Hopkins (41) established that sheep farmers were poor estimators of live weight, leading to 85% of farmers underdosing their sheep for nematode control. Eighty-six percent of cattle farmers also underestimated live weight but by a greater margin than in sheep: 47% compared with 18% underestimation (41, 42).

In this study, 50% of survey respondents estimated weight and used average weights to determine flukicide doses (**Figure 6C**). Underdosing is likely to be prevalent within the dairy industry, given that only 40% were weighing the heaviest animal, weighed each animal, or used weigh tapes to determine dose volume (**Figure 6C**).

CONCLUSION

Seventy-two percent of the respondents who completed the survey wanted more information on *F. hepatica* control strategies. The evidence generated from this survey has identified several areas where *F. hepatica* management in Victoria could be optimized and has identified what IPM strategies need to be communicated to dairy farmers. Our key findings are as follows: (1) diagnostic tests are underutilized to inform flukicide timing and management of *F. hepatica* in replacement animals, (2) flukicide doses were not accurately determined and underdosing is likely to be prevalent within the dairy industry, (3) there was an overreliance on single flukicide actives and OXY was rarely used to treat *F. hepatica*, and (4) non-chemical approaches were not effectively utilized and animals had considerable access to high-risk *F. hepatica* areas on-farms. Coyne et al. (43) identified that the three biggest barriers to change on sheep farms with confirmed TCBZ resistance were overcoming habitual practices, economic feasibility, and the increased complexity in implementing IPM strategies. The best way forward for the dairy industry in Victoria would be, firstly, to do a more extensive (regionally representative) survey to establish regional differences in the management of *F. hepatica* to generate the evidence base for a tailored extension and control program. Secondly, we recommend that an economic study should be performed on the financial returns of implementing an IPM strategy on dairy farms in Victoria (44). These steps will generate the evidence base needed to encourage dairy farmers to overcome the barriers to change and implement IPM strategies on their farms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by La Trobe University Science, Health and Engineering (SHE) College Human Ethics Sub-Committee (CHESC). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JK and TS: conceptualization and writing — original draft preparation. JK, MS, and TS: methodology, formal analysis, and investigation. JK, TS, MS, GR, and TB: writing — review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.669117/full#supplementary-material

Supplementary Datasheet 1 | A copy of the hardcopy survey that was disseminated to dairy producers in Victoria, Australia.

Supplementary Datasheet 2 | Flukicide products registered for use in cattle in Australia have been listed.

REFERENCES

1. Penny J. *Report of Royal Commission on Fluke. Launceston Examiner (Tas.: 1842 - 1899)* (1869).
2. Watt G. An approach to determining the prevalence of liver-fluke in a large region. In: *Proceedings of the 2nd International Symposium on Veterinary Epidemiology and Economics 7-11 May 1979*, Canberra, Australia: Australian Government Publishing Service. Retrieved from An approach to determining the prevalence of liver fluke in a large region | SciQuest (1979). p. 152–5.
3. McCausland I, Vandegraaff R, Nugent L. Fascioliasis in dairy cows on irrigated pasture. *Aust Vet J.* (1980) 56:324–6. doi: 10.1111/j.1751-0813.1980.tb05739.x
4. Schweizer G, Braun U, Deplazes P, Torgerson P. Estimating the financial losses due to bovine fasciolosis in Switzerland. *Vet Record.* (2005) 157:188–93. doi: 10.1136/vr.157.7.188
5. Oakley G, Owen B, Knapp N. Production effects of subclinical liver fluke infection in growing dairy heifers. *Vet Record.* (1979) 104:503–7. doi: 10.1136/vr.104.22.503
6. Dairy Australia. Dairy farm guidelines for BJD control. In: *Best Practice Recommendations for Managing the Risk of BJD in Australian Dairy Herds.* (2015). Available online at: https://www.dairyaustralia.com.au/resource-repository/2020/07/09/dairy-farm-guidelines-for-bjd-control#.YCTIQmgzYuU (accessed February 13, 2021).
7. Moran JB, Drysdale GR, Shambrook DA, Markham NK. A study of the key profit drivers in the Victorian dairy industry. In: Stone GM, editor. *Proceedings*

of 9th Congress of the Asian-Australasian Association of Animal Production. (2000). p. 54–7.
8. Watson P, Watson D. *Sustainability Framework NRM Survey*. (2015). Available online at: http://www.dairyingfortomorrow.com.au/wp-content/uploads/Australian-Dairy-Sustainability-Framework-NRM-Survey-2015.pdf: Dairy Australia (accessed February 14, 2021).
9. Lean I, Westwood C, Playford M. Livestock disease threats associated with intensification of pastoral dairy farming. *New Zealand Vet J*. (2008) 56:261–9. doi: 10.1080/00480169.2008.36845
10. Brockwell YM, Elliott TP, Anderson GR, Stanton R, Spithill TW, Sangster NC. Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle. *Int J Parasitol Drugs Drug Resist*. (2014) 4:48–54. doi: 10.1016/j.ijpddr.2013.11.005
11. Elliott TP, Kelley JM, Rawlin G, Spithill TW. High prevalence of fasciolosis and evaluation of drug efficacy against *Fasciola hepatica* in dairy cattle in the Maffra and Bairnsdale districts of Gippsland, Victoria, Australia. *Vet Parasitol*. (2015) 209:117–24. doi: 10.1016/j.vetpar.2015.02.014
12. Kelley JM, Rathinasamy V, Elliott TP, Rawlin G, Beddoe T, Stevenson MA, et al. Determination of the prevalence and intensity of *Fasciola hepatica* infection in dairy cattle from six irrigation regions of Victoria, South-eastern Australia, further identifying significant triclabendazole resistance on three properties. *Vet Parasitol*. (2020) 277:109019. doi: 10.1016/j.vetpar.2019.109019
13. Hort C. A survey of *Fasciola hepatica* infection of sheep and cattle in NSW and Victoria. In: *Proceedings of the Australian Sheep Veterinary Society Conference: The Australian Veterinary Association*. (1998). p. 61–4.
14. Boray JC, Hutchinson G, Love S. *Liver Fluke Disease in Sheep and Cattle*. NSW Agriculture (1999). Available online at: http://www.wormboss.com.au/sheep-goats/files/pages/worms/flukes/liver-fluke/Prime_Fact_446_Liver_fluke_disease_in_sheep_and_cattle.pdf (accessed February. 16, 2021).
15. Bloemhoff Y, Danaher M, Forbes A, Morgan E, Mulcahy G, Power C, et al. Parasite control practices on pasture-based dairy farms in the Republic of Ireland. *Vet Parasitol*. (2014) 204:352–63. doi: 10.1016/j.vetpar.2014.05.029
16. Selemetas N, Phelan P, O'Kiely P, de Waal T. The effects of farm management practices on liver fluke prevalence and the current internal parasite control measures employed on Irish dairy farms. *Vet Parasitol*. (2015) 207:228–40. doi: 10.1016/j.vetpar.2014.12.010
17. Easton S, Pinchbeck GL, Bartley DJ, Hodgkinson JE, Matthews JB. A survey of experiences of UK cattle and sheep farmers with anthelmintic prescribers; are best practice principles being deployed at farm level? *Prevent Vet Med*. (2018) 155:27–37. doi: 10.1016/j.prevetmed.2018.04.009
18. Economics Reference Committee. *Australia's Dairy Industry: Rebuilding Trust and a Fair Market for Farmers*. (Senate Printing Unit, Parliament House, Canberra.: Commonwealth of Australia) (2017). Available online at: https://www.aph.gov.au/Parliamentary_Business/Committees/Senate/Economics/Dairyindustry/Report (accessed February 07, 2021).
19. Dairy Australia. Dairy situation and outlook June 2018. In: *National Dairy Farmer Survey*. (2018). Available online at: https://www.dairyaustralia.com.au/resource-repository/2020/09/01/dairy-australia-situation-and-outlook-june-2018#.YCtxdmgzbct (accessed February 07, 2021).
20. Dairy Australia. *Cow & Farms Data*. (2021). Available online at: https://www.dairyaustralia.com.au/en/industry-statistics/cow-and-farms-data#.YCrUf2gzbct (accessed February 16, 2021).
21. State of Victoria. *Postcode Boundaries*. Department of Environment, Land, Water and Planning (2021). Available online at: https://discover.data.vic.gov.au/dataset/postcode-boundaries-polygon-vicmap-admin (accessed January 02, 2021).
22. State of Victoria. *Irrigation District*. Department of Environment, Land, Water and Planning (2018). Available online at: https://discover.data.vic.gov.au/dataset/irrigation-district (accessed January 30, 2021).
23. Salimi-Bejestani M, Daniel R, Felstead S, Cripps P, Mahmoody H, Williams D. Prevalence of *Fasciola hepatica* in dairy herds in England and Wales measured with an ELISA applied to bulk-tank milk. *Vet Record English Ed*. (2005) 156:729–31. doi: 10.1136/vr.156.23.729
24. Schirmer J, Yabsley B, Peel D, Mylek M, Hanigan I, Brown K. *Wellbeing of Dairy Farmers: Findings From the 2014 Regional Wellbeing Survey Farmers and Agriculture Report*. Centre for Research and Action in Public Health: University of Canberra (2014). Available online at: https://www.canberra.edu.au/research/institutes/health-research-institute/regional-wellbeing-survey/survey-results/reports#2014 (accessed February 07, 2021).
25. Department of Agriculture and Water Resources. Answers to questions on notice. In: *Budget Estimates May 2017*. Estimates Committees: Commonwealth of Australia (2017). Available online at: https://www.aph.gov.au/Parliamentary_Business/Senate_estimates/rratctte/estimates/bud1718/AG/index (accessed February 07, 2021).
26. Khan S, Abbas A, Rana T, Carroll J. *Dairy Water Use in Australian Dairy Farms: Past Trends and Future Prospects*. Melbourne, VIC: CSIRO Water for a Healthy Country National Research Flagship (2010).
27. Schirmer J, Mylek M, Yabsley B. *Wellbeing, Community Connections and Resilience of Dairy Farmers: Findings From the 2015 Regional Wellbeing Survey*. Centre for Research and Action in Public Health: University of Canberra (2015). Available online at: https://www.canberra.edu.au/research/institutes/health-research-institute/regional-wellbeing-survey/survey-results/reports#2014 (accessed February 07, 2021).
28. Overend D, Bowen F. Resistance of *Fasciola hepatica* to triclabendazole. *Aust Vet J*. (1995) 72:275–6. doi: 10.1111/j.1751-0813.1995.tb03546.x
29. Alves D, Carneiro M, Martins I, Bernardo C, Donatele D, Pereira Júnior O, et al. Distribution and factors associated with *Fasciola hepatica* infection in cattle in the south of Espírito Santo State, Brazil. *J Venomous Anim Toxins Includ Trop Dis*. (2011) 17:271–6. doi: 10.1590/S1678-91992011000300006
30. Kuerpick B, Conraths FJ, Staubach C, Froehlich A, Schnieder T, Strube C. Seroprevalence and GIS-supported risk factor analysis of *Fasciola hepatica* infections in dairy herds in Germany. *Parasitology*. (2013) 140:1051–60. doi: 10.1017/S0031182013000395
31. Olsen A, Frankena K, Toft N, Thamsborg SM, Enemark HL, Halasa T. Prevalence, risk factors and spatial analysis of liver fluke infections in Danish cattle herds. *Parasites Vectors*. (2015) 8:160. doi: 10.1186/s13071-015-0773-x
32. Harris R, Charleston W. An examination of the marsh microhabitats of *Lymnaea tomentosa* and *L. columella* (Mollusca: Gastropoda) by path analysis. *New Zeal J Zool*. (1977) 4:395–9. doi: 10.1080/03014223.1977.9517964
33. Takeuchi-Storm N, Denwood M, Hansen TVA, Halasa T, Rattenborg E, Boes J, et al. Farm-level risk factors for *Fasciola hepatica* infection in Danish dairy cattle as evaluated by two diagnostic methods. *Parasites Vectors*. (2017) 10:555. doi: 10.1186/s13071-017-2504-y
34. Takeuchi-Storm N, Denwood M, Petersen HH, Enemark HL, Stensgaard AS, Sengupta ME, et al. Patterns of *Fasciola hepatica* infection in Danish dairy cattle: implications for on-farm control of the parasite based on different diagnostic methods. *Parasites Vectors*. (2018) 11:674. doi: 10.1186/s13071-018-3248-z
35. Mezo M, González-Warleta M, Castro-Hermida JA, Ubeira FM. Evaluation of the flukicide treatment policy for dairy cattle in Galicia (NW Spain). *Vet Parasitol*. (2008) 157:235–43. doi: 10.1016/j.vetpar.2008.07.032
36. Hume W. *A Review of Management Strategies for Control of Fasciola hepatica in Cattle in South Eastern Australia*. (Honours thesis) La Trobe University (2018).
37. Bullen S. Anthelmintic resistance in gastrointestinal nematode parasites of dairy cattle in the Macalister Irrigation District of Victoria. *Aust Vet J*. (2016) 94:35–41. doi: 10.1111/avj.12407
38. Kelley JM, Elliott TP, Beddoe T, Anderson G, Skuce P, Spithill TW. Current threat of triclabendazole resistance in *Fasciola hepatica*. *Trends Parasitol*. (2016) 32:458–69. doi: 10.1016/j.pt.2016.03.002
39. Cornelius M, Jacobson C, Besier R. Factors likely to influence the adoption of targeted selective treatment strategies by sheep farmers in Western Australia. *Prevent Vet Med*. (2015) 121:325–31. doi: 10.1016/j.prevetmed.2015.08.004

40. Easton S, Bartley DJ, Hotchkiss E, Hodgkinson JE, Pinchbeck GL, Matthews JB. Use of a multiple choice questionnaire to assess UK prescribing channels' knowledge of helminthology and best practice surrounding anthelmintic use in livestock and horses. *Prevent Vet Med.* (2016) 128:70–7. doi: 10.1016/j.prevetmed.2016.04.010
41. Besier R, Hopkins D. Farmers' estimations of sheep weights to calculate drench dose. *J Dept Agric Western Aust Ser.* (1989) 4:120–1.
42. Machila N, Fèvre EM, Maudlin I, Eisler MC. Farmer estimation of live bodyweight of cattle: implications for veterinary drug dosing in East Africa. *Prevent Vet Med.* (2008) 87:394–403. doi: 10.1016/j.prevetmed.2008.06.001
43. Coyne LA, Bellet C, Latham SM, Williams D. Providing information about triclabendazole resistance status influences farmers to change liver fluke control practices. *Vet Record.* (2020) 187:357. doi: 10.1136/vr.105890
44. Torres-Acosta J, Mendoza-de-Gives P, Aguilar-Caballero A, Cuéllar-Ordaz J. Anthelmintic resistance in sheep farms: update of the situation in the American continent. *Vet Parasitol.* (2012) 189:89–96. doi: 10.1016/j.vetpar.2012.03.037

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